PRODUCTION AND CHARACTERIZATION OF HUMAN-HUMAN HYBRIDOMA AUTOANTIBODIES FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

© Hélène Massicotte, B.Sc., M.Sc.
Department of Medicine
Division of Experimental Medicine
McGill University, Montreal

August, 1985

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

A François...

TABLE OF CONTENTS

SOMMAIRE

La maladie lupique (SLE) se caractérise par la production d'autoanticorps et par la présence de diverses anomalies biologiques, d'origine inconnue. L'étude de la diversité de ces autoanticorps fut entreprise à l'aide de la production d'anticorps monoclonaux et anti-idiotypiques. Le meilleur rendement en hybridomes (7.5%) et en anticorps anti-DNA (17%) fut obtenu par l'emploi d'un rapport cellulaire 1:1 et d'une densité cellulaire de $4x10^5$ cellules/ml. L'étude de la capacité de liaison antigénique des anticorps monoclonaux a révélé que 70% d'entre eux étaient polyspécifiques et que 55% de ceux-ci avaient en plus une activité anti-IgG (RF) et/ou anti-coagulante (PTT). Ce fait a démontré qu'un seul anticorps peut avoir à la fois deux activités biologiques distinctes, restreignant ainsi le nombre d'anticorps requis pour expliquer les diverses manifestations cliniques observées chez les lupiques. Les 8 antisérums anti-idiotypiques élevés contre 8 anticorps anti-DNA monoclonaux ont démontré la présence d'idiotypes communs chez les autoanticorps anti-DNA monoclonaux seulement (7 à 58%). Cependant, un antisérum reconnaissait un idiotype commun chez les anticorps anti-DNA provenant de lupiques, de personnes atteintes de polyarthrite rhumatoïde (RA) et normales. D'où, l'étude de l'idiotypie a permis de distinguer les autoanticorps anti-DNA de maladies différentes.

ABSTRACT

The origin of autoantibodies, as well as the mechanisms which account for the diversity of serological abnormalities in SLE, is unknown. The antibody diversity in SLE was analyzed here by producing hybridoma antibodies and raising anti-idiotype antisera to these hybridoma antibodies. The optimal yield of 7.5% hybridomas and 17% anti-DNA antibodies occurred with the use of a PBL to GM 4672 cell ratio of 1:1 and a cell plating density of $4x10^5$ cells/ml. Eighteen percent of all hybridoma antibodies produced had an SLE-related antibody activity and all were of the IgM class. Each antibody had its own pattern of reactivity: 69% had multiple antigen-binding specificities and 55% of these had in addition RF and/or PTT activity. Thus, the same antibody may be responsible for several observed clinical manifestations in SLE, and consequently, restrict the number of antibodies needed. Anti-idiotype antisera raised against 8 SLE hybridoma anti-DNA antibodies detected between 7 to 58% shared idiotypes among SLE hybridoma anti-DNA antibodies. Only one antiserum detected shared idiotypes on DNA binding and non-DNA binding hybridoma antibodies from RA, SLE, and normal individuals. Consequently, the study of the expression of idiotypes allowed the differentiation of anti-DNA antibodies derived from different disease states.

ACKNOWLEDGEMENT

I would like to thank Dr H. L. Goldsmith for having accepted me in the PhD program and department and acknowledge Drs H. Tannenbaum and J. Rauch for their contribution to, and critical review of, all aspects of the work presented in this thesis.

I would like to thank Drs H. Tannenbaum and D. Danoff for the use of their patients' sera.

I would like to acknowledge the technical assistance and friendship of Antonia Schiavone, Marion Tannenbaum and especially Joanne Wild in assisting me with this research, and Dorothy McKelvey in the early preparation of this manuscript.

I am thankful to Dr D. A. Bell for granting me time from my research to write the present report and for his moral and intellectual support.

I am most grateful to my husband, François Paquin, who supported me through all the years of study and for the preparation of the final manuscript through our first year of marriage.

I am also grateful to all my friends and family who have endured and supported my work.

Statement of Originality

1. Human-human hybridoma antibodies were successfully produced by the fusion of peripheral blood lymphocytes from SLE patients in different states of their disease with the GM 4672 lymphoblastoid cell line.

2. An evaluation of the effect of the cell ratio and cell density on the yield of hybridoma antibodies was done. Cell ratios of 1:1 and 5:1 lymphocyte(s):GM 4672 cell(s) and cell plating densities of $1x10^5$, $2x10^5$ and $4x10^5$ cells/ml were compared. The optimal yield of both hybridoma antibodies and anti-DNA hybridoma antibodies was obtained when a cell ratio of one lymphocyte to one GM 4672 cell line and a cell plating density of $4x10^5$ cells/ml were used.

3. Hybridoma antibodies with SLE-related autoantibody activities were successfully produced. Eighteen percent of all hybridoma antibodies produced had anti-dDNA, anti-nDNA, anti-cardiolipin, RF and/or PTT activities. They were all of the IgM class. The IgM production of anti-DNA hybridoma antibodies was not different from the IgM production of non-DNA binding hybridoma antibodies.

4. The antigen-binding specificities of the SLE-related hybridoma autoantibodies was analysed by direct binding

and inhibition of dDNA binding assays. The direct binding assay revealed that of the 46 hybridoma autoantibodies with SLE-related antibody activities, 6 had strict RF activity, 3 had strict PTT activity, 4 had both RF and PTT activities, 5 had strict anti-dDNA activity while the rest of them combined anti-dDNA, anti-nDNA, anti-cardiolipin, RF and/or PTT activities. Thus, the direct binding analysis revealed that one hybridoma autoantibody may have two distinct antibody activities which restricted the number of antibodies required to explain the diverse clinical manifestations observed in SLE. The inhibition binding study revealed that each antibody had its own pattern of reactivity: 69% had multiple antigen-binding specificities. They must recognize a similar epitope in the different molecules which inhibited their dDNA binding activity.

5. Anti-idiotype antisera were raised in rabbits against 8 anti-DNA hybridoma antibodies. They were rendered anti-idiotype specific by multiple absorptions on Sepharose-HIgM and HIgG columns. The absorbed antisera did not bind significantly to dDNA, HIgM, and HIgG, but specifically to their immunogen.

6. The location of the idiotypes on the immunogens recognized by each antiserum was derived from two assays. The idiotype was situated in the antigen-binding site of the antibody if the homologous anti-idiotype antiserum inhibited the

dDNA binding of the antibody and if the binding of the idiotype to its anti-idiotype antiserum was antigen inhibitable. Using these 2 types of inhibition assays, it was possible to demonstrate the antigen-binding-site location of the idiotype for 5 anti-idiotype antisera and the non-antigen-binding site location of the 1305 idiotype. For 2 idiotype/anti-idiotype systems, only one type of inhibition assay could be used, which demonstrated that the 100-1 idiotype system was antigen-binding site related while the 600 idiotype system was not.

7. Study of the idiotype expression on the monoclonal lupus autoantibodies revealed that they shared idiotypes in a range of 7 to 58%. These shared idiotypes (except 134 idiotype) were only found on SLE hybridoma antibodies and not on RA or normal hybridoma antibodies. Moreover, these shared idiotypes were only found on SLE hybridoma antibodies with an anti-DNA activity.

8. Preliminary data on the expression of these idiotypes in sera of patients with SLE and RA, and of normal individuals suggested that some shared idiotypes were mainly expressed in patients with SLE disease, while others were equally and highly expressed in normal patients as well as in those with RA disease.

VITAE

The items of each following lists are the one resulting from this thesis only:

List of Awards:

1984-1986	Arthritis Society Fellowship in Basic Science
1984	Alberta Lupus Society Student Award
1984	Canadian Society for Clinical Investigation Student Award
1983	McGill Graduate Faculty Summer Research Fellowship

List of Abstracts:

Massicotte H., Rauch J., and Tannenbaum H.: Human hybridoma autoantibodies to DNA. Proceedings of XIV Laurentian Conference of Rheumatology. October 1-2, 1982, Val David, Quebec.

Rauch J., Massicotte H., and Tannenbaum H.: Human hybridoma autoantibodies to DNA. Northeastern American Rheumatism Association Meeting. October 28-29, 1982, Boston.

Rauch J., Massicotte H., and Tannenbaum H.: Anti-idiotype antibodies detect shared idiotypes on SLE hybridoma anti-DNA autoantibodies. Annual Meeting of the Royal College of Physicians and Surgeons of Canada. September 1922, 1983, Calgary, Alberta.

Massicotte H., Rauch J., and Tannenbaum H.: Anti-idiotype antibodies detect shared idiotypes on SLE hybridoma anti-DNA autoantibodies. Proceedings of XV Laurentian Conference of Rheumatology. September 30-October 1, 1983, Val David, Quebec.

Massicotte H., Rauch J., and Tannenbaum H.: Anti-idiotype antibodies detect shared idiotypes on SLE hybridoma anti-DNA autoantibodies. Fourth Annual Research Seminar of Experimental Medicine Division of Montreal General Hospital, November 2nd, 1983, Montreal, Quebec.

Rauch J., Massicotte H., Tannenbaum H.: Anti-idiotype antibodies detect shared idiotypes on SLE hybridoma anti-DNA autoantibodies. Third International Seminar on the Treatment of Rheumatic Diseases November 13-20, 1983, Tel-Aviv and Jerusalem, Israel.

Rauch J., Straaton K., Massicotte H., Tannenbaum H.: Monoclonal human hybridoma autoantibodies manifesting both rheumatoid factor and anti-DNA reactivities. ASPC, 1984.

Massicotte H., Rauch J., Tannenbaum H.: Specific and shared idiotypes found on hybridoma anti-DNA autoantibodies derived from RA and SLE patients. 1984 Annual Meeting of the Canadian Society for Clinical Investigation. September 11th, Montreal, Quebec.

Massicotte H., Rauch J., Tannenbaum H.: Specific and shared idiotypes found on hybridoma anti-DNA autoantibodies derived from RA and SLE patients. Proceedings of XVI Laurentian Conference of Rheumatology. October 19-20, 1984, Val David, Quebec.

List of Publications:

Shoenfeld Y., Rauch J., Massicotte H., Datta S.K., André-Schwartz J., Stollar B.D., and Schwartz R.S.: Polyspecificity of monoclonal lupus autoantibodies. N. Eng. J. Med. 308: 414-420, 1983.

Massicotte H., Rauch J., Shoenfeld Y., and Tannenbaum H.: Influence of fusion cell ratio and cell plating density on production of human-human hybridomas secreting anti-DNA autoantibodies from patients with systemic lupus erythematosus. Hybridoma 3: 215-222, 1984.

Rauch J., Massicotte H., and Tannenbaum H.: Hybridoma anti-DNA autoantibodies from patients with rheumatoid arthritis and systemic lupus erythematosus demonstrate similar nucleic acid binding characteristics. J. Immunol. 134: 180-186, 1985.

Rauch J., Massicotte H., Wild J., and Tannenbaum H.: Sensitive solid phase radioimmunoassay for the detection of anti-DNA autoantibodies. J. Rheum. 12: 482-486, 1985.

Rauch J., Tannenbaum H., Straaton K., Massicotte H., and Wild J. Human-human autoantibodies with both anti-DNA and rheumatoid factors. J. Clin. Invest. 77: 107-112, 1986.

Rauch J., Massicotte H., and Tannenbaum H.: Specific and shared idiotypes found on hybridoma anti-DNA autoantibodies derived from rheumatoid arthritiis and systemic lupus erythematosus patients. J. Immunol. 135: 2385-2392, 1985.

List of Abbreviations

ANA:	anti-nuclear antibody
ARA:	American Rheumatism Association
ATP:	adenosine triphosphate
Bis:	N,N'-methylenebisacrylamide
BSA:	bovine serum albumin
BUdr:	5'-deoxyribose bromouracile
°C:	degree centigrade
$CaCl_2$:	calcium chloride dihydrate
CFA:	complete Freund's adjuvant
CH_{50}:	whole complement titer which is the reciprocal of the dilution of serum that lyses 50% of a standard suspension of erythocyte
CLL:	chronic lymphocytic leukemia
CNBr:	cyanogen bromide
CO_2:	carbon dioxide
cpm:	counts per minute
CTP:	cytidine triphosphate
CVI:	common variable immunodeficiency disease
dH_2O:	distilled water
ddH_2O:	double dH_2O
DMSO:	dimethyl sulphoxide
DNA:	deoxyribonucleic acid
dDNA:	denatured DNA
DMEM-H21:	Dulbecco's modified eagle medium H-21 £430-2100
DNCB:	2,4-dinitrochlororobenzene
ds:	double-stranded
DTT:	dithiothreitol
dUDP:	deoxy UDP
dUMP:	deoxyuridine monophosphate
EBNA:	Epstein-Barr nuclear antigen
ECG:	electrocardiogram
ECGS:	endothelial cell growth supplement
ESR:	erythrocytes sedimentation rates
FBS:	fetal bovine serum
FTA:	fluorescent treponemal absorption
GDP:	guanosine diphosphate
GM-to-HAT:	HAT medium without HAT
GMP:	guanosine monophosphate
GTP:	guanosine triphosphate
HAT medium:	hypoxanthine-aminopterin-thymidine RPMI medium
HCl:	hydrochloric acid
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethane
HGPRT:	hypoxanthine guanosine phosphoribosyl transferase
HIgG:	human IgG
HIgM:	human IgM
HMy2:	LICR-LON-HMy2 human cell line
hrs:	hours
IEF:	isoelectrofocusing
IFA:	incomplete Freund's adjuvant
Ig:	immunoglobulin

IgA:	immunoglobulin A
IgG:	immunoglobulin G
IgM:	immunoglobulin M
IMP:	inosine monophosphate
KCN:	potassium thiocyanate
KI:	potassium iodide
KPO_4:	potassium phosphate buffer
LE cell:	lupus erythematosus cells
M:	molar or mole per liter
mg:	milligram
ml:	milliliter
mM:	millimolar
MW:	molecular weight
Na:	sodium
NaCl:	sodium chloride
$NaHCO_3$:	sodium carbonate
NaN_3:	sodium azide
NaOH:	sodium hydroxide
nDNA:	native DNA
$(NH_4)_2SO_4$:	ammonium sulphate
nmoles:	nanomoles
NP:	nucleoprotein
OD_{280}:	optical density at 280 nanometer
PBL:	peripheral blood lymphocytes
PBS:	phosphate buffered saline
PEG:	polyethylene glycol
PLL:	poly-L-lysine
PMW:	pokeweed mitogen
poly (I):	polyinosinic acid
poly (dT):	polydeoxythymidylic acid
PP:	phosphoric acid
PRPP:	5-phosphoribosyl-1-pyrophosphate
PTT:	prolonged partial thromboplastin
RF:	rheumatoid factor
RIA:	radioimmunoassay
RNA:	ribonucleic acid
RNP:	ribonucleoprotein
rpm:	rotation per minute
RT:	room temperature
S.D.:	standard deviation
SFM:	serum free medium
SLE:	systemic lupus erythematosus
Sp2/0:	Sp2/0-Ag-14 mouse myeloma cell line
SRBC:	sheep red blood cells
ss:	single-stranded
Staph A:	<u>Staphylococcus Aureus</u>
TAC:	tris-ammonium chloride
TBS:	tris-saline buffer
TCA:	trichloroacetic acid
TDP:	thymidine diphosphate
TEMED:	N,N,N',N'-tetramethylethylenediamine
TMP:	thymidine monophosphate
TNP-SRBC:	2,4,6-trinitro phenylated SRBC
TTP:	thymidine triphosphate

UDP:	uridine diphosphate
VDRL:	venereal disease research laboratory test
µg:	microgram
µl:	microliter
v/v:	volume/volume
w/v:	weight/volume
zDNA:	brominated poly(dG-dC)
%:	percent

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Anti-DNA antibody production is characteristic of SLE (Koffler 1971, Nakamura 1978). These autoantibodies have been implicated in the immune complex nephritis associated with this disease (Agnello 1973, Koffler 1974). However, the presence of antibodies to nucleic acids is seen, to a lesser extent, in several other conditions (Koffler 1971, Bell 1975, Nakamura 1978). In what respect(s) do the anti-DNA antibodies differ from one disease to the next such that they are pathogenic in SLE?

Until recently, the only way to study anti-DNA antibodies was to use the serum of a patient as a source despite the fact that heterogeneous population of antibodies was present. The advent of hybridoma technology (Kohler 1975) rendered possible the production and study of homogeneous preparations of anti-DNA antibodies (Andrzejewski 1980, 1981, Littman 1983, Kozbor 1983). The purpose of this study was to produce and study human-human hybridoma autoantibodies expressed in SLE.

Part I. Historical Review

Chapter 1. PROBLEM

1.1 History of SLE

The interpretation of the pathogenicity of SLE has evolved through the years. In the early stage of its history, SLE was first identified in dermatology. It was named lupus erythematosus for the distinctive red, wolf-like rash often found on the bridge of the nose and cheeks of those afflicted with this disease (Cazenave 1851). Many years later, when it appeared that internal organs such as joints, blood, vessels, heart, lungs, brain and kidneys were also involved, its name changed from lupus erythematosus to systemic lupus erythematosus (Kaposi 1872). From a dermatological disease, which SLE was regarded for a century, it became classified as a collagen-vascular type disease following the observation that the histology of postmortem SLE kidneys revealed thickened loops of Henlé (Baehr 1935, Klemper 1941). A few years later, the biological era of lupus began with two discoveries: the LE cells by Hargraves (1948) and the immunofluorescence technique by Coons (1951). Friou (1957, 1958a,b,c) applied the immunofluorescence technique to blood and tissues of SLE patients and observed the staining of the nuclear material. The ANA test was then discovered. Simultaneously, Cepilinni (1957) and Seligmann (1957) described the existence of anti-DNA antibodies in lupus sera. Based on these observations

of the presence of autoantibodies to nuclear antigens, SLE was finally classified as a disorder of the immune system, that is, as an immune complex and autoimmune disease (Dubois 1974, Koffler 1980).

1.2 Theories of Autoimmunity

The premise of the science of immunology is based on the ability of an organism to recognize and react to foreign antigens while remaining tolerant to "self"-antigens. However, under some circumstances, a breakdown in the tolerance towards "self"-antigens may occur and lead to benign or possibly pathological consequences (Talal 1978), phenomena designated as autoimmunity. Ehrlich (1900) used the term "horror autotoxicus" to emphasize that an individual would not react against "self" components.

Autoimmune reactions can be manifested by autoantibody production and/or by cellular immune mechanisms; thus, tissue damage can result from immune complexes (complement mediated) and/or cellular immune processes.

The production of autoantibodies may arise from: (1) the introduction into the organism of exogeneous antigens that are cross-reactive with autoantigens, (2) changes in the physical structure of autoantigens, thus rendering these molecules immunologically reactive or, (3) exposure to previously sequestered antigens.

The "sequestered antigens" theory proposed that certain antigens (e.g. thyroglobulin, spermatozoa, or lens proteins) are normally secluded from contact with lymphocytes by virtue of

their sequestration in a non-vascular site. Autoimmunity would arise when, for whatever reason, these antigens suddenly came in contact with immunocompetent cells. There is, however, no evidence for antigen sequestration since many normal individuals possess traces of thyroglobulin and other sequestered antigens as well as the presence of B lymphocytes with specificity for thyroglobulin and other autoantigens (Bankhurst 1973, 1975, and Cohen 1980).

As mentioned above, modified cross-reactive or related antigens can terminate a state of specific immunological unresponsiveness. For example, mice made tolerant to bovine serum albumin, can be induced to make anti-bovine serum albumin antibodies by the injection of chemically modified serum albumin or a cross-reactive antigen, human serum albumin (Talal 1980). In a similar manner, the appearance of autoimmunity following viral infection could be due to the generation of modified or cross-reactive antigens as a result of the presence of the virus. For example, there is the immunological cross-reaction between streptococcal and human cardiac antigens, which has been postulated to explain the development of rheumatic fever after streptococcal infection (Kaplan 1962, 1964).

Unresponsiveness of the organism to self-antigens led Burnet (1959) to propose, at the cellular level, the "Clonal Selection Theory" which has also been called the "Clonal Abortion Theory" (Stocker 1976, Nossal 1977). This theory postulated that a cell capable of reacting against self-antigens would be eliminated during ontogeny in order to prevent

autoimmunity. That process would function both in the newborn and in the adult human since immature B cells are released throughout life (Klinman 1976). This theory was no longer valid when it was found that healthy subjects had low concentrations of autoantibodies and that polyclonal cell activators induced normal B cells to produce a variety of autoantibodies both in vitro and in vivo (Izui 1977, Cohen 1977, Fishbach 1978). The broad spectrum of autoantibodies found in most autoimmune diseases (Dubois 1974) tends to argue against this idea as well.

The concept of disordered immunological regulation is now the most attractive theory to explain many examples of autoimmunity. According to this theory, the potential for autoimmunity present in normal individuals is not expressed because of the normal functioning of the immunological regulation mechanism. This is due to the interaction of various T lymphocyte subpopulations (Claman 1966, 1972, Taylor 1969). A disequilibrium resulting either in the generation of helper T cells or in a deficiency of suppressor T cells could trigger these potentially autoreactive B cell clones into autoantibody production. The influence of immune response genes and idiotype networks can be incorporated into this theory of autoimmunity.

The idiotype network theory proposed by Jerne (1974) sees the immune system as a network of interacting idiotypes. This theory views individual immunoglobulin molecules as expressing two critical sets of sites through which interaction with other elements of the immune system occurs. The first

site is the antigen-combining site, designated as paratope (p). The corresponding antigenic determinants of an antigen, to which the paratope binds, is called the epitope (e). The second site is also situated in the variable regions of the Fab fragment. Each immunoglobulin molecule expresses, in that region, antigenic determinants unique to it or shared by only a relatively limited number of Ig molecules. These idiotypical determinants were termed idiotopes (i). Jerne proposed that for each antibody expressing a given p_1 and i_1, there is a complementary antibody possessing a p_2 capable of binding i_1, and expressing its own sets of idiotypes i_2. Similarly, there is a complementary antibody to this second antibody and so on. Jerne had limited his concept to B cells and their products, antibodies. It is now clear that T cells also participate in idiotype-based regulatory systems and that the network theory is not open-ended (Talal 1980). The equilibrium between clones based on idiotype interaction is very sensitive to perturbation, by antigens, idiotypes and anti-idiotypes. The perturbation of this balance may lead to autoimmunity.

1.3 Manifestations of SLE

SLE patients bear diverse clinical manifestations during the course of their illness. These clinical features, listed in Table 1, do not occur in all patients. Some patients show numerous manifestations of the disease while others have relatively few of them during their life. However, virtually all SLE patients exhibit at least once, one or more of the serologic abnormalities

listed in Table 2. The sickness in most individuals is in fact characterized by exacerbations and remissions occurring over a period of many years. These different observations have rendered it difficult to classify a patient as having lupus. For these reasons, the American Rheumatism Association (ARA) established 11 criteria (revised in Tan 1982), based on more than 10 years of clinical data, to help diagnose SLE. These criteria (listed in Table 3), taken individually, are not all exclusive of SLE disease (Tan 1982). However, the presence of any 4 or more of these 11 criteria, serially or simultaneously, during any interval of patient observation has rendered the diagnosis of SLE for a patient valid in 95% of the cases.

Although the diagnosis of SLE is now facilitated, the cause of SLE is still unknown. Genetic, hormonal and environmental influences have been implicated. For example: 1) SLE may be exacerbated by ultraviolet light or certain drugs (Tan 1976a and b, Hahn 1972); 2) SLE usually prevails in women and black people rather than in males and white people (Dubois 1974); 3) there are aberrations in the T cell, B cell, as well as accessory cell compartments related to the function, interaction and regulation of these cells (Hahn 1975, Butler 1972, Sakane 1978, Kuntz 1979, Schneider 1975). The outcome of all these abnormalities is a B cell hyperactivity and the production of abnormal levels of autoantibodies. The autoantibodies lead to tissue destruction as illustrated in Figure 1.

As a result, one alternative for the management of SLE resides in the use of immunosuppressive drugs to decrease the production of autoantibodies. However, these drugs also affect the production of other kinds of antibodies and cause undesirable side effects. Consequently, treated SLE patients are often predisposed to opportunistic infection. There is thus a need for a new kind of therapy.

The efforts of many researchers are directed toward the study of serum anti-DNA antibodies, since they have been implicated in the immune complex nephritis associated with this disease (Agnello 1973, Koffler 1974). The levels of anti-native or double-stranded DNA antibodies in serum often correlate closely with disease activity: high levels in active SLE and low or undetectable levels in remission periods (Koffler 1971, Steiman 1978, Bell 1980). However, serum studies are limited in terms of defining the different specificities of the autoantibodies.

The advent of hybridoma technology (Kohler 1975) has rendered possible the study of single monoclonal antibodies. The characterization of hybridoma autoantibodies would thus indicate the homogeneous or, conversely, the heterogeneous nature of the anti-DNA population. Moreover, the study of individual autoantibodies could lead to the identification of a common idiotype on these antibodies. This approach can then lead to the production of anti-idiotype antibodies which may be prove useful in the diagnosis and/or the treatment of SLE patients.

1.4 History of Fusion Technology

Hybridoma technology resulted from the need to study the immunoglobulin (Ig) structure, secretion and function as well as Ig genes. This technique originated accidentally from the joint application of two independently derived biological tools (Kearney 1984, Tom 1983).

The first tool was the availability of myeloma cells (Potter 1972). These cells represent monoclonal malignancies of B cell lineage which secrete a unique species of Ig. With time, it has been possible to, first, transplant them *in vivo* and, finally, to adapt them to *in vitro* culture (Pettengill 1967, Horibata 1970). One remarkable characteristic of myeloma cells is the fact that they grow indefinitely *in vivo* or *in vitro*. Thus, these cell lines became a continuous source of a homogeneous population of Ig from which new information on Ig was acquired.

The second technology developed was that of cell fusion in which two somatic cells were fused, resulting in the production of hybrid cells. Cell fusion products - multinucleated cells - were observed as early as 1828-1858 in vertebrate tissue and lesions and in 1912-1927 in *in vitro* culture (Harris 1970, Ringertz 1976). However, it was only many years later that hybrid cells could be isolated from non-hybrid cells (Barski 1960). The production and isolation of hybrid cells were subsequently facilitated by the use of selective media (Littlefield 1964) and of new fusion-inducing agents such as Sendai virus (Okada

1962, 1965, Harris 1965), lysolecithin (Poole 1970), polyethylene glycol (Pontevorco 1976) and the combination of polyethylene glycol and dimethyl sulphoxide (DMSO) (Norwood 1976).

It appeared that when 2 Ig-producing myeloma cells were fused together, the resultant hybrid cells retained the characteristics of both parents. They expressed both sets of Ig light and heavy chains, resulting in the formation of hybrid molecules in which random association of both sets of parental light and heavy chains occurred (Cotton 1973, Schwaber 1974). No new chains were produced and no scrambling of V and C regions were observed (Schwaber 1974, Kohler 1975). With that result in mind and the need to obtain monoclonal antibodies of predefined specificity, Kohler and Milstein (1975) tried to fuse a myeloma cell line to sheep red blood immunized splenic cells (SRBC). Their fusion was successful and anti-SRBC hybridoma antibodies were obtained. The revolution began. Investigators, fusing mouse x mouse, mouse x rat, mouse x human and finally human x human cells, obtained a broad spectrum of antibodies. The limits of the production of monoclonal antibodies are a suitable myeloma fusion partner and specific stimulated B lymphocytes.

1.5 Purpose of the Present Study

The primary objective of the present investigation was to analyse differences between individual anti-DNA autoantibodies in patients with SLE. This was accomplished by the analysis of human-human monoclonal anti-DNA antibodies

synthesized from different patients with SLE, and by the determination of the presence of dominant idiotypes which might distinguish between pathogenic and non-pathogenic anti-DNA antibodies. Thus, this study consisted of grosso modo three parts. The first part involved the production of human-human hybridoma monoclonal antibodies. The second involved the study of these monoclonal antibodies by determining their antigen-binding specificities and cross-reactivities along with determining their functional activities. Finally, the last part involved the production and study of anti-idiotype antibodies raised against anti-DNA hybridoma antibodies both in rabbits and mice. This part also included the assessment of cross-reacting idiotypes among the hybridoma autoantibodies.

The fusion procedure used is the technique of Kohler and Milstein (1975), adapted to the human cell, and is illustrated in Figure 2. The availability of a human lymphoblastoid cell line, GM 4672, has rendered such an application possible. The GM 4672 cell line originates from a patient's myeloma and produces an IgG2k of unknown specificity. It was selected for resistance to 6-thioguanine, an analog of guanine, a base involved in the formation of DNA and RNA. The incorporation of 6-thioguanine in the medium of these cells selected the cells deficient in the hypoxanthyl-guanosine-phosphoribosyl transferase enzyme (HGPRT), which did not allow the utilization of the salvage pathway of DNA and RNA synthesis. Subsequently, if these cells were placed in a medium containing

aminopterin, which is an analog of the dihydrofolic acid, these cells would die rapidly since both the main and salvage pathways for DNA and RNA synthesis were blocked, as illustrated in figure 3.

Using these selective criteria, human-human fusions are effected in which the GM 4672 cell gives the hybrid cell longevity and the human lymphocyte donates the HGPRT enzyme. The fused cells are grown in a medium containing aminopterin as well as thymidine and hypoxanthine, compounds required for the utilization of the salvage pathway (Figure 3). In such a medium, only the hybrid cells survive. Non-malignant human cells do not survive for long periods of time in tissue culture. The source of human cells used in this study are the B lymphocytes of SLE patients. As previously discussed, the lymphocytes of these patients are in a hyperactive state, which involves in the production of a wide variety of autoantibodies. Thus, the hybrid cells are expected to produce the IgG2K of the myeloma cell line and the autoantibodies of the SLE PBL, since the hybrid cells retain the capacity of producing the Ig of both parental cells (Kohler 1975, Schwaber 1974).

1.6 <u>What is an Antibody ?</u>

The protein molecules that resulted from the presence of, and combined specifically with, an antigen (chemical substances capable of inducing a specific immune response) were termed antibodies; collectively, all proteins with antibody activity are referred to as immunoglobulins.

Two kinds of function are characteristic of every

antibody molecule, 1) specific binding of one or a few of an almost limitless variety of ligands, and 2) regardless of this ligand-binding specificity, participation in a limited number of general or effector reactions e.g. complement fixation. The former function is attributed to the Fab (antigen-binding fragment) portion of an Ig molecule while the latter functions are attributed to the Fc (crystallizable fragment) portion. Both fragments were identified from the study of Ig protease cleavage products.

All immunoglobulin molecules have a common structure consisting of 4 polypeptide chains, two large (450 to 550 amino acid residues) and two small (211 to 217 amino acid residues). The large chain is termed the heavy or H chain; the smaller one is termed the light or L chain. Analysis of the amino acid sequences of both chains showed linear periodicity which suggested that L and H chains have repeating domains, each with about 110 amino acid residues and an approximately 60 membered-SS-bonded loop. Thus, the L chain has 2 domains while H chain has 4 or 5 domains. Critical examination of extensive sequence data revealed that, with the exception of minor genetic polymorphism, variability in H or L chains seemed to be confined to the amino terminal half of Fab fragment, the carboxy terminus being constant for a given immunoglobulin type. From that, the terms variable (V) and constant (C) region arose, with V_L and C_L signifying the domains of light chains and V_H and C_H those of heavy chains. Moreover, within each V_L and

V_H there is profound variability within regions called hypervariable (HV) regions; conversely, the relatively conserved portions are called framework (FW) regions. In the sequences of some H chains, the presence of a section of varying length was noted between the Fc and Fab portions and was called the "hinge" region. Indeed, the Fab portions of an antibody molecule can apparently pivot about this hinge to a remarkable degree (Werner 1972, Cathou 1970, Valentine 1967).

In addition, there are other diversity segments at the joining junction of the V and C regions of both chains; for the L chain, it is called J_L and for H chain, J_H and D. Again, studies of amino acid sequences imply different J and D segments for both chains.

Figure 1A illustrates the model of the prototypical immunoglobulin molecule and Figure 1B illustrates the linear periodicity with the position of amino acids involved in disulfide bond.

Three categories of antigenic determinants are used to classify Igs: 1) Those that differentiate among the main Ig classes are the same in all normal individuals of a given species: they are called isotypic determinants or isotypes. Various isotypes are associated with different effector functions, but they are essentially unrelated to ligand-binding activity. Thus, antibodies of diverse Ig classes can be specific for the same antigen. Human Igs are divided into 5 principal classes which are IgG, IgA, IgM, IgD, IgE; their respective heavy chains are γ, α, μ, δ, and ε.

There are two types of light chains, kappa (k) and lambda (λ). Only 1 type of H and L chain is found in a single Ig molecule. Thus, for example, an IgGλ will be formed by 2Lλ and 2Hγ. 2) Other Ig determinants reflect regular small differences between individuals of the same species in the amino acid sequences of their otherwise similar Igs. The differences are specified by allelic genes and are called allotypes. These markers are not associated with particular ligand-binding or effector functions. Figure 1C illustrates the organization of k, λ, and H genes with their isotypes and allotypes. 3) Antigenic determinants of a third kind, idiotypes, are unique to the Ig molecule produced by a given clone of Ig-producing cells related to the antigen which induced their production or related to the individual (or groups of individuals) who produced it.

The idiotypic determinants are located in the Fab fragment, specifically in the V region, along with the antigen-combining site. The combining site specificity as well as its three dimensional structure is the result of non-covalent interactions between the variable regions of the light and heavy chain (specifically HV 1, 2 and 4 for H chain and 1 and 3 for L chain (Capra 1977)) of Ig molecules which represent a segment of 5-34A° (Bona 1981). An idiotype may or may not be associated with the combining site. One antibody molecule may express one or several idiotypes. An idiotype found in only one antibody molecule is called a private or individual idiotype (IdI), while an idiotype found

on several antibody molecules of the same individual or a group of individuals is called a public or shared idiotype (IdX). An idiotype may be found in the FW regions or in the HV regions of an Ig.

The specificity of a binding site results from: 1) the shape of the site, which is concave and complementary to that of the antigen in order to expose a large area of surface to the antigen and 2) the amino acid side chains within the cavity which are precisely positioned to take full advantage of electrostatic, hydrogen-binding and Van der Waals interactions, all of which are weaker than covalent interaction (Capra 1977).

The diversity observed in antibodies may arise from 4 mechanisms: 1) different combinatorial pairing between V regions of H and L chains, 2) combinatorial sequences of V_L and J_L, and of V_H, D, J_H gene segments, 3) somatic mutation in one or a number of positions in any of these gene segments and 4) the splicing between the joining ends of each gene segment, which is often imprecise and results in additional variations at junction positions (Jeske 1984). These events contribute to the formation of extended numbers of different combining sites and influence the expression of a particular idiotype.

TABLE 1

CLINICAL MANIFESTATIONS OF SLE DURING ENTIRE COURSE OF ILLNESS

manifestations	percent of patients
Musculoarticular	95
Hematologic/reticuloendothelial	85
Cutaneous disease	80
Fever without other cause	80
Easy fatiguability	80
Neuropsychiatric disease	60
Pulmonary disease	60
Renal disease	55
Cardiac	50
Gastrointestinal	45

(Hahn 1980)

TABLE 2

LABORATORY ABNORMALITIES IN SLE

Test	percent positive during course of disease
ANA by indirect immunofluorescence (includes anti-NP, anti-DNA, antihistone, anti-RNP, anti-Sm)	95
Hypocomplementemia (includes CH_{50}, C1q, C4, C2, C3, properdin, factor B)	80
Elevated ESR	80
Anemia	80
of chronic disease	70
hemolytic	10
Antilymphocyte antibodies	70
Antibodies to Sm and/or RNP	67
Antibodies to native DNA	65
Positive direct Coomb's tests	65
Leukopenia	65
LE cells	60
Renal abnormalities (elevated serum, creatinine, abnormal urinalysis, elevated 24-hour urine protein)	60
Rheumatoid factors	50
Polyclonal increase in gamma globulins (especially IgG)	50
Cryoglobulins	35
Thrombocytopenia	15
Circulating anticoagulants	10
False positive VDRL	10
False positive FTA	10

(Hahn, 1980)

TABLE 3

THE 1982 REVISED CRITERIA FOR CLASSIFICATION OF SYSTEMIC LUPUS ERYTHEMATOSUS

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis - convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion OR b) Pericarditis - documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed OR b) Cellular casts - may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	a) Seizures - in the absence of offending drugs or known metabolic derangements; e.g. uremia, ketoacidosis, or electrolyte imbalance OR b) Psychosis - in the absence of offending drugs or known metabolic

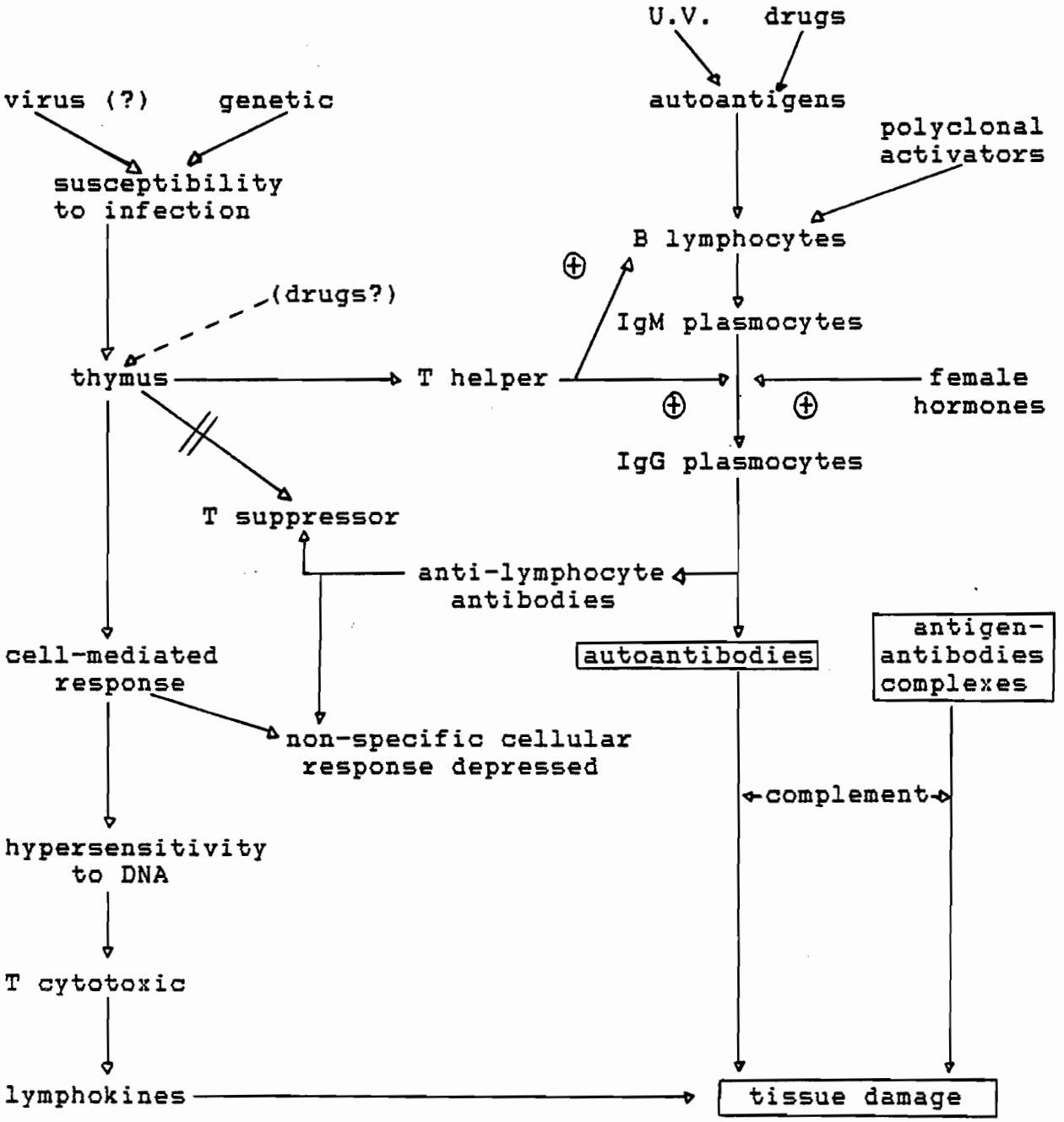
TABLE 3 (continued...)

	derangements; e.g. uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia - with reticulocytosis OR b) Leukopenia - less than 4,000/mm^3 total on 2 or more occasions OR c) Lymphopenia - less than 1,500 /mm^3 on 2 or more occasions OR d) Thrombocytopenia - less than 100,000/mm^3 in the absence of offending drugs
10. Immunologic disorder	a) Positive LE cell preparation OR b) Anti-DNA: antibody to native DNA in abnormal titer OR c) Anti-Sm: presence of antibody to Sm nuclear antigen OR d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

(Tan, 1982)

FIGURE 1

SCHEMATIC ILLUSTRATION OF THE INTERPLAY OF GENETIC, HORMONAL, AND ENVIRONMENTAL INFLUENCES PRODUCING THE IMMUNOLOGIC ABNORMALITIES CHARACTERISTIC OF SLE



(Meyer, 1982)

FIGURE 2. FUSION PROCEDURE

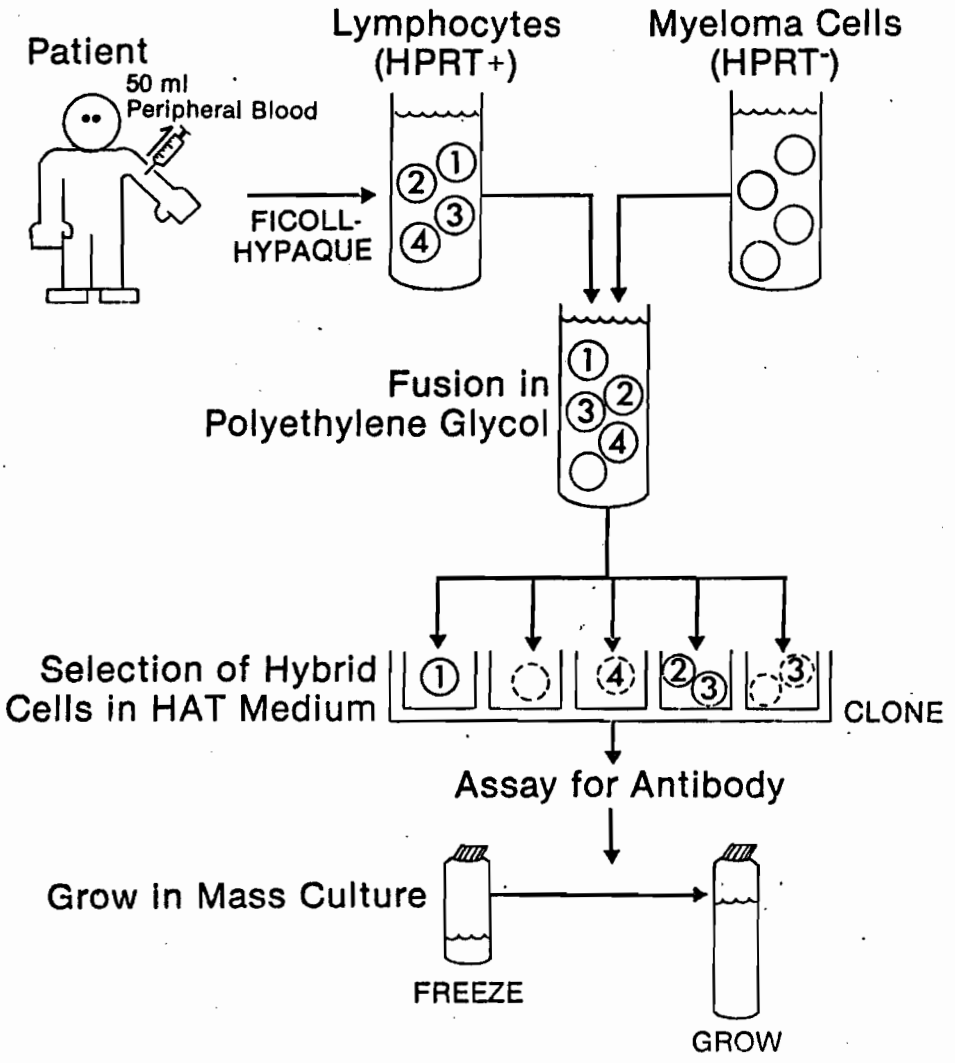
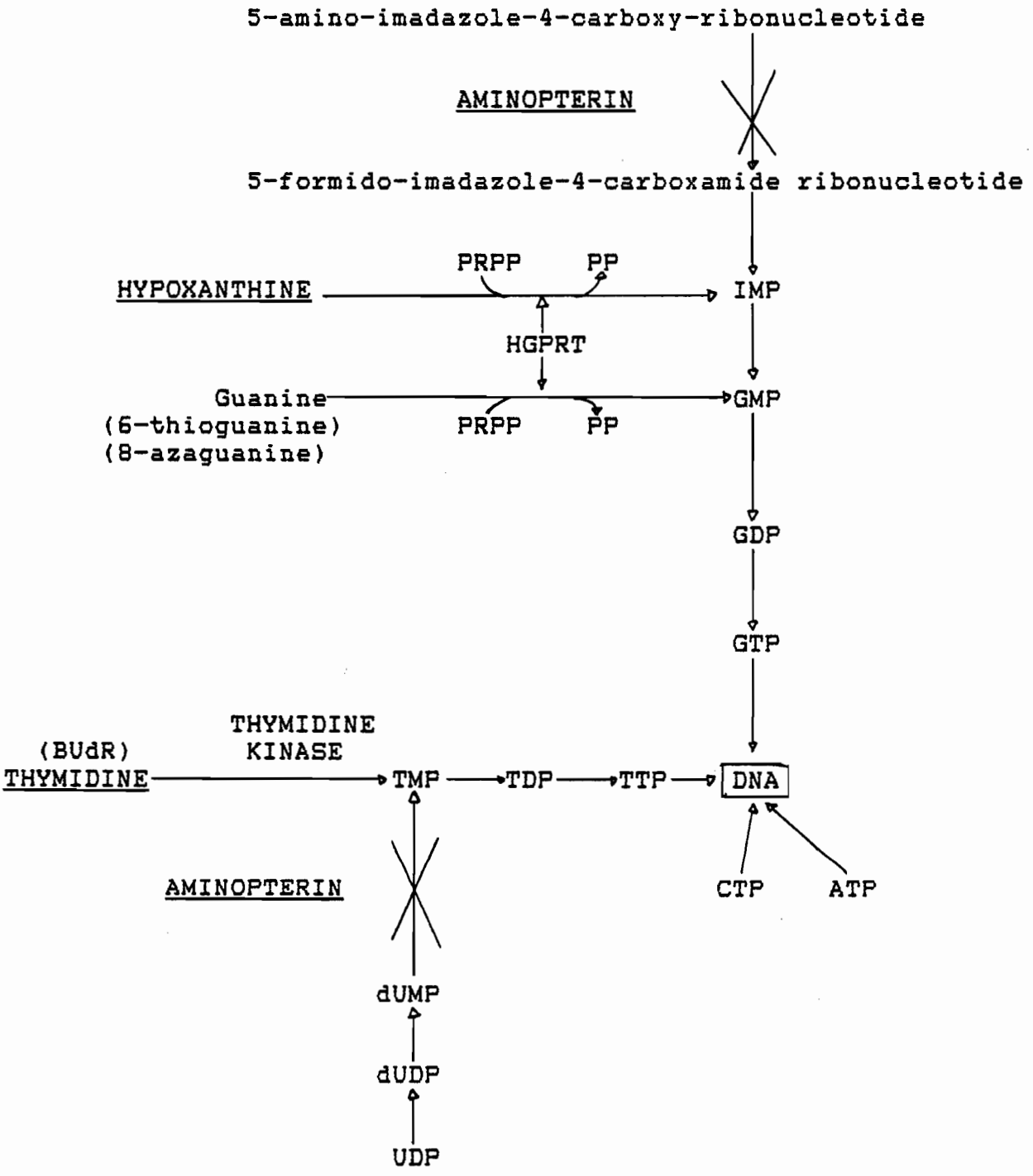
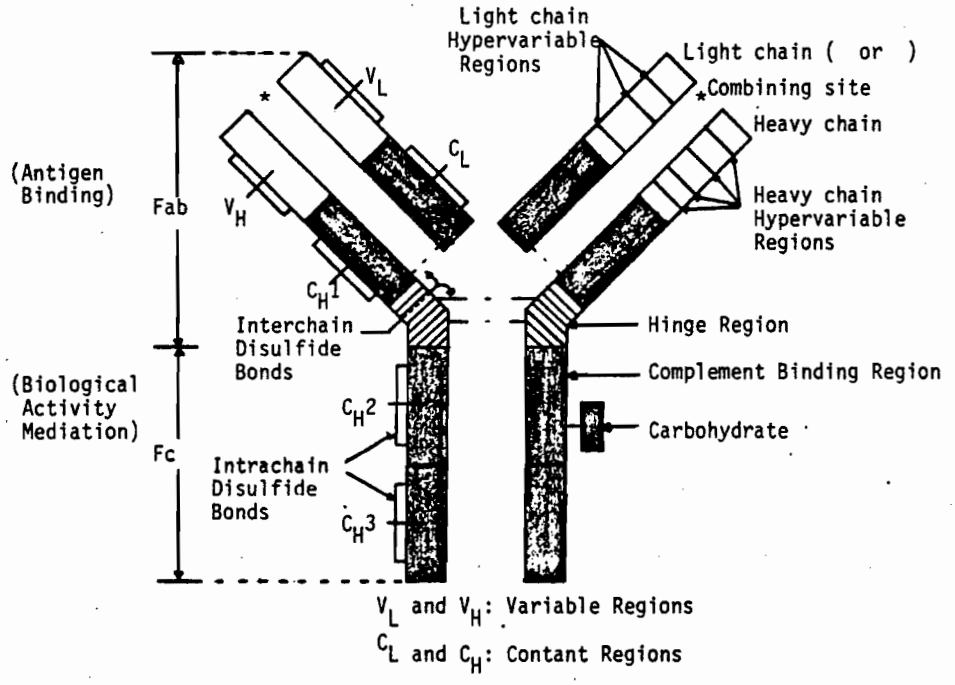


FIGURE 3. MAIN AND SALVAGE BIOSYNTHETIC PATHWAYS



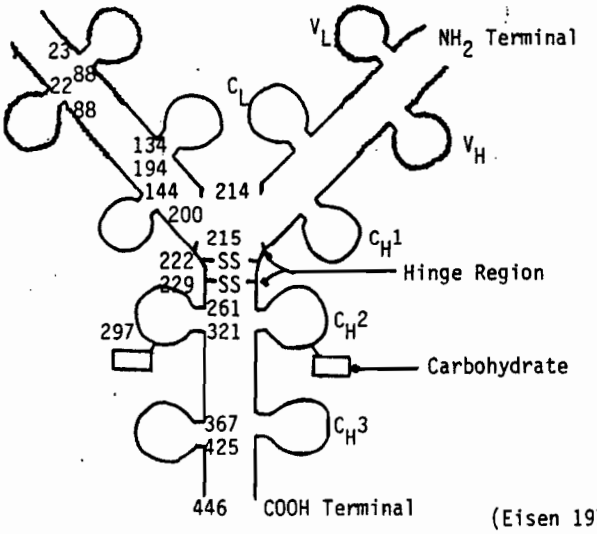
(Littlefield 1964)

FIGURE 1A. MODEL OF THE PROTOTYPICAL IMMUNOGLOBULIN MOLECULE



(Andrews 1980)

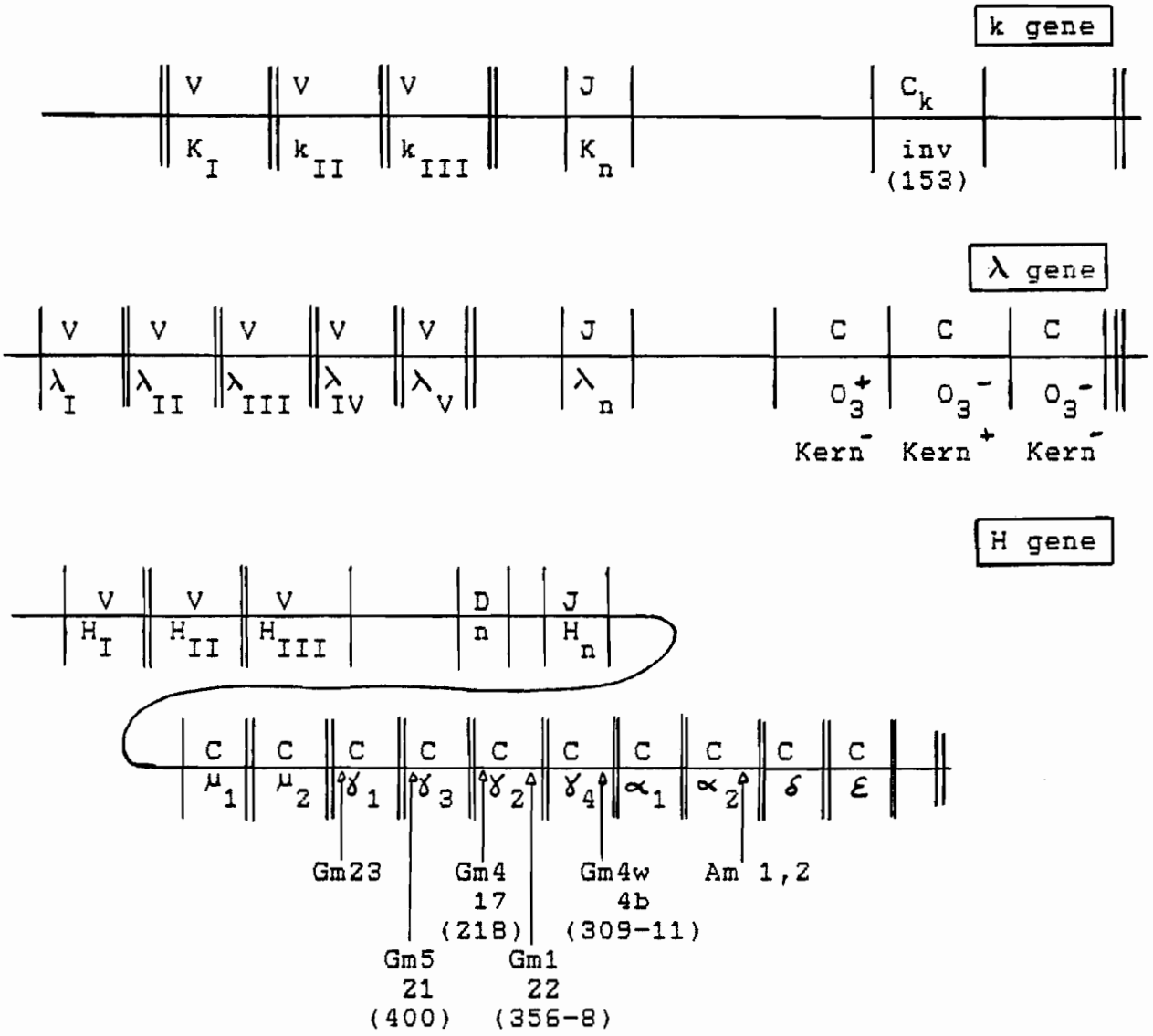
FIGURE 1B. LINEAR PERIODICITY IN AMINO ACID SEQUENCES OF IMMUNOGLOBULINS



(Eisen 1974)

FIGURE 1C

ORGANIZATION OF Ig GENES ALONG WITH ALLOTYPE SPECIFICITIES



(de Preval, 1982)

There are 3 variable regions (V), an unknown number of joining sequences (J) and one constant region (C) in the K gene that are used to produce all the L_λ chains observed. Similarly, there are 5 V regions, an unknown number of J regions and 3 C regions in the λ gene that encode for all L chains. There are 3 V regions, an unknown number of D & J regions and 10 C regions in the H gene that produce all H chains.

PART II. Material and Methods

Chapter 2. PRODUCTION OF HUMAN-HUMAN HYBRIDOMAS

2.1 GM 4672 Cell Line

The human lymphoblastoid cell line, GM 4672, originally described by Croce (1980), was obtained from the Cell Repository Institute of Medical Research, Camden, NJ. The GM 4672 cell is an IgG_2K producing mutant cell line which was selected for hypoxanthine guanosine phosphoribosyl transferase deficiency. This allows the cell line to be sensitive to the hypoxanthine-aminopterin-thymidine (HAT) medium in which the cells cannot survive. The GM 4672 cells were grown in 10 ml of GM 4672 medium periodically (every 2-3 passages) supplemented with 20 µg/ml of 6-thioguanine in order to prevent revertants. This GM 4672 medium was made up of RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10 mM Hepes, 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 10% fetal bovine serum (FBS), 0.2% gentamicin, 2.5 µg/ml fungizone and 0.5% non-essential amino-acids.

Four days prior to fusion, the GM 4672 cell line was staged up by distributing the 10 ml GM 4672 flask into four 75 cm^2 flasks (1.5 ml per flask, Corning Glass Works, Corning, NY. 14830) already containing 20 ml of GM 4672 medium without 6-thioguanine. The volume of these flasks was doubled 2 days later, resulting in 10^7-10^8 myeloma cells in the log phase. On the day of fusion, the cells were washed twice

with 10 ml of SFM before mixing with the lymphocytes.

2.2 Human Fusion Procedure

Heparinized (Hepararin Sodium, Allen and Hanburys, Montreal) venous blood (50 ml) from each of 18 SLE patients who satisfied the revised ARA criteria for the classification of SLE (Tan 1982) was diluted 1:1 with serum-free RPMI 1640 medium, supplemented with 10 mM Hepes, 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 1% Na pyruvate, 0.2% gentamicin and 0.5% non-essential amino-acids (SFM). The lymphocytes were isolated on a Ficoll-Hypaque density gradient (Ficoll-Paque, Pharmacia). 18 ml of Ficoll-Hypaque solution was overlayed with 25 ml of diluted blood and centrifuged at room temperature for 20 minutes at 400g. The upper part of the Ficoll-Hypaque density gradient, the plasma, was kept at -20°C for further tests. The lymphocytes obtained from the interphase layer were washed twice in SFM.

$2x10^7$ lymphocytes were fused with $0.4X10^7$ or $0.2x10^7$ GM 4672 cells to give cell ratios of 5:1 or 1:1 respectively, in the presence of polyethylene glycol (PEG) 1540 (molecular weight 1300-1600, J.T. Baker Chemical Co., Phillipsburg, NJ.) (Gefter 1977). Lymphocytes and GM 4672 cells were copelleted by centrifugation at 300g for 10 minutes. The cell pellet was resuspended in 0.5 ml of PEG 44% (w/w in SFM) which was centrifuged for 3 minutes at 300g, followed immediately by the addition of 10 ml of SFM and by a centrifugation at 300g for 10 minutes. The cells were resuspended in 40 ml of post-recovery fusion medium. The cell suspension was then divided

into two 25 cm^2 tissue culture flasks and incubated for 24 hours (37°C, 5% CO_2). The post-recovery fusion medium was made up of RPMI 1640 medium supplemented with 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 1% Na pyruvate, 15% FBS and 0.2% gentamicin.

The following day, the cells were pelleted and resuspended in hypoxanthine-aminopterin-thymidine (HAT) medium (Littlefield 1964) (RPMI 1640 medium supplemented with 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 100 mM Na pyruvate, 0.5% non-essential amino-acids, 15% FBS, 0.2% gentamicin, 10^{-4} M hypoxanthine, $1.6x10^{-5}$ M thymidine, $4x10^{-7}$ M aminopterin, $2.9x10^{-6}$ M glycine, 10mM Hepes, 2.5 µg/ml fungizone and 5% NCTC 135 (Gibco)). Equal numbers of cells were plated at 3 cell concentrations ($1x10^5$, $2x10^5$ and $4x10^5$ cells per well (cells/well)). The first 2 concentrations ($1x10^5$ and $2x10^5$ cells/well) were seeded in 0.3 ml of HAT into 0.3 ml wells of 96-well tissue culture plates (Costar, Data Packaging, Cambridge, MA), while $4x10^5$ cells/well were plated in 1.0 ml of HAT into 2.0 ml well of 24-well tissue culture plates (Costar), using only the inner wells of every plate. The peripheral wells were filled with sterile distilled water because of the rapid evaporation in those wells.

The HAT medium was replaced every 5 days by removing half of it by suction and adding back fresh medium. When hybridomas were observed macroscopically (generally 4-6 weeks after fusions), feeding was continued with

GM-to-HAT medium, which is the HAT medium without hypoxanthine-aminopterin-thymidine, sodium pyruvate and with 10% FBS. One to two weeks later, the supernatants were tested for antibody production. When hybridoma clones covered the wells and the cells were confluent, the clones were staged up in GM-to-HAT medium in the following sequences: from a 0.3 ml well to a 2 ml well to a 25 cm^2 flask and, finally, to a 75 cm^2 flask (Corning), at dilutions of 1/3, 1,10 and 1/2, respectively.

2.3 Cloning Procedure

The hybridoma clones which were positive for anti-DNA antibody production were cloned by limiting dilution at a concentration of one cell per well. A cell count by the Trypan blue exclusion method (Gorer 1956) was performed on an aliquot taken from a 2 ml confluent culture well. One ml from the same tissue culture well was diluted in GM-to-HAT medium according to the cell count to yield a stock suspension of 5 cells per ml. Two hundred ul of this stock suspension was plated in each of the inner wells of 96-well microtiter plates. The peripheral wells were filled with sterile distilled water. The plates were incubated at 37°C in 5% CO_2 and fed after 3 weeks, and every 7 days thereafter, with fresh GM-to-HAT medium.

2.4 Freezing and Storage of Human Hybridoma Cells

Approximately 10^7-10^8 cells were centrifuged at 1200 rpm for 6 minutes and resuspended in 1 ml of cool-down medium (RPMI 1640 supplemented with 10 mM Hepes, 100 µg/ml penicillin-streptomycin, 50% (v/v) FBS, and 200 mM L-glutamine). The

cells were then allowed to sit on ice for at least 1 hour. One ml of serum-free freezing medium (RPMI 1640 supplemented with 10 mM Hepes, 100 µg/ml penicillin-streptomycin, and 20% (v/v) DMSO) was then added and 1 ml of the cells was distributed per freezing vial (Cryotube 38x12, Nunc, Denmark) and stored for 1-3 days at -70°C. The vials were then transferred to a liquid nitrogen freezer for long-term storage.

2.5 Thawing Procedure

The vials of frozen cells were quickly thawed in a 37°C water bath. The cells were immediately transferred to a centrifuge tube containing 10 ml of GM-to-HAT medium and centrifuged at 1200 rpm for 6 minutes. The cells were washed once, resuspended in 5 ml of GM-to-HAT medium supplemented with 15% FBS and 50 µg/ml of Endothelial Cell Growth Supplement (ECGS, Biomedical Products Division, Lexington, MA), then transferred to a 25 cm^2 flask (Corning), and incubated overnight at 37°C, 5% CO_2. The following day, the cells were centrifuged, resuspended in 1 ml of fresh GM-to-HAT medium supplemented with 50 µg/ml ECGS and 15% FBS, and then transferred into 2.0 ml wells. The cells were checked every 2-3 days thereafter, fed and staged up to larger volume as needed. As soon as the cells were staged up, ECGS was withdrawn.

Chapter 3. CHARACTERIZATION OF HUMAN HYBRIDOMAS

3.1 Screening for Antibodies to dDNA, nDNA, and Cardiolipin

Polystyrene test tubes were coated with either dDNA, nDNA or cardiolipin overnight at 4°C (see Sections 9.1, 9.2, 9.3). The tubes were washed once with 0.1M potassium phosphate buffer (KPO_4) pH 7.0, containing 0.1% bovine serum albumin (BSA, Sigma, St-Louis, MO) and 0.01% Tween 20 (0.01% Tween buffer) and incubated in the same buffer for 45 minutes at 37°C to saturate any unoccupied sites of polystyrene tubes. Seventy-five µl of hybridoma culture fluid was then added to duplicate tubes and incubated for 1 hour at 37°C. The negative control included tubes with buffer and GM 4672 supernatant, while the positive one included an SLE serum with high anti-DNA and anti-cardiolipin antibodies. After aspiration of the sample and 3 washes with the 0.01% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified sheep anti-human IgM+IgG antibodies (100,000 cpm/tube) (see Sections 9.4 and 9.6) was added and the tubes were incubated overnight at 4°C. The tubes were then washed three times with the buffer and were counted for bound ^{125}I-radioactivity for one minute in a Beckman gamma counter. Antibodies were considered to be positive for binding to a particular antigen if the cpm bound to the antigen exceeded 3 standard deviations above the mean of the negative controls.

3.2 Class Determination of Hybridoma Anti-DNA Antibodies

The culture fluids containing anti-DNA antibodies were incubated on dDNA coated tubes, as described in the screening

assay. In one set of tubes, the antibody label used was ^{125}I-rabbit-anti-human IgM (mu-chain specific, see Sections 9.6 and 9.4) while in the second set of tubes, ^{125}I-rabbit-anti-human IgG (gamma-chain specific, see Sections 9.6 and 9.4) was used.

3.3 Quantitation of Immunoglobulins

Polystyrene test tubes were coated with 200 µl of rabbit anti-human IgM or rabbit anti-human IgG at 2.5 µg/ml in 0.05M Na Borate, pH 8.6 overnight at 4°C. After the tubes had been washed once with 0.1M KPO_4 containing 0.1% BSA and 0.05% Tween 20 (0.05% Tween buffer), and incubated in the same buffer for 45 minutes at 37°C, 75 µl of dilutions of hybridoma culture fluid was added to duplicate tubes. The tubes with the samples were incubated for one hour at 37°C. After aspiration of the sample and 3 washes with the 0.05% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM or anti-human IgG was added and the tubes were incubated overnight at 4°C. The tubes were then washed three times with the buffer; the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter. The concentration of the immunoglobulins was determined from a standard curve ranging from 0.02 to 1 µg/ml. The standard curve, employing known quantities of commercially available HIgM (Cappel Laboratories, Cochranville, P.A.), was constructed for each experiment.

3.4 Inhibition of dDNA Binding of Hybridoma Anti-DNA Antibodies by Polynucleotides and Cardiolipin

Hybridoma anti-DNA culture fluids were titrated on dDNA

coated tubes to determine the dilutions which gave 50-60% of maximum binding. This corresponds to the linear portion of the binding curves. Twice this dilution was used to correct for the following 1:1 dilution of culture fluid with the competitors. One hundred µl of the dilution of anti-DNA antibodies was preincubated in a small test glass tube at 37°C for 1 hour, with 100 µl of competitors which have similarities with dDNA. Seventy-five µl of the mixture was then added to duplicate dDNA coated tubes which had been washed once with 0.01% Tween buffer and incubated in the same buffer for 45 minutes at 37°C. This mixture was incubated on the dDNA coated tubes for 1 hour at 37°C and then washed three times with 0.01% Tween buffer. One hundred µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM was added and the tubes were incubated overnight at 4°C. The tubes were then washed three times with the buffer and the remaining ^{125}I-radioactivity was counted for 1 minute in a Beckman gamma counter. The results were presented as % binding, which was calculated as the ratio of cpm bound observed in the presence of competitors over the cpm bound in the absence of competitors times 100. The competitors used were polynucleotides such as polydeoxythymidylic acid (poly (dT), P.L. Biochemical Inc., Milwaukee, Wis.), polyinosinic acid (poly (I), Collaborative Research, Waltham, Ma), yeast RNA (Sigma), nDNA as well as dDNA itself and a left-handed form of DNA, called zDNA (brominated poly (dG-dC), a gracious gift of Dr. B.D. Stollar, Boston) and the phospholipid, cardiolipin.

3.5 Screening for Rheumatoid Factor Activity

Polystyrene test tubes were coated with 200 µl of a 30 µg/ml solution of human IgG (purified from human Cohn fraction II (Sigma) by chromatography on DEAE cellulose in 0.01M sodium phosphate buffer, pH 8.0) (Reif 1969) in 0.025M TBS overnight at 4°C. Tubes coated with 30 µg/ml BSA in TBS served as controls for non-specific hybridoma antibody binding. After the tubes had been washed once with 0.01% Tween buffer, incubated in the same buffer for 1 hour at room temperature and had received a second wash, 75 µl of hybridoma culture fluid was added to duplicate tubes and incubated overnight at 4°C. The following day, after aspiration of the sample and 3 washes with 0.01% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM (100,000 cpm/tube) was added and the tubes incubated overnight at 4°C. The tubes were washed three times with the buffer and the remaining ^{125}I-radioactivity was counted for 1 minute in a Beckman counter. To further investigate the specificity of the rheumatoid factor activity, hybridoma supernatants were incubated on tubes coated with Fc and Fab fragments purified from papain digested DEAE purified human IgG (Section 9.8). The assay was performed exactly as described above for the radioimmunoassay on human IgG coated tubes. A monoclonal rheumatoid factor isolated from serum was kindly provided by Dr. Jack Karsh (Ottawa General Hospital) and served as the positive control in all assays.

3.6 Screening for Prolonged Partial Thromboplastin Time

Fifty µl of culture fluid was diluted with an equal

volume of freshly reconstituted Verify Normal Citrate (General Diagnostics, N.J.) which contains fresh normal human plasma enriched with Factors I, V, VIII of bovine origin. In the meantime fresh Thrombofax (activated, Ortho Diagnostic, Baritan, NJ.) diluted 1:64 in 0.025M TBS (25mM tris, 140mM NaCl) pH 7.4 and 0.025M calcium chloride dihydrate ($CaCl_2$, Fisher) were both preincubated at 37°C for at least 5 minutes. Thrombofax contains phospholipids for optimal platelet-like activity and a plasma activator for optimal activation of the contact phase of coagulation. The whole reaction was done in a Haake circulating water bath at 37°C. After the diluted culture fluid had been warmed at 37°C for a few seconds, 0.1 ml of Thrombofax was added. Four minutes later, 0.1 ml of Kaolin (2 mg/ml in 0.1M Tris pH 7.6, Fisher), the particulate surface on which the coagulation would proceed, was added. The sample was then shaken 3 times at two-minute intervals. Six minutes after the Kaolin, 0.1 ml of $CaCl_2$ was added and a stopwatch was started to read the time of coagulation by the tilt tube technique, in which the tube was shaken briefly to mix the contents and kept at 37°C for 25 to 30 seconds; it was then removed from the water bath and gently titled back and forth until a gel clot formed. The watch was immediately stopped and the time of coagulation recorded. A positive prolonged partial thromboplastin time activity was recorded when the time of coagulation of sample was 5 times greater than the time of coagulation of the control GM 4672

supernatant.

3.7 Purification of Hybridoma Anti-DNA Antibodies

Anti-DNA hybridoma antibodies were affinity-purified on a rabbit-anti-human-IgM-Sepharose column (Section 9.5). At least 300 ml of hybridoma culture fluid was passed over the column at room temperature. The column was then washed with PBS (phosphate buffered saline: 0.9% NaCl, 0.02M potassium phosphate pH 7.3) until the OD_{280} was 0. The antibody was eluted from the column with 0.1M glycine-HCl pH 2.3. All tubes containing protein, as determined by OD_{280}, were pooled and dialyzed against PBS overnight at 4°C. The purified antibody was distributed in a volume of 2-3 ml and stored at -20°C.

Chapter 4. MONOCLONALITY

4.1 ^{14}C-Leucine Incorporation into Hybridoma Cells

Ten ml of a cell suspension was centrifuged (400 g, 5 minutes) and resuspended in fresh GM-to-HAT medium, 12 hours prior to the biolabelling day, in order to have the cells in log phase. The cells were centrifuged, resuspended in 1.5 ml of RPMI 1640 medium without leucine (Gibco), supplemented with 200 mM L-glutamine and 100 µg/ml of penicillin-streptomycin (named RPMI-leu medium), and transferred to a freezing vial. The cells were centrifuged again, resuspended in 1.0 ml of RPMI-leu medium supplemented with 10% FBS, and then incubated for 2 hours at 37°C with 5% CO_2 to get rid of leucine contained in the cells. Then the cells were centrifuged, resuspended in a 1 ml of ^{14}C-leucine RPMI medium (20 µl of 1 mCi ^{14}C-leucine (Amersham-Searle) plus 80 µl of RPMI-leu medium supplemented with 10% FBS), and incubated 8 hours at 37°C, 5% CO_2. One hundred ul of RPMI 1640 medium supplemented with 200 mM L-glutamine, 100 µg/ml of penicillin-streptomycin and 10% FBS was then added to the cells, which were incubated for an additional 12-hour period.

The following day, the cells were centrifuged, and the supernatant collected. The cells were resuspended in 1.0 ml PBS, centrifuged and the supernatant collected and mixed with the previous one. The immunoglobulins were precipitated from the cell's supernatant by the addition of an equal volume (0.3 ml) of 3.5M ammonium sulfate ($(NH_4)_2SO_4$) pH 7.3 and incubation on ice for 30 minutes. After centrifugation at 1680

g for 15 minutes, the precipitate was redissolved in 0.2 ml of PBS and the precipitation repeated. The pellet was finally dissolved in 0.2 ml of freshly de-ionized 8M urea-0.2M Tris buffer, pH 8.2 (Fisher). The de-ionization was done by passage of the urea buffer through a column of Amberlite resin (BDH Chemicals, Montreal).

4.2 Complete Reduction and Alkylation of Immunoglobulins

Complete reduction of the ^{14}C-biolabelled immunoglobulins (Section 4.1) was performed at 37°C for 30 minutes by the addition of 20 µl of dithiothreitol (DTT) (15 mg/ml in urea buffer) (Boehringer Mannheim, W. Germany). Alkylation was then conducted at 37°C for 30 minutes by the addition of 20 µl of iodoacetamide (40 mg/ml) (Sigma). The reaction was finally terminated by the addition of 20 µl of DTT, and the proteins were precipitated free of urea and reagents by the addition of 0.52 ml of methanol and by incubation at -20°C for 30 minutes. After centrifugation at 1680 g for 15 minutes, the precipitate was dissolved in 20 µl of sample buffer for urea formate gel (0.01M NaOH, 2.2% formic acid, 8M urea, 5% glycerol, a small amount of methyl green for colour) pH between 2.8 and 3.5.

4.3 Urea Formate Gel Electrophoresis

Heavy and light chains from completely reduced and alkylated immunoglobulins (Section 4.2) were separated in a urea formate gel. The gel was prepared as follows: a) 14.4 g urea, 7.5 ml 30% acrylamide (Fisher)-0.8%N,N'-Methylene bisacrylamide (Bis) (Eastman), 3 ml buffer 10x (0.1M NaOH, 22%

formic acid, pH 3.0) and 8.4 ml dH_2O were mixed and stirred on a hot plate until completely dissolved; b) the mixture was filtered on 1M paper (Eastman) and degassed 5 minutes; c) 30 mg ammonium persulfate (BioRad Laboratories, California) and 60 µl N,N,N',N'-tetramethylethylenediamine (TEMED) (BioRad) were added to polymerize the gel. Electrophoresis was performed using a Bio-Rad slab gel system Model 220 with 20 samples per slab. The power supply used was an LKB 2197. Electrophoresis was performed at 170V for 17 hours with a pre-run at 150V for 2 hours. Heavy and light chains were localized by staining a guide strip with boiling hot Coomassie blue stain (0.25% w/v Coomassie brillant blue R-250 from Bio-Rad in 45% methanol, 10% acetic acid), and destaining in 7.5% acetic acid and 25% methanol for half an hour.

4.4 Isoelectric Focusing Electrophoresis

Acrylamide gel was prepared by mixing 45 ml of freshly de-ionized 8M urea-0.2M tris, 3 ml of ampholines pH 3.5-10 (LKB Instruments, Rockville, MD) and 0.4 ml of freshly made riboflavin (4 mg/100ml, Sigma). Acrylamide gel isoelectric focusing was conducted at 10°C using an LKB 2217 Ultrophor electrophocusing unit, an LKB 2197 power supply, and an Haake G cooling system. The zone containing the light chain of the urea formate gel (Section 4.3) was placed directly on the surface of the isoelectric focusing gel at the cathode end. The entire surface of the isoelectric focusing gel was then covered with a layer of melted petrolatum (Fisher), to prevent drying of the acrylamide gel strip. The focusing

was done over the length of the gel. Electrophoresis was performed at 1300V for 5 to 6 hours, for a total of 6200 volt-hours (volt x hour). The petrolatum was removed and kept for further use. The gel was then fixed and stained with TCA-Coomassie (0.005% (w/v) Coomassie blue in 20% (w/v) Trichloroacetic acid (TCA, Fisher)) at 60°C for 15-30 minutes followed by a transfer to the first water wash bath (500 ml, overnight). The first wash was discarded and the gel was then washed with several successive changes of water at 20 minute intervals, until the petrolatum was completely removed. Finally, the gel was soaked in 1.0% glycerol for 30 minutes and transferred to a sheet of Whatman No. 1 paper and to a BioRad gel slab dryer model 224 for drying. Saran wrap was placed over the gel to prevent the gel from sticking to the dryer apparatus. The gel was dried for 4 hours at 160-165°C. The dried gel was then placed against Kodak XOMAT film and was autoradiographed for varying periods of time.

Chapter 5. PRODUCTION AND CHARACTERIZATION OF RABBIT ANTI-IDIOTYPE ANTISERA

5.1 Immunization Procedure

Anti-idiotype antibodies were raised against 8 hybridoma anti-DNA antibodies: 100-1, 103-1, 134, 600, 604, 1206, 1305,and 1400. Purified anti-DNA antibodies (Section 3.7) were injected into New Zealand white rabbits (50 µg of antibody per rabbit). The first injection was done according to the multiple intradermal injection method of Vaitakaitis (1971). For the first injection, the antibody was mixed with an equal volume of complete Freund's adjuvant (Difco). Thereafter, the immunizations were given in incomplete Freund's adjuvant (Difco) and injected intramuscularly into the rabbits' hind limbs. The rabbits were bled by venipuncture of the ears ten days after the third booster (4th injection) and thereafter, 10 days after each booster.

5.2 Absorption and Specificity of Antisera

The rabbit antisera were rendered idiotype-specific by extensive absorptions on a pooled normal human IgM-Sepharose column and on a human IgG (from the GM 4672 cell line) Sepharose column (Section 9.5). The specificity of the antisera was tested by direct binding of the sera to HIgM (chromatographically purified from Cappel), HIgG (purified from the GM 4672 cell line), and idiotype coated tubes. Polystyrene tubes were coated with 200 ul of either HIgM, HIgG or idiotype (purified hybridoma antibody) at 2.5 µg/ml in 0.05M Na Borate, pH 8.6 overnight at 4°C. Seventy five µl of diluted absorbed sera (1/20, 1/40,

1/80) was incubated in duplicate on each type of coated tubes for 1 hour at 37°C, after these tubes had been preincubated with 0.05% Tween buffer for 45 minutes at 37°C. The contents of the tubes were then aspirated and the tubes washed three times with 0.05% Tween buffer. One hundred µl of ^{125}I-radiolabelled affinity-purified goat anti-rabbit IgG (Section 9.6 and 9.4) was added and the tubes were incubated overnight at 4°C. The tubes were washed three times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter. Normal rabbit serum was absorbed in the same manner as the anti-idiotype sera and was used as a negative control. The sera were defined to be idiotype-specific when their binding to HIgM and HIgG coated tubes was less than or equal to the binding absorbed normal rabbit sera, while their binding to their idiotype coated tubes still remained high.

5.3 Inhibition of dDNA Binding of Anti-DNA Antibodies by Anti-Idiotype Antibodies

Hybridoma anti-DNA culture fluids were titrated on dDNA coated tubes (0.5 µg/ml) to determine the dilution which gave 50-60% of maximum binding. Twice that dilution was used to correct for the following 1:1 dilution of culture fluid by anti-idiotype sera. One hundred µl of the selected dilution of anti-DNA antibodies was incubated with 100 µl of varying dilutions of anti-idiotype sera, ranging from 0.0008 to 0.1 mg/ml, according to OD_{280}, in a small test glass tube at 37°C for 1 hour. Seventy-five µl of the mixture was then added to

duplicate dDNA coated tubes, which had been washed once with 0.01% Tween buffer and incubated in the same buffer for 45 minutes at 37°C. This mixture was incubated on dDNA coated tubes for 1 hour at 37°C. The tubes were then washed 3 times with 0.01% Tween buffer; and 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM was added and the tubes were incubated overnight at 4°C. The tubes were then washed 3 times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter. The results were presented as % binding, which was calculated as the ratio of cpm bound observed in the presence of anti-idiotype sera over the cpm bound in the presence of absorbed normal rabbit serum diluted at the same concentration of anti-idiotype sera times 100.

5.4 dDNA Binding of Anti-Idiotype Antisera

Seventy-five µl of dilutions of anti-idiotype antibody or normal rabbit sera, varying from 0.0125 mg/ml to 0.1 mg/ml according to OD_{280}, was added to dDNA coated tubes which had been washed once with 0.01% Tween buffer and preincubated with the buffer for 45 minutes at 37°C. The samples were incubated on the tubes for 1 hour at 37°C. After the tubes had been washed three times, 100 µl of ^{125}I-radiolabelled affinity-purified goat anti-rabbit IgG was added and the tubes were incubated overnight at 4°C. The remaining ^{125}I-radioactivity was counted after the tubes had been washed three times with 0.01% Tween buffer.

5.5 Effect of the dDNA Binding of Anti-134 Antiserum on the dDNA Binding of Clone 134

Seventy-five µl of dilutions of anti-idiotype antibody or normal rabbit sera, varying from 0.007 mg/ml to 0.1 mg/ml according to OD_{280} or 134 culture fluid, was first added to dDNA coated tubes which had been washed once with 0.01% Tween buffer and preincubated with the buffer for 45 minutes at 37°C. These samples were allowed to incubate on the tubes for 1 hour at 37°C. The tubes were then washed three times and 75 µl of culture fluid or anti-idiotype antibody or normal rabbit sera, at the same concentration as mentioned earlier, was added to the tubes in a manner to have 134 antibody/sera and sera/134 antibody sequences on the tubes; these tubes were incubated for 1 hour at 37°C. After 3 washes, 100 µl of ^{125}I-radiolabelled rabbit anti-human IgM was added and incubated on the tubes overnight at 4°C. The remaining ^{125}I-radioactivity was counted after three washes of the tubes with the buffer.

5.6 Inhibition of Binding of Anti-DNA Antibodies to their Anti-Idiotype Antibodies by Polynucleotides, Cardiolipin, or Anti-Idiotype Antibodies

Polystyrene test tubes were coated with 200 µl of 1/3 serial dilutions from 1/100 to 1/243000 of anti-idiotype antibodies in 0.05M Na Borate pH 8.6, overnight at 4°C, to choose the best coating concentration of anti-idiotypes. After the tubes had been washed once with 0.05% Tween buffer and incubated in the same buffer for 45 minutes at 37°C, 75 µl of a 1/2 dilution of idiotype was added and incubated for 1 hour at

37°C. After aspiration of the sample and 3 washes with 0.05% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM was added and the tubes were incubated overnight at 4°C. The tubes were then washed 3 times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter. The best concentration of anti-idiotype coated tubes which gave maximum binding of the idiotype was chosen for the inhibition assay. The idiotype was then titrated on these anti-idiotype coated tubes to choose the dilution which gave 50-60% of maximum binding. Twice that dilution was used to account for the following 1:1 dilution of the idiotype with polynucleotides or with the anti-idiotype antibodies. One hundred ul of the idiotype was preincubated in a small test glass tube at 37°C for 1 hour, with 100 µl of either polynucleotides or anti-idiotype antibodies (ranging from 0.0007 mg/ml to 0.1 mg/ml). Seventy-five µl of the mixture was then added in duplicate to the anti-idiotype coated tubes which had been washed once with 0.05% Tween buffer and incubated in the same buffer for 45 minutes at 37°C. The rest of the experiment was the same as previously described for the inhibition assays with ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM. The results were presented as % binding, which was calculated as the ratio of cpm bound in the presence of polynucleotides or anti-idiotype over the cpm bound in the absence of polynucleotides for the former, and the presence of equivalent concentrations of normal rabbit serum, for the

latter times 100.

Chapter 6. ANALYSIS OF A PANEL OF HYBRIDOMA ANTIBODIES FOR DOMINANT CROSS-REACTIVE IDIOTYPES

6.1 Inhibition of Binding of Anti-Idiotype Antibodies to their Homologous Idiotype by Anti-DNA Antibodies

Polystyrene test tubes were coated overnight at 4°C with 200 µl of dilutions of idiotype in 0.05 Na Borate pH 8.6, ranging from 0.25 µg/ml to 2.5 µg/ml, to choose the best coating concentration of idiotype. After the tubes had been washed once with 0.05% Tween buffer and incubated in the same buffer for 45 minutes at 37°C, 75 µl of a 1/200 dilution of anti-idiotype serum was added and incubated for 1 hour at 37°C. After aspiration of the sample and three washes with 0.05% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified goat anti-rabbit IgG was added and the tubes were incubated overnight at 4°C. The tubes were then washed three times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter. The lowest concentration of idiotype which still gave good binding was chosen for the inhibition assay. The anti-idiotype serum was then titrated on these coated tubes to choose the dilution which gave 50-60% of maximum binding. Twice that dilution was used to account for the following 1:1 dilution of anti-idiotype sera with the antibodies. One hundred µl of that dilution of anti-idiotype sera was preincubated in a small test glass tube at 37°C for 1 hour with 100 µl of purified antibodies, ranging from 0.001 µg/100 µl to 1 µg/100 µl. A panel of 18 different purified antibodies was studied.

Seventy-five µl of the mixture was then added in duplicate to the idiotype coated tubes, which had been washed once with 0.05% Tween buffer and incubated in the same buffer for 45 minutes at 37°C. The rest of the procedure was the same as that of the previously described inhibition assays, with the exception of the use of ^{125}I-radiolabelled affinity-purified goat anti-rabbit IgG in this assay. The results were presented as % binding, which was calculated as the ratio of cpm bound observed in the presence of antibodies over the cpm bound in the absence of antibodies times 100.

6.2 Direct Binding of Hybridoma Antibodies or Sera to Anti-Idiotype Antibodies

Polystyrene test tubes were coated with 200 µl of dilutions of anti-idiotype antisera in 0.05M Na Borate pH 8.6 overnight at 4°C. Dilutions were 1:4000 for anti-134 antiserum (1.4 µg/tube), 1:4000 for anti-103-1 antiserum (1.06 µg/tube), 1:4000 for anti-600 antiserum (2.35 µg/tube), 1:2700 for anti-604 antiserum (0.79 µg/tube), 1:24300 for anti-1206 antiserum (0.12 µg/tube), 1:2700 for anti-1305 antiserum (0.78 µg/tube), 1:4000 for anti-100-1 antiserum (0.35 µg/tube), and 1:900 for anti-1400 antiserum (0.3 µg/tube). As control, normal rabbit serum similarly absorbed was used at a dilution of 1:4200 (1.32 µg/tube). After the tubes had been washed once with 0.05% Tween buffer and incubated in the same buffer for 45 minutes at 37°C, 75 µl of anti-DNA culture fluid or serum diluted 1/100 with 0.05% Tween buffer was added and incubated for 1 hour at 37°C. After

aspiration of the sample and three washes with 0.05% Tween buffer, 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-human IgM or of ^{125}I-radiolabelled affinity-purified sheep anti-human IgG+IgM was added and the tubes were incubated overnight at 4°C. The tubes were washed three times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter.

CHAPTER 7. PRODUCTION OF MOUSE ANTI-IDIOTYPE HYBRIDOMAS

7.1 Sp2/0 Cell Line

The mouse plasmacytoma cell line, Sp2/0-Ag-14 (Sp2/0), was grown and maintained in tissue culture in 10 ml of Sp2/0 medium (DMEM-H21 medium (Gibco), supplemented with 10 mM Hepes, 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 10% FBS, 0.2% gentamicin, 0.5% non-essential amino-acids and 20 µg/ml 8-azaguanine). Four days prior to fusion, the Sp2/0 cells were staged up by distribution of the 10 ml Sp2/0 flask into four 75 cm^2 flasks containing 20 ml of Sp2/0 medium without 8-azaguanine. The volume of these flasks was doubled 2 days later to end up with 10^8-10^9 myeloma cells in the log phase. All mouse cell flasks were incubated horizontally.

7.2 MPC-11 Cell Line

The MPC-11 mouse myeloma cells were grown and maintained in tissue culture in 10 ml of MPC-11 medium (the same medium as described in Section 7.1 for the Sp2/0 medium, with a replacement of 8-azaguanine by 6-thioguanine at the same concentration). This cell line was treated similarly to the Sp2/0 cell, four days prior to fusion.

7.3 Mouse Immunization

Purified anti-DNA antibodies (Section 3.7) were injected into BALB/C mice (Jackson Laboratories, Bar Harbor) (25 µg/mouse) to raise anti-idiotype antibodies. All injections (in total volume not greater than 0.3 ml) were given intraperitoneally. Three experiments were performed. The first experiment involved two groups of four female mice.

The first group of mice received 103-1 antibody. For the first immunization, two mice received 103-1 antibody bound to Staph A, while the two other mice of the group received 103-1 antibody mixed with an equal volume of CFA. The same procedure was repeated with the second group, which received 100-1 antibody. Thereafter, the injections of the idiotype for these groups were given in saline at two week intervals. The second experiment was composed of two groups of three female mice. The first group received 103-1 antibody while the second group received 134 antibody. For the two groups, the first injection of the idiotype was done in CFA and thereafter in saline. Finally, the third experiment involved 3 groups of 3 male mice. The first group received 1400 antibody, the second 1206 antibody, and the last one 1305 antibody. CFA was used for the first injection of antibody, while IFA was used for the following ones.

Five days after the third booster (4th injection), the mice were sacrificed, their blood was collected separately, their spleens were removed, and the splenic cells of mice from one group were mixed and fused to raise hybridoma anti-idiotype antibodies.

7.4 Removing the Spleen

First, each mouse was etherized and bled by slitting the neck with a sharp razor blade. The mouse was then killed by severing the spinal cord. The mouse was placed on its back (spleen side up) on non-sterile gauze. Its body was washed (torso, side and tail) three

times with non-sterile gauze saturated with betadine. The corpse was finally placed on sterile gauze in a sterile hood and wiped twice, nose to tail (spleen side up), with two 70% alcohol swabs. With sterile scissors and forceps, a flap of mouse's skin was cut, rolled onto the forceps and peeled back over the head. The scissors and forceps were washed and resterilized (to get rid of hair) in alcohol and flamed. The mesentary was then cut and the spleen removed and placed in a sterile petri dish filled with 20 ml of warm serum-free media (DMEM-H21 medium supplemented with 10mM hepes, 200 mM L-glutamine, 100 µg/ml penicillin-streptomicin, 1% Na pyruvate and 0.2% gentamicin).

7.5 Mouse Fusion Procedure

In a sterile laminar flow tissue culture hood, the excised spleen in the petri dish (Section 7.4) was cut into small bits (all far fragments removed) using sterile scissors and forceps. The spleen bits were then minced between the frosted sides of two sterile microscopic slides in order to get all the splenic cells into suspension in SFM. The cells in suspension were transferred to a 50 ml centrifuge tube and the petri dish rinsed with fresh SFM which was added back to the centrifuge tube. The suspension was allowed to sediment for 5 minutes at RT. The supernatant, which contained the splenic cells, was carefully removed from the sediment, which contained splenic fat and debris, and put into 2 centrifuge tubes. These tubes were centrifuged for 5 minutes at RT at 250g (1000 rpm).

The supernatant was aspirated and the cells resuspended in 10 ml of TAC (tris-ammonium chloride buffer to lyse red blood cells) and incubated for 10 minutes at RT. Then, 10 ml of post-fusion recovery medium (DMEM-H21 medium supplemented with 200 mM L-glutamine, 100 µg/ml penicillin-streptomycin, 1% Na pyruvate, 15% FBS, and 0.2% gentamicin) was added and the cells were centrifuged for 5 minutes at 250g. The cells were then washed twice by resuspending the pellet with 10 ml of SFM and recentrifuging. At the second wash, the cells from the 2 centrifuge tubes were pooled. A live cell count was determined by the trypan blue exclusion method.

The Sp2/0 myeloma cells were washed twice with 10 ml of SFM. After a cell count of the myeloma cell suspension, 10^8 splenic cells were fused with 10^7 Sp2/0 cells (10:1 cell ratio) in the presence of PEG. Splenic and myeloma cells were copelleted by centrifugation at 250g for 10 minutes at RT. The cell pellet was resuspended in 0.5 ml of 44.4% (w/w in SFM) PEG, which was centrifuged at 500g for 3 minutes, followed immediately by the addition of 10 ml of SFM and by centrifugation at 300g for 5 minutes. The cell pellet was then resuspended in 40 ml of post-fusion recovery medium and transferred into 2 small flasks (25 cm^2) for an overnight incubation at 37°C in 5% CO_2.

The MPC-11 cells were centrifuged at 250g for 5 minutes and resuspended in an adequate volume of HAT medium (DMEM-H21 medium supplemented as the HAT medium used

for human cell fusions (Section 3.2), but without NCTC 135) to give a suspension of $5x10^4$ cells per ml. One hundred µl of this suspension of MPC-11 cells were plated in the inner wells of 96 well tissue culture plates to serve as a feeder layer for the fused cells. The peripheral wells were filled with dH_2O. These plates were incubated overnight at 37°C in 5% co_2.

The following day, the fused cells were centrifuged and resuspended in an appropriate volume of HAT medium to give a cell suspension of $3.3x10^6$ cells per ml. Two hundred µl of this suspension was distributed to each well which already contained the feeder layer (MPC-11). The plates were then incubated at 37°C in 5% CO_2 to allow the hybridomas to grow. The HAT medium of these plates was replaced every five days by fresh media.

When hybridomas were observed macroscopically (generally 2 weeks after the fusion), feeding was continued with Hy Pass Medium (DMEM-H21 medium supplemented with 200 mM L-glutamine, 15% FBS, 0.5% non-essential amino-acids, 100 µg/ml penicillin-streptomycin, 0.2% gentamicin, 10 mM Hepes and 2.5 µg/ml fungizone). As the cells became confluent and the clones covered the well, the clones were staged up (scaled up) in Hy Pass Medium in the following sequence: from a 0.3 ml well to a 2 ml well to a 25 cm^2 flask, and finally to a 75 cm^2 flask, at dilutions of 1/3, 1/10 and 1/2 respectively. Each hybridoma culture fluid was tested for anti-idiotype antibody production (Section 7.6) just before their staging up to the 25 cm^2 flask.

7.6 Screening for Anti-Idiotype Hybridomas

Polystyrene test tubes were coated with 200 µl of a 2.5 µg/ml of either HIgM, HIgG or the idiotype used as immunogen in 0.05M Na Borate, pH 8.6, overnight at 4°C. After the tubes had been washed once with KPO_4-FBS buffer (0.1M KPO_4, 0.5% FBS, heat-inactivated at 56°C for 30 minutes, 0.2% NaN_3) and incubated in the same buffer for 45 minutes at 37°C, 75 µl of neat hybridoma culture fluid was added and the tubes incubated for 1 hour at 37°C. As control, 75 µl of neat culture fluid of MPC-11 or Sp2/0 was used. After aspiration of the sample and 3 washes with KPO_4-FBS buffer, 100 µl of ^{125}I-radiolabelled affinity-purified rabbit anti-mouse immunoglobulin was added and the tubes incubated overnight at 4°C. The tubes were then washed 3 times with the buffer and the remaining ^{125}I-radioactivity was counted in a Beckman gamma counter for 1 minute.

7.7 Cloning Procedure

The mouse hybridoma clones which were positive for anti-idiotype antibody production were cloned by limiting dilution at a concentration of one cell per well, as described for the human hybridoma cloning procedure (Section 2.3), but with the use of Hy Pass Medium instead of the GM-to-HAT medium.

7.8 Freezing and Storage of Mouse Hybridoma Cells

The same procedure as described in Section 2.4 for the human clones was used, except that Hy Pass medium was used in place of GM-to-HAT medium.

7.9 Thawing Procedure

Frozen mouse hybridoma cells were thawed similarly to the technique used for frozen human cells, except that Hy Pass medium was used and there was no addition of ECGS (Section 2.5).

CHAPTER 8. CHARACTERISTICS OF SLE PATIENTS

8.1 Patient Selection

No criteria were established prior to selecting the SLE patients fused. They were patients regularly seen in the lupus clinic of the Montreal General Hospital. Most of them were picked because of a recent severe exacerbated period of their illness according to their physician, whatever the current manifestation of the disease was. Both the physician and the patient had to consent to the withdrawal of the blood.

Four patients were specifically selected for fusion. The lymphocytes of these patients were used in fusions 6, 8, 14, and 26, respectively. Two were chosen because they were male SLE patients, while the other 2 had a history of positive VDRL and PTT tests, respectively. These two tests are frequently associated with circulating anticoagulant. The purpose of the selection of these four patients was to see, first, if anti-DNA antibodies from males and females were similar, and second, if hybridoma anticoagulant antibodies could be obtained.

8.2 SLE Serum Screening

The sera of SLE patients fused and of the others seen weekly in the lupus clinic of the Montreal General Hospital, as well as some sera of RA patients and normal individuals, were screened by RIA for anti-DNA and anti-cardiolipin activities. The procedure used was the same described in Section 3.1, with the following specifications: each serum was tested at three

dilutions (1/100, 1/400, and 1/1600) with the use of 0.05% Tween buffer. The first dilution of each serum (1/100) was heat-inactivated at 56°C for 30 minutes and then used to make the following dilutions (1/400, 1/1600). As controls, pooled normal human sera from persons in the laboratory and a high anti-DNA binding serum was used in each assay. To detect bound serum antibodies, ^{125}I-radiolabelled affinity-purified sheep anti-human IgG+IgM was used.

Chapter 9. MISCELLANEOUS PROCEDURES

9.1 Preparation of Native and Denatured DNA Stock Solutions

A 1 mg/ml stock solution of DNA was prepared by dissolving calf thymus DNA (Millipore Corporation, Freehold, NJ) in 0.015M saline sodium citrate buffer pH 7.0 by slowly stirring it on a magnetic stirrer for 16 hours at 4°C. Denatured DNA (dDNA) was prepared by placing the stock DNA solution in a boiling water bath for 15 minutes, followed by rapid cooling in an ice-water bath. Native DNA (nDNA) was prepared enzymatically by treating the stock DNA solution with S1 nuclease (Miles Laboratories Inc., Elkhart, IN). Ten ml of the 1 mg/ml stock solution of DNA was first dialysed overnight at 4°C against acetate buffer, pH 5 (14.8 ml of 0.2M acetic acid mixed with 35.2 ml of 0.2M Na acetate and brought up to a volume of 100 ml with dH_2O). Then, 100 µl of 0.01M $ZnCl_2$ and 50 µl of S1 nuclease (250,000 units/ml) were added and the mixture was incubated at 37°C for 45 minutes. The reaction was stopped by the addition of 1.5 ml of 0.06M KPO_4-0.03M EDTA, pH 8. The S1 nuclease digested DNA was then purified on a Sephacryl S-200 column (Pharmacia. Uppsala, Sweden) in 0.01M phosphate buffered saline pH 7.3 (PBS) at 4°C.

9.2 Preparation of Cardiolipin Stock Solution

Eighty µl of 50 mg/ml of cardiolipin (Bovine, Sigma) in ethanol was evaporated to dryness with nitrogen and then diluted to 2.0 ml with sodium saline citrate buffer pH 7.0. The solution was then vortexed and incubated at 37°C for 15 minutes. The solution was pipetted up and down and vortexed

before storage at -20°C. The final concentration was 4 mg/ml.

9.3 Coating of Polystyrene Tubes with nDNA, dDNA, and Cardiolipin

12x75 mm polystyrene tubes (Stockwell Scientific, Monterey Park, CA) were coated overnight at 4°C with 200 µl of either 2.5 µg/ml of dDNA in saline sodium citrate buffer pH 7.0, 2.5 µg/ml of nDNA in TBS (0.025M Tris, 0.14M NaCl, pH 7.4), or 2.5 µg/ml of cardiolipin in TBS. Control tubes were coated with 2.5 µg/ml of BSA in TBS to determine the non-specific binding of the hybridoma antibodies to an irrelevant antigen.

9.4 ^{125}I-Labelling of Immunoglobulins and Proteins

The immunoglobulins and proteins were labelled by the chloramine T method (McConahey, 1966). All solutions used for labelling were made in 0.05M sodium phosphate buffer pH 7.5 and stored at 4°C. The chloramine T solution was freshly prepared for each labelling experiment. A column of Sephadex G25 Coarse in 0.05M sodium phosphate buffer pH 7.5 was packed in a 10 ml disposable pipet. Fifty µl of the protein solution (1-1.5 mg/ml) to be labelled, 10 µl of potassium iodide (KI) (1.1 mg/l), 5 or 10 µl 1 mCI ^{125}I (Amersham-Searle) and 10 µl of chloramine T (9 mg/5ml) were added to a small glass tube on ice and swirled on ice for 5 minutes. Then, 10 µl of sodium metabisulfite (38 mg/5ml), 10 µl of KI (110 mg/l) and 860 µl of bovine serum albumin (BSA) (2 mg/ml) were added to the tube, and stirred for one minute on ice. The mixture was immediately applied to the Sephadex G25

column and eluted from the column with 0.05M sodium phosphate buffer pH 7.5. Twenty one-ml fractions were collected and 10 µl of each fraction were counted in a Beckman gamma counter for 0.1 minute. The peak tubes were kept at 4°C. Each label was used in concentration of 100,000 cpm per 100 µl.

9.5 Coupling of Protein to CNBr Sepharose

The amount of protein to be coupled was determined by OD_{280} and dialized overnight against a coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3) at 4°C. The amount of CNBr-Sepharose (Pharmacia) required was weighed out, based on 5 mg of protein per ml of gel and each g of gel giving 3.5 ml of swollen gel. The gel was swelled on a sintered glass funnel with 0.001M HCl using 200 ml/g of gel, and washed subsequently with 2 liters of dH_2O and 100 ml/g of gel of coupling buffer. The gel was then transferred to a 50 ml centrifuge tube (Corning) and the protein solution was added to the gel. The mixture of gel and protein was rotated continuously on an end-over-end rotator (Roto-Torque, Cole Parmer Instrument Company, Illinois) for 2 hours at room temperature, or overnight at 4°C. The tube was centrifuged, the supernatant was removed and the percentage of protein coupled was calculated from the protein remaining in the supernatant by taking an OD_{280} of the supernatant. The gel was washed once with PBS pH 7.3 and then incubated and rotated with 1M glycine pH 8.0 overnight at 4°C to block remaining active groups. The gel was then centrifuged to remove the glycine and washed twice

with PBS. The gel was packed into a column to continue the washing. The gel was washed with PBS until the OD_{280} was zero. Then, the gel was washed alternately with a 100 ml of 0.1M glycine-HCl pH 2.3, PBS, and finally with PBS with NaN_3 and stored at 4°C. After each column use, the column was washed with 3M KSCN pH 7.0 and then put back in PBS with NaN_3 and stored at 4°C.

9.6 Affinity-Purification of Anti-Immunoglobulin Reagents

9.61 Rabbit Anti-human IgM

Rabbit anti-human IgM (mu, specific; Cappel Laboratories, Cochranville, PA) was positively absorbed on Sepharose-protein A column (Pharmacia). The purified rabbit anti-human IgM was then radioiodinated (Section 9.4). For the detection of RF activity, this rabbit anti-human IgM fraction was further purified prior to labelling. It was negatively absorbed on a Sepharose DEAE purified human IgG Cohn fraction II column and on a Sepharose IgG (from the GM 4672 cell line) column.

9.62 Rabbit Anti-Human IgG

Rabbit anti-human IgG (heavy and light chains; Cappel) was positively absorbed on a Sepharose-protein A column. The purified rabbit anti-human IgG was then radioiodinated (Section 9.4).

9.63 Sheep Anti-Human IgG+IgM

Sheep anti-human immunoglobulins were positively absorbed on a Sepharose normal human IgG+IgM column and then

radioiodinated (Section 9.4).

9.64 Goat Anti-Rabbit IgG

Goats were immunized with rabbit IgG (7S) in Dr. B.D. Stollar's lab. The goat anti-rabbit immunoglobulin antisera obtained from them was affinity-purified on a sepharose normal rabbit immunoglobulin column. The purified antibody was then radioiodinated (Section 9.4).

9.65 Rabbit Anti-Mouse IgG+IgM

Rabbits were immunized with pooled mouse hybridoma immunoglobulins (IgM+IgG) in Dr. R.S. Schwartz's lab. The rabbit anti-mouse immunoglobulin antisera obtained from them was affinity-purified on a Sepharose normal mouse immunoglobulin column. The purified antibody was then radioiodinated (Section 9.4)

9.7 DEAE Cellulose Purification of IgG

The preparation for a γ-globulin fraction from serum was done by a DEAE-cellulose cut off according to Reif (1969). The dry cellulose DE52 was weighed (1.5g dry cellulose per ml of serum or 50g of preswollen cellulose per 12ml of serum), preswollen with 0.01M KPO_4 buffer, pH 8.0 in a sintered glass buchner funnel and then transferred to a beaker and allowed to equilibrate with the buffer overnight at 4°C. The next day, the buffer was aspirated and the cellulose washed once. The serum, diluted with 3 times its volume of dH_2O, was added and mixed with the cellulose at 4°C. Every 10 minutes for one hour, the mixture of cellulose-serum was stirred. Then, the supernatant was removed and put aside. The cellulose was

washed 3 times with 0.01M KPO_4 buffer pH 8.0, each time with a volume equal to a third of the wet weight of cellulose. The washes were combined with the serum effluent of the previous step. The effluent containing the γ-globulin fraction of the serum was finally concentrated by ultrafiltration and was ready for use.

9.8 Purification of Fab and Fc Fragments of Human IgG

One hundred and fifty mg of DEAE purified human IgG in 0.1M potassium phosphate buffer, pH 7.0, was digested with 1.5 mg crystallized papain (Worthington, Freehold, NJ) for 16 hours at 37°C (Stanworth, 1973). The digest was applied to a 20 ml column of Sephadex G25 in 0.1M sodium phosphate buffer, pH 7.6, in order to exchange buffers. The digested IgG was then fractionated on a 2.5x20 cm CM-Sepharose (Pharmacia) column equilibrated in 0.1M sodium phosphate buffer, pH 7.6, using a stepwise gradient elution of 0-0.4M NaCl (Stanworth, 1973). The material which was eluted prior to the NaCl gradient was pooled and concentrated, and applied to a 5.0x100 cm Sephacryl S-200 (Pharmacia) column in PBS, pH 7.3, to separate the whole molecule from digested IgG fragments. The fraction containing Fab and Fc fragments was applied to a 2.5x24 cm DEAE cellulose (Whatman, Maidstone, Kent) column in 0.1M sodium phosphate buffer, pH 8.0, and eluted with a stepwise gradient of 0 to 0.3M NaCl. The material which was eluted with 0.1M sodium phosphate buffer, pH 8.0, was pooled, concentrated and applied to a 5 ml protein A-Sepharose (Pharmacia) column. The protein A-Sepharose-bound material was eluted with 0.1M glycine-HCl,

pH 2.3, and dialyzed against 2 changes of PBS, pH 7.3, at 4°C. The Fc containing fraction was finally isolated from a rabbit anti-human IgG (Fc-specific) Sepharose immunoabsorbent column by elution with 0.1M glycine-HCl, pH 2.3, and dialyzed against PBS, pH 7.3. Purified Fab fragments were isolated from the DEAE cellulose fraction above by negative absorption on the rabbit anti-human IgG (Fc specific) Sepharose column. Isolated Fab and Fc fragment preparations were checked for purity in Ouchterlony immunodiffusion plates against rabbit anti-human-Fc and rabbit anti-human-Fab specific antisera (Cappel).

9.9 Choice of Buffer Systems for Radioimmunoassays

9.91 Effect of the Addition of Tween 20 to the RIA Buffer

Four concentrations (0, 0.01, 0.025, and 0.05%) of Tween 20 (Fisher Scientific Company, Fairlawn, NJ.) in 0.1M potassium phosphate (KPO_4) buffer, pH 7.0, containing 0.1% bovine serum albumine (BSA) (Gibco, Grand Island, NY.) were used to assess the binding of the hybridoma anti-DNA antibodies to uncoated tubes and dDNA coated tubes.

9.92 Effect of Different Buffers

Three buffers (0.1M KPO_4 buffer, pH 7.0; 0.025M Tris-buffered saline (TBS), pH 7.4; and 0.01M phosphate-buffered saline (PBS), pH 7.3), all containing 0.1% BSA and 0.01% Tween 20, were used to test the binding of the hybridoma anti-DNA antibodies to dDNA coated tubes.

9.93 Effect of PLL Pretreatment of Polystyrene tubes

Tubes coated with PLL alone or PLL+dDNA, as decribed below, at PLL concentrations of 0, 5, 25, 50 and 100 µg/ml,

were used to assess the effect of the PLL precoating concentration on the binding of hybridoma anti-DNA antibodies to PLL and PLL+dDNA coated tubes. The optimal buffer (0.1M KPO_4 -0.1% BSA -0.01% Tween 20), determined previously, was used in these experiments.

Polystyrene tubes were coated with 200 µl of poly-L-lysine (PLL) (Sigma) for 30 minutes at RT. The PLL solution was then aspirated and the tubes washed 3 times with 0.025M TBS. The tubes were incubated overnight at 4°C with TBS alone (PLL coated tubes) or dDNA or nDNA (2.5 µg/ml in TBS) (PLL plus dDNA and PLL plus nDNA coated tubes, respectively). The following morning, all tubes were washed 3 times with TBS, and a solution of poly-L-glutamic acid (Sigma) (50 µg/ml in TBS) was added for 2 hours at RT to neutralize excess positive charges from PLL pretreatment. The contents were aspirated and the tubes washed twice with TBS, and left in TBS at 4°C until used in the RIA. Uncoated tubes were left dry and untreated.

9.94 Coating of Polystyrene Tubes with ^{125}I-Radiolabelled DNA

Five µl of ^{125}-radiolabelled DNA (New England Nuclear, Boston, MA) was added to 2.5ml of TBS. Two hundred µl of this solution was added to both uncoated polystyrene tubes and PLL pretreated tubes. For coating with denatured ^{125}I-radiolablelled DNA, the same amount of ^{125}-DNA was diluted in TBS, boiled for 15 minutes, cooled in an ice-water bath, and then applied to the tubes. All tubes were coated for

16-18 hours at 4°C, washed 3 times with RIA buffer, and counted for 1 minute in a Beckman gamma counter.

9.95 Binding of Hybridoma and Serum Anti-DNA Antibodies to dDNA and nDNA Coated Tubes With and Without PLL Pretreatment

Using the optimal buffer (0.1M KPO_4 -0.1% BSA -0.01% Tween) and PLL concentration (50 µg/ml), tubes were coated with PLL alone, PLL+dDNA, PLL+nDNA, dDNA, nDNA, or left uncoated. IgM hybridoma anti-DNA antibodies 134 and 103-1 were diluted to give an equivalent concentration of IgM (as quantitated by a solid phase RIA for IgM content). Spent culture media from the GM4672 cell line and from one non-DNA binding hybridoma antibody were included at an equivalent concentration as negative controls.

To observe whether the findings with spent culture media were applicable to detection of anti-DNA antibodies in the sera of SLE patients, the following experiments were performed: sera were diluted 1/100 in buffer (0.1M KPO_4 -0.1% BSA -0.05% Tween) and heat-inactivated for 30 minutes at 56°C. A concentration of 0.05% Tween was found to be necessary for serum experiments to eliminate the high non-specific binding of sera. Sera were titrated at dilutions of 1/100, 1/400, and 1/1600 on PLL, PLL+dDNA, PLL+nDNA, dDNA, nDNA, and uncoated tubes.

PART III. Results and Analysis

Chapter 10. PRODUCTION OF HYBRIDOMAS

10.1 Fusion Results

The total number of lymphocytes obtained from SLE patients, the number of wells plated, and clones produced from the eighteen fusions, are summarized in Table 4. The wells plated and clones obtained are noted regardless of which plate, cell ratio or cell density was used.

The number of lymphocytes fused varied from 1.6×10^7 cells to 1.1×10^8 cells. A mean of 299 wells were plated per fusion. Initial growth was observed between the 5^{th} and 6^{th} week of incubation of fused cells. The earliest growth was seen as small growing clusters of cells near the periphery in some wells and by a change of colour of the medium well from red to yellow. Clones continued to show up during the following four weeks. Each clone was staged up as soon as the cells covered the bottom of the well. Usually, three months after the cell fusions, no more clones were growing and the plates were discarded.

Bacterial or yeast contamination often occurred between the 3^{rd}-7^{th} week of incubation. Fusions 2, 3, and 4 became contaminated by bacteria (Acinetobacter species) before clones arose (3 weeks after the fusion), whereas fusions 6 and 7 became contaminated by yeast (Candida species) at the time of clonal growth (6th week after fusion). The results of only 10 fusions

(fusion 1, 10, 11, 12, 13, 14, 15, 16, 19, 23) were used for determining the optimal cell ratio and cell plating density to yield the maximum number of clones (Sections 10.2 and 10.3). Three fusions (5, 8, 26) are not represented above. The myeloma cell partner of fusion 5 did not show sensitivity in the HAT medium and continued to grow. This was most likely due to the fact that the myeloma cells were not previously exposed to 6-thioguanine (Section 2.1) prior to this particular fusion. The lymphocytes from fusion 8 were used to test the production of clones obtained from two different myeloma cell partners; GM 4672 and LICR-LON-HMy2 (HMy2, obtained from Dr. M.J. O'Hare, England), using only the 5:1 cell ratio because of the limited number of myeloma cells available. No clones were obtained with the HMy2 partner, while 48 clones were produced using the same lymphocytes with the GM 4672 myeloma partner. The lymphocytes from fusion 26 were used to test the production of clones obtained with and without pokeweed mitogen (PWM, Gibco) stimulation, also using only the 5:1 ratio for the same reason as mentioned above. Sixteen clones were obtained from the non-stimulated cell fusions. The PWM-stimulated cell fusions became contaminated before clones could be seen and staged up. At that point, no more fusions were performed; thus, PWM-stimulation was not retested.

The sensitivity of the myeloma cells to HAT medium (as in fusion 5) was checked at each fusion. Fusion of myeloma cells with themselves was done simultaneously with the fusion of myeloma cells with

SLE lymphocytes. A total of 1487 wells were seeded with fused myeloma cells from all the eighteen fusions. If one excludes fusion 5, only 20 of 1487 wells (1.4%) showed growth. In fusion 5, 100% of the myeloma seeded wells and all the other wells showed continued growth in the HAT medium. Without an effective selective medium all the plates of fusion 5 were thrown out and no clones were recorded.

A total of 261 clones arose from the eighteen SLE fusions. This represents a percentage growth varying from 1.1% to 40%, with a mean of 8.5%. The first fusion was called 1, the clones arising from it were assigned a number. Thus, if fusion 1 generated 36 clones, these would be identified as 100, 101, 102, ..., 135. Similarly, fusion 6 had 6 clones, noted as 600, 601, 602, ..., 605. The supernatant from each clone was screened for anti-DNA antibody production (Section 3.1) as soon as cell growth was confluent in 2 ml wells. Hybridomas which were negative in the screening test, were staged up in 10 ml volume, retested and frozen down (Section 2.4). From the 261 clones which arose, thirty-three produced anti-DNA antibodies. They are listed in Table 5 with their respective fusion. Almost all anti-DNA antibody-producing clones were stable for more than 10 months (i.e. their activity remained). Occasionally some clones lost their binding activity very rapidly (2-4 months). The latter clones had to be thawed more frequently to ensure activity.

Cells from all 261 clones were frozen so that

these could be thawed and tested for other antibody activities at some later date and also in case the growing clones were lost. The supernatants of these clones were also tested for rheumatoid factor and lupus anticoagulant activities, which will be discussed in sections 15.1 and 15.2.

10.2 Cloning Results

Anti-DNA producing and some non-DNA binding clones were cloned by limiting dilution at a concentration of one cell per well (Section 2.3). The clones arising from each subcloning procedure (called subclones) were designated by their parental hybridoma name followed by a hyphen and a number. Thus, if clone 103 generated 8 subclones, these would be identified as 103-1, 103-2, ..., 103-8. Similarly, clone 134 had 2 subclones and these were noted as 134-1 and 134-2. The other anti-DNA hybridomas cloned: 100, 600, 604, 1108, 121, 112, 128, 1202, 1206, and 1305 had, respectively, 2, 15, 1, 14, 1, 5, 0, 13, 2, and 1 subclone(s). Three non-DNA binding clones 109, 110, and 113 were also cloned. The 2 former clones became infected before subclones arose and were not recloned. The last one, 113, had 15 subclones. In each cloning procedure, 120 wells were seeded. Thus the efficiency of cloning varied from 0% to 13%. However, only the anti-DNA producing hybridomas gave rise to anti-DNA subclones. All 15 subclones of 113 were negative. The subclone 134-2 was recloned by limiting dilution and gave rise to 5 subclones, identified in the same manner as the other subclones, that is the parental clone's name followed by a hyphen and a number (134-2-1, 134-2-2, ..., 134-2-5).

No other clones were cloned twice.

10.3 Lymphocyte to GM 4672 Cell Ratio

Table 6 compares the frequency of clones arising from the fusion of lymphocytes with GM 4672 cells at ratios of 5:1 and 1:1 for 10 fusions. At the lymphocyte to GM 4672 cell ratio of 1:1, successful growth of clones occurred in 7.5% (124/1660) of the wells seeded, compared with a yield of 4.1% (62/1529) at the 5:1 cell ratio. The hybridoma yield from the 1:1 cell ratio was significantly greater ($p<0.05$) than the yield from fusions done at 5:1 cell ratio (see Appendix I). 17% of the clones formed at a 1:1 cell ratio produced anti-DNA autoantibodies, while only 11% of the hybridomas produced at the 5:1 cell ratio secreted anti-DNA antibodies. The anti-DNA hybridoma yield from the 1:1 cell ratio was also significantly greater ($p<0.05$) than the yield from fusions done at 5:1 cell ratio. The optimal cell ratio condition was thus 1:1.

10.4 Cell Plating Density

The well size and cell plating concentration affected the growth of hybridoma clones. Table 7 compares the yields of hybridomas obtained at 3 cell plating densities in the same 10 fusion experiments as in Section 10.2, using the optimal lymphocyte to GM 4672 cell ratio of 1:1. The maximal yield of hybridomas was obtained in 2.0 ml wells plated with $4x10^5$ cells (16.3% of the wells demonstrated clones). Smaller wells (0.3 ml) plated with $2x10^5$ and $1x10^5$ cells/well produced clones in 6.6% and 5.5% of the wells, respectively.

Hybridoma yields differed significantly ($p<0.05$) among the 3 cell plating densities. However, the yield of anti-DNA hybridoma antibodies was not significantly different ($p>0.05$) among the 3 cell plating densities. The optimal plating condition was thus $4x10^5$ cells per 2.0 ml well under which 13% of clones produced secreted anti-DNA antibodies.

10.5 Quantitation of IgM and IgG Production in Culture Fluid

The amount of IgM and IgG produced in each hybridoma culture fluid was determined by the incubation of diluted culture fluid on either rabbit anti-human IgM or anti-human IgG coated tubes (Section 3.3). A standard curve was established in each assay using purified human IgM with ^{125}I-rabbit labelled anti-human IgM or using purified human IgG with ^{125}I-rabbit labelled anti-human IgG with the corresponding tubes.

The amount of IgM produced by the anti-DNA antibody producing clones is given in Table 8 in the last column. The IgM production varied from 0.26 µg/ml to 19.9 µg/ml of culture fluid with a mean of 5.11 µg/ml. No detectable IgG was synthesized by the anti-DNA antibody producing clones.

The amount of IgM and IgG produced by twenty non-DNA binding clones is given in Table 9. The IgM production varied for the IgM non-DNA binders from 0.59 µg/ml to 8.75 µg/ml with a mean of 3 µg/ml. There is no statistical difference in the level of IgM produced by the DNA and non-DNA binding hybridoma antibodies (Appendix II).

Four clones produced only IgG (0.06 µg/ml to 0.78 µg/ml of culture fluid). The amount of IgG produced by the myeloma cell line GM 4672 was 0.26 µg/ml of culture fluid. Thus some clones produced higher amounts of IgG than the myeloma cell fusion partner. It appeared that clones produced only one class of antibody, but, as it will be discussed in Section 11.3, a clone produces both IgM and IgG antibodies only when it is switching class or, more accurately, is losing its anti-DNA activity. Some clones were recorded with no production of IgM and IgG.

10.6 Comparison of Clones Produced at the 5:1 and 1:1 Cell Ratios

The thirty-three anti-DNA antibody producing clones were divided arbitrarily into those producing weak or strong anti-DNA antibodies depending on the direct binding of their neat and diluted culture fluid to dDNA coated tubes. If the culture fluid lost its anti-DNA activity rapidly upon dilution, the antibody was declared weak, otherwise it was classified strong. Table 8 lists all the anti-DNA antibodies with their corresponding cell ratio and cell density origins, their anti-DNA activity and their individual production of IgM. There is no noticeable difference in the production of weak or strong anti-DNA activity at the different cell fusion ratios (5:1/1:1 : 56-44/46-54 % weak-strong antibody) nor in the production of IgM (5:1/1:1 : 6.05/4.78 µg/ml).

TABLE 4

TOTAL NUMBER OF LYMPHOCYTES OBTAINED, WELLS PLATED AND CLONES PRODUCED IN EACH FUSION

Fusion	Number of Lymphocytes	Total Number of Wells Plated	Total Number of Clones Obtained	Growth Yield (%)*
1	3.6×10^7	315	36	11.4
2	3.6×10^7	315	0	-
3	2.6×10^7	203	0	-
4	3.7×10^7	332	0	-
5	4.5×10^7	437	0	-
6	2.9×10^7	245	6	2.5
7	1.8×10^7	153	3	2.0
8	3.8×10^7	120	48	40.0
10	3.1×10^7	229	3	1.3
11	4.1×10^7	338	8	2.4
12	2.9×10^7	242	8	3.3
13	1.6×10^7	133	21	15.8
14	2.6×10^7	189	25	13.2
15	3.7×10^7	329	36	10.9
16	6.2×10^7	539	37	6.9
19	7.8×10^7	681	12	1.8
23	4.0×10^7	365	4	1.1
26	1.1×10^8	221	14	6.3

TOTAL:18 TOTAL:261 MEAN:8.5

* Growth yield: $\frac{\text{number of wells with growth} \times 100}{\text{total number of wells plated}}$

TABLE 5

ANTI-DNA ANTIBODY-PRODUCING CLONES OBTAINED FROM SLE FUSIONS

Fusions	Names	Total Clones Producing Anti-DNA Antibodies
1	100, 103, 134, 112, 121, 128	6
6	600, 601, 604	3
11	1101, 1108	2
12	1202, 1204, 1206	3
13	1305, 1311	2
14	1400, 1401, 1402, 1403, 1404, 1405, 1406, 1407, 1412, 1414, 1415, 1418, 1422	13
19	1900, 1905	2
26	2607, 2608	2
		TOTAL: 33

Table 6

OPTIMAL LYMPHOCYTE TO GM 4672 CELL RATIO

Cell ratio	Number of Fusions	Number of wells	Number of Clones	Yield (%)**	% of Clones Producing Anti-DNA
5:1	10	1529	62	4.1*	11% (7/62)
1:1	10	1660	124	7.5*	17% (21/124)

* $p<0.05$

** Yield (%) = $\frac{\text{number of wells with growing clones} \times 100}{\text{total number of wells plated}}$

TABLE 7

OPTIMAL CELL PLATING DENSITY

Cell Ratio	Cell Number/ Well *	Number of Wells Plated	Number of Clones	Yield (%)	% of clones producing Anti-DNA
1:1	$4x10^5$	246	40	16.3**	13 (5/40)
	$2x10^5$	563	37	6.6***	24 (9/37)
	$1x10^5$	851	47	5.5***	15 (7/47)

* $4x10^5$ cells were plated in 1.0 ml HAT in a 2.0 ml well. $2x10^5$ and $1x10^5$ cells were plated in 0.3 ml HAT in a 0.3 ml well.

** $p < 0.05$

*** $p > 0.05$

TABLE 8

CLASSIFICATION OF THE ANTI-DNA ANTIBODIES INTO WEAK OR STRONG ANTIBODIES ALONG WITH THEIR CELL RATIO AND CELL DENSITY ORIGIN, AND LIST OF THEIR PRODUCTION OF IgM

Cell Ratio	Total of Anti-DNA Clones	Cell Density (cells/well)	Anti-DNA Clones	Anti-DNA Activity	Quantity of IgM in µg/ml of Culture fluid
5:1	9	$4x10^5$	121	Weak	0.26
			1905	Weak	4.35
			2607	Strong	5.70
		$2x10^5$	128	Weak	N.D.*
			2608	Strong	6.30
		$1x10^5$	1412	Weak	N.D.
			1305	Weak	5.65
			1400	Strong	15.80
			103	Strong	4.30
1:1	24	$4x10^5$	1108	Weak	2.05
			1202	Weak	0.32
			1414	Weak	10.50
			1206	Strong	0.38
			1418	Strong	4.68
		$2x10^5$	100	Weak	7.10
			1405	Weak	N.D.
			1415	Weak	N.D.
			1204	Weak	1.46
			1311	Weak	2.91
			1401	Strong	1.05
			1402	Strong	3.20
			1406	Strong	10.60
			1422	Strong	7.15
			600	Strong	2.36
			604	Strong	0.41
		$1x10^5$	112	Weak	N.D.
			1101	Weak	0.38
			601	Weak	N.D.
			1900	Weak	2.64
			1404	Weak	7.70
			1407	Strong	6.25
			134	Strong	4.60
			1403	Strong	19.90

* not determined

TABLE 9

QUANTITY OF IgM AND/OR IgG IN SOME NON-DNA BINDING SUPERNATANTS

Clones	IgM (µg/ml)	IgG (µg/ml)
GM 4672	0.0	0.26
1411	3.36	0.0
1410	3.18	0.0
1003	2.15	0.0
1104	1.65	0.0
1109	4.44	0.0
801	1.35	0.0
1500	1.70	0.0
1520	3.33	0.0
1600	0.59	0.0
2300	1.35	0.0
2301	8.75	0.0
2613	1.12	0.0
2602	5.75	0.0
1311	3.20	0.0
1417	0.0	0.78
824	0.0	0.06
1607	0.0	0.11
1203	0.0	0.44

Chapter 11. CHARACTERIZATION OF HUMAN ANTI-DNA HYBRIDOMAS

11.1 Class Determination of Anti-DNA Antibodies

The immunoglobulin class determination for three clones, 100, 103, 134, is shown in Table 10. In this assay, the 3 anti-DNA antibodies (culture fluids) were first incubated on dDNA coated tubes which were then divided into 2 groups. An anti-human IgG label was added to one set of tubes and an anti-human IgM label was added in the other set of tubes (Section 3.2). As a negative control, the non-DNA binding antibody (IgG2K) (culture fluid) produced by the GM 4672 cell line, the cell partner used for fusion was used. Consequently, no anti-DNA activity was observed from it, whatever the use of either the anti-human IgM or anti-human IgG labels.

Table 10 shows that the 3 clones tested had a higher bound radioactivity using the labelled rabbit anti-human IgM than the labelled rabbit anti-human IgG. So these three clones, 100, 103, and 134, are anti-DNA antibodies of the IgM class. The class determination of all 24 anti-DNA hybridoma antibodies showed them all to be anti-DNA antibodies of the IgM class. No anti-DNA IgG hybridomas were found, but non-DNA binding IgG and IgM hybridomas were found (Section 10.5).

11.2 Long-term Production of Antibodies by Clone 134

The quantity of IgM and IgG antibodies produced by clone 134 was followed over a period of 8 months. Table 11 shows the results at different periods of time during tissue culture. The 134 clone produced only IgM antibodies, no IgG,

and the amount of IgM fluctuated between 2 to 124 µg/ml of culture fluid. The direct binding of each of these culture fluids to dDNA, nDNA and cardiolipin coated tubes was studied. Figure 4 shows the titration of each culture fluid on dDNA coated tubes. All the titration curves are S-shaped and look similar. Only the culture fluid of 2/27/83 showed lower binding to dDNA at 1 ug/ml than the other supernatants, which suggests the production of an anti-DNA antibody of lower affinity occurred during that period of time. The culture fluid of 4/28/83 was titrated to one nanogram as shown in Figure 4.

The binding levels to nDNA did not relate to the amount of IgM found in each culture fluid (Table 11). The highest binding was found with the culture fluid having only 12.7 µg/ml of IgM. In order to ensure that the culture fluids of clone 134 were not all different, these culture fluids were diluted to the same concentration of 2 µg of IgM per ml of culture fluid and their binding to dDNA, nDNA and cardiolipin coated tubes were compared (Figure 5). Once again, the binding to dDNA coated tubes (first bar of each date) of these culture fluids, diluted at the same concentration of 2 µg/ml, looked very similar. As in the titration analysis, only the 2/27/83 culture fluid had a slightly lower binding to dDNA. If the bindings of these culture fluids to nDNA are compared (second bar of each date), 3 culture fluids showed lower binding than the others. They were 2/27/83, 3/21/83, and 4/11/83. The binding to cardiolipin

(last bar) was low for all the culture fluids except for the 2/27/83 culture fluid, which was much higher than the others. However, the binding to dDNA, nDNA and cardiolipin of the last culture fluid of clone 134 on 4/28/83 was the same as with the first culture fluid on 8/17/82, 8 months earlier.

11.3 Antibody Class Switch

The fluctuating amount of IgM produced by clone 134 with time (Section 11.2) is representative of the production of antibodies by any hybridoma. Nevertheless, as it can be seen with clone 1311 (Table 12), it appeared that some clones might be stable and produce equivalent amounts of antibodies over a period of eight months. Clone 1311 produced non-DNA IgM binding antibodies, at a constant level of 2.94 ± 0.32 µg/ml.

In contrast, Table 12 also shows the antibody "class switch" accompanied by a loss of anti-DNA activity by clones 134-2 and 103-1. Initially, both clones produced anti-DNA IgM antibodies with no detectable IgG antibody. After 5 months of tissue culture, both clones were no longer producing IgM antibodies, but rather IgG antibodies. The binding to dDNA shown in Table 12 was observed using a rabbit anti-human IgM label. A repetition of the experiment with the use of a rabbit anti-human IgG label did not show any anti-DNA activity. It seems that these clones had their production of anti-DNA IgM antibodies turned off, while the production of non-DNA binding IgG antibodies was turned on. Moreover, the switch of the class and antigen binding capacity of the antibody produced also affected the presence of idiotype

on the hybridoma antibody. Idiotypes 134 and 103-1, respectively, present on 134-2 IgM antibody and 103-1 IgM antibody, were not found on the 134-2 IgG and 103-1 IgG antibodies following the switch. This strongly confirms an antibody turned off rather than a real class switch.

Table 12 also shows that at least 2 clones (1501, 1908) were not producing any IgM or IgG antibodies and consequently had no anti-DNA activity detected by anti-IgM or anti-IgG labels.

11.4 Comparison of Direct Binding of Anti-DNA Antibodies to dDNA, nDNA and Cardiolipin

The direct binding activity of 15 different anti-DNA antibodies (culture fluids) to dDNA, nDNA and cardiolipin coated tubes was compared at a similar concentration of 2 ug of IgM per ml of culture fluid. These results are shown in Figure 6, after subtraction of the buffer background and the non-specific binding to BSA coated tubes. The results of each clone are represented as 3 bars with binding to dDNA, nDNA and cardiolipin, in that order. Binding to dDNA was highest in each case except for 1305. The binding characteristics varied considerably between dDNA and nDNA. Four clones were strong nDNA binders (over 7000 cpm, 103, 134, 1400, 1407) and all of these bound strongly to dDNA coated tubes. However, there were 7 other strong dDNA-binders (over 7000 cpm, 1401, 1403, 1406, 1418, 1422, 600, 604), which bound only moderately or weakly to nDNA. There were no nDNA binders which failed to bind to dDNA.

Only one clone (103) showed moderate binding (over 2000 cpm) to cardiolipin. The four other clones (1414, 1900, 1305, 1202) were poor binders to dDNA at this concentration of 2 μg/ml. Four clones (1403, 1401, 1900, 1414) did not (or very little) demonstrate anti-cardiolipin activity. 1414 was the only clone presented in that figure which demonstrated strict anti-dDNA activity.

Each clone demonstrated its own pattern of reactivity with dDNA, nDNA and cardiolipin. Moreover, each fusion gave clones of different reactivities. For example, 103 showed strong binding to all 3 antigens while 134 showed strong binding to only dDNA and nDNA, although both clones came from the same fusion (fusion 1). Different profiles of binding can also be seen with clones 1400, 1401, 1403, 1406, etc. which all came from fusion 14 (see Table 5). Overall, 33% of the anti-DNA antibodies demonstrated activity to dDNA, nDNA and cardiolipin; 49% demonstrated anti-dDNA and anti-nDNA activities; and 18% demonstrated anti-dDNA activity only. No strict anti-nDNA or anti-cardiolipin antibodies, or anti-dDNA and anti-cardiolipin antibodies without anti-nDNA activity were produced.

11.5 Comparison of Direct Binding of Clone 103 and its Subclones to dDNA, nDNA and Cardiolipin

The 103 anti-DNA clone was subcloned, resulting in eight subclones (Sections 2.3 and 10.2). Table 13 shows the amount of IgM produced by each subclone and by the parental cell, 103. The amount of IgM ranged from 1.9 μg/ml to 18.2

µg/ml of culture fluid. Figure 7 shows the binding and titration of these clones (culture fluid) on dDNA coated tubes. The dotted line shows the curve for the parental cell, 103. Almost all of the subclones of 103, except for 103-3, showed better binding to dDNA coated tubes than 103 itself. Figure 8 gives the binding of these clones diluted at the same concentration of 2 µg/ml to dDNA, nDNA and cardiolipin. All clones bound very well to dDNA, the preferred antigen. The binding to nDNA and cardiolipin was more variable. Binding to nDNA for 4 subclones (103-1, 103-2, 103-3, 103-8) approached the binding of clone 103, but was much lower for the 4 other subclones. In the case of the binding to cardiolipin, the binding of 3 subclones (103-2, 103-6, 103-8) was higher than that of clone 103 and the 5 other subclones demonstrated similar binding characteristics to cardiolipin.

11.6 Inhibition of dDNA Binding of Clones 103, 1401, and 1406 by Polynucleotides and Cardiolipin

Competition assays were preformed to analyze the binding specificity of the antigen binding site of 3 anti-DNA antibodies. The competitors used all have similarities with dDNA, the antigen used for the screening of anti-DNA antibody production. They included polynucleotides such as poly (I), poly (dT), dDNA itself, zDNA, and a phospholipid such as cardiolipin. The primary structures of these competitors are compared in Figure 9. The shadowed square in Figure 9 stresses the similarity among the competitors, especially the sugar phosphate backbone.

The antibody was incubated first with each of these competitors and then assayed for residual binding to dDNA. Inhibition of the binding indicated that the antibody recognized antigenic determinants shared by both the competitor and dDNA. The results with three representative anti-DNA antibodies are shown in Figures 10, 11, and 12. Each graph shows that as the amount of competitor was increased, the degree of anti-DNA binding decreased, depending on the affinity of the anti-DNA antibody for the competitor. The concentration of competitor required for fifty percent inhibition (dotted horizontal line in each graph) reflected the relative avidity of the antibody for the competitor. This value was chosen to establish the preference of each clone for a given competitor.

In the competition assay, clones 103, 1406, and 1401 reacted with at least 4 of the 7 competitors used. Clone 103 reacted preferentially with poly (dT) (Figure 10), which was followed by poly (I), zDNA, and equally by cardiolipin and dDNA. Less than 15% of inhibition of dDNA binding of 103 was observed with both nDNA and RNA.

The competition assay for 1401 (Figure 11) showed a different profile. No competition was seen with poly (dT). The best competitor was zDNA, the left-handed double helix followed by poly (I), dDNA and cardiolipin. Again only 20% of inhibition of dDNA binding of 1401 was observed with nDNA and RNA.

The competition assay of 1406 (Figure 12) shows that the concentration required to achieve 50% inhibition for several competitors can be very similar. Clone 1406 required 0.27, 0.5, and 0.6 nanomoles (nmoles) of phosphorus dDNA, zDNA, and poly (I), respectively to achieve 50% inhibition. 1406 is the only clone shown of 19 competition assays (see Section 11.7) which reacted preferentially with dDNA, the screening antigen. No inhibition of dDNA binding of 1406 was observed with poly (dT), nDNA and RNA.

11.7 Binding Preference of 19 Anti-DNA Clones to Polynucleotides and Cardiolipin

Table 14 summarizes the results of competition assays performed with 19 anti-DNA antibodies, listing the amount of each competitor required to achieve 50% inhibition of binding to dDNA coated tubes. All the anti-DNA antibodies cross-reacted with at least 4 of 7 competitors used. Table 14 shows the clones in progressive order of the amount of dDNA required to achieve 50% inhibition. This value varied from 0.18 to greater than 10.7 nmoles of phosphorus, the highest concentration of dDNA used, which represents a range of 100 fold. The range of poly (dT) and zDNA required to achieve 50% inhibition was 10,000 fold (0.0001 to 2 nmoles of phosphorus), that for poly (I) was 100 fold (0.01 to 45), and for cardiolipin, 10 fold (4 to 40). The best competitor was poly (dT) for 50% (9/18) of the clones and zDNA for 38% (7/18). Six percent (1/18) did not see any difference between zDNA and poly (dT) and only one clone, 1406 (6%), was best inhibited by dDNA, closely followed by zDNA

and poly (I). In no case were nDNA and RNA strong inhibitors, with inhibition always less than 30%. Cardiolipin was always last in order of the competitors which inhibited the binding of clones to dDNA coated tubes. Appendix III gives all the graphics from which the values of Table 14 were taken.

11.8 Inhibition Profiles of dDNA Binding of Clone 103 and its Subclones by Polynucleotides and Cardiolipin

Table 15 summarizes the results of competition assays of 103 and its subclones, giving again the amount of each competitor required to achieve 50% inhibition of dDNA binding. All the subclones and the parental cells were best competed by poly (dT). The amount required for each competitor was within a 10 fold range. The order of competition for each subclone was quite similar, the amount of zDNA and poly (I) required were overlapping. The subclones often required less competitor than the parental cell. For example, the amount of dDNA required to achieve 50% inhibition was less for all the subclones than for the parental cell 103.

11.9 Inhibition of nDNA binding of Clones 103-4 and 1407 by Polynucleotides and Cardiolipin

A competition assay was performed on nDNA coated tubes for two clones. The procedure followed was the same described in Section 3.4, except for the use of nDNA coated tubes instead of dDNA coated tubes. The binding of clones 103-4 and 1407 to nDNA coated tubes was inhibited by the presence of polynucleotides and cardiolipin. Table 16 gives the nanomoles phosphate of each competitor required to achieve 50% inhibition

of antibody binding to nDNA coated tubes. Both clones were competed at least by five of the seven competitors used. Clone 1407 was the only one of the two clones tested which was inhibited by nDNA. The order of the competitors was the same for the two clones if one excepts nDNA: dDNA, poly (dT), zDNA, poly (I) and cardiolipin. No inhibition of antibody binding was observed in both cases with RNA. The order of competitors observed here is quite different from what was seen with the inhibition of binding of the same clone to dDNA by polynucleotides and cardiolipin (Tables 14 and 15). The main differences were that the best competition was observed in both cases with dDNA, that the inhibition of binding was observed for one clone with nDNA, and that the order of competition was very similar in both cases.

11.10 Purification of Hybridoma Antibodies by Sepharose Anti-IgM Affinity Column

Figure 13 shows the titration curves on dDNA coated tubes of eleven hybridoma antibodies which were affinity-purified over a Sepharose rabbit anti-human IgM column. Clones 128, 1108, 121, and 112-2 were anti-DNA antibodies that were very weakly positive in culture fluid form. The purification step concentrated them and rendered them workable. Immediately after this procedure, neat purified stock of 128, 1108, and 100-1 had a concentration of 39.9, 61.1, and 52.5 µg/ml of IgM, respectively, and similar binding strengths to dDNA. Clones 601, 1207, and 1307 were purified as negative control IgM antibodies. The

purification and concentration step revealed that clone 601 was in fact an anti-DNA antibody of very low affinity which appeared negative in culture fluid. However, the purified clones 1207 and 1307 remained negative. The IgM contents determined by OD_{280} of the purified stock varied from 9.8 to 141.4 µg/ml. The back calculations of the IgM contents in culture fluid from the volume and IgM concentration of the purified stock were similar to the amounts of IgM found by radioimmunoassay (Section 10.5), ranging from 1.16 to 8.56 µg of IgM per ml of culture fluid. Subsequently, purified clones 128 and 1108 lost their anti-DNA activity (Figure 13) after one and 5 months of storage at -20°C, respectively.

11.11 Comparison of Direct Binding of Purified Clones to dDNA, nDNA and Cardiolipin

Figure 14 shows the direct binding to dDNA, nDNA, and cardiolipin coated tubes of 9 purified clones diluted at the same concentration of 9.8 µg/ml. The purified clones acted similarly to the unpurified culture fluids, but with lower binding. All clones bound to the three antigens tested. The The preferred antigen was dDNA. The purified clone 1305 showed to prefer dDNA to nDNA, which contrasts with what was observed in culture fluid (Section 11.4).. Similarly, the purified subclone 103-1 had much higher binding to the three antigens than the binding of its purified parental clone 103 (Section 11.5). The binding to dDNA of purified subclones 134-1 and 134-2 was much higher than the binding of the purified parental clone 134. However, when the binding to

nDNA and cardiolipin of these subclones were assessed, one subclone (134-2) bound higher to both antigens, while the other subclone (134-1) bound less to both antigens than the parental clone 134.

11.12 Electrophoresis

Preliminary electrophoresis was done to see the homogeneity ("monoclonality") of each hybridoma product. The Ig produced by an hybridoma was biosynthetically labelled, purified, reduced and alkylated as described (see Chapter 4). Then, the H chain was separated from the L chain in an urea formate gel. The homogeneity of the L chain was subsequently checked in an IEF gel. Figure 15 illustrates the result of an urea formate gel while Figure 16 illustrates the radiogram of two IEF gels, one done in Sherbrooke where the author learned the gel techniques in Dr. D. Gibson's laboratory, and another done in Montreal by the author. The L chains were localized on the urea formate gel by a guide strip which contained normal HIgM and HIgG, a L chain from a patient with a light chain myeloma, and some standard molecular weights. The box in Figure 15 shows the strip cut and applied on IEF gel, which contained the L chain. The H chain remained.

It can be seen in Figure 16, that the following hybridomas antibodies 1400, 1401, 1206, 2608, 130-6, 1311, and 2600 were monoclonal while 2614, 1414, and 1206-1 were not. The two last bands (arrows, Figure 16) were seen repeatedly in all hybridomas and are unidentified. More gels are required to asses the monoclonality of all the hybridomas produced here.

TABLE 10

CLASS DETERMINATION OF HYBRIDOMA ANTI-DNA ANTIBODIES

CLONES (Culture fluid)	cpm bound to dDNA-coated tubes	
	^{125}I-Anti-Human IgG	^{125}I-Anti-Human IgM
100	347	7905
103	167	8855
134	199	11522
GM 4672 (myeloma)	401	397

TABLE 11

nDNA BINDING AND IgM CONTENT OF ANTI-DNA ANTIBODY-PRODUCING CLONE 134 CULTURE FLUIDS OVER A PERIOD OF 8 MONTHS

Date of culture fluid	Amount of IgM in µg/ml	Binding to nDNA in cpm
08/17/82	12.7 *	11830
11/01/82	27.3	8430
11/30/82	15.4	5840
02/27/83	2.0	2800
03/21/83	4.6	3240
04/11/83	9.9	9020
04/28/83	124.1	8820

* Quantitation of IgM and nDNA binding were performed on diluted and neat culture supernatants of the cells respectively.

FIGURE 4. dDNA BINDING CURVE OF 134 ANTI-DNA ANTIBODY CULTURE FLUIDS OVER A PERIOD OF 8 MONTHS

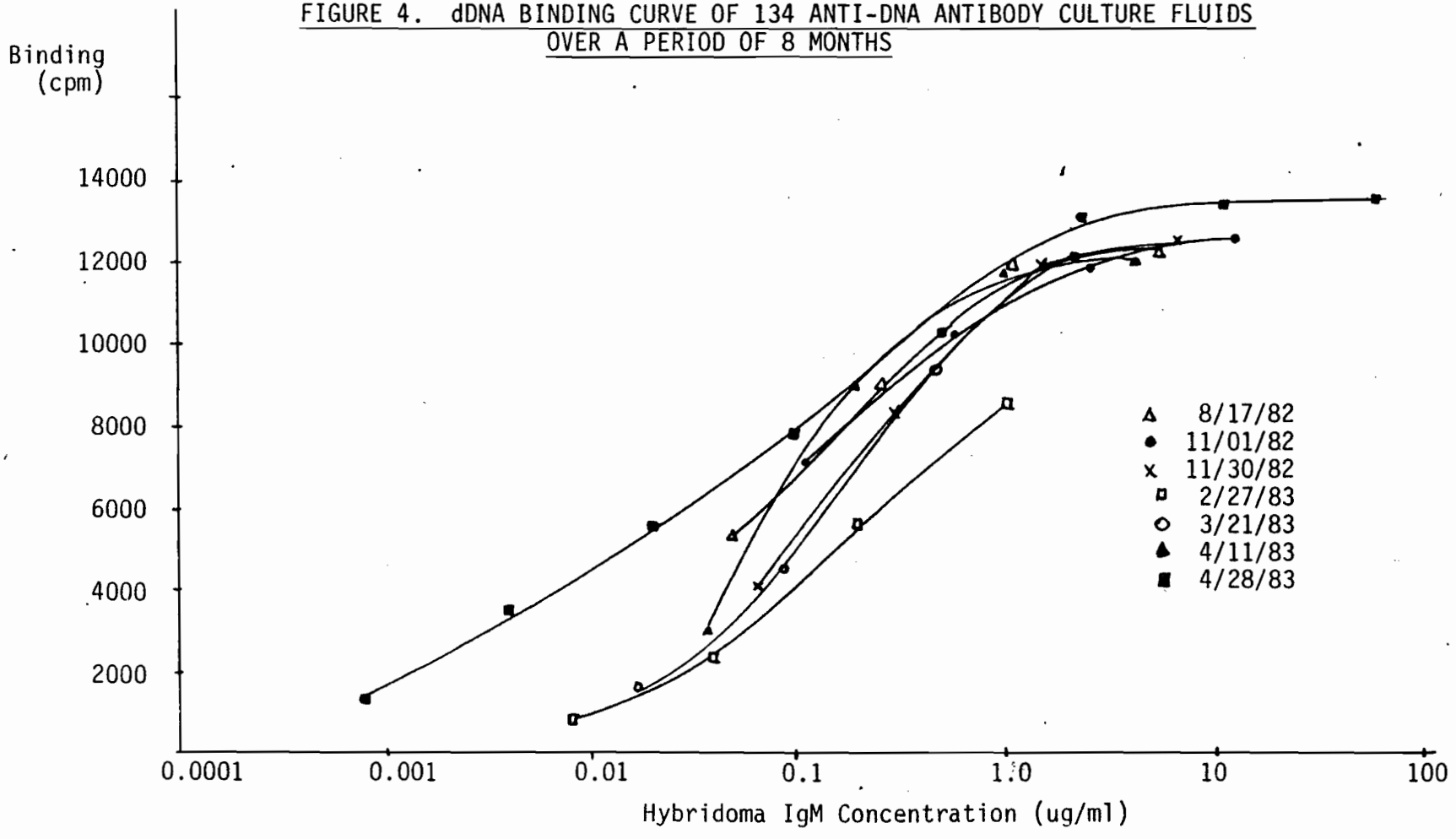


FIGURE 5. dDNA, nDNA, CARDIOLIPIN BINDING OF 134 ANTIBODY CULTURE FLUIDS DILUTED TO 2 ug OF IgM/ml

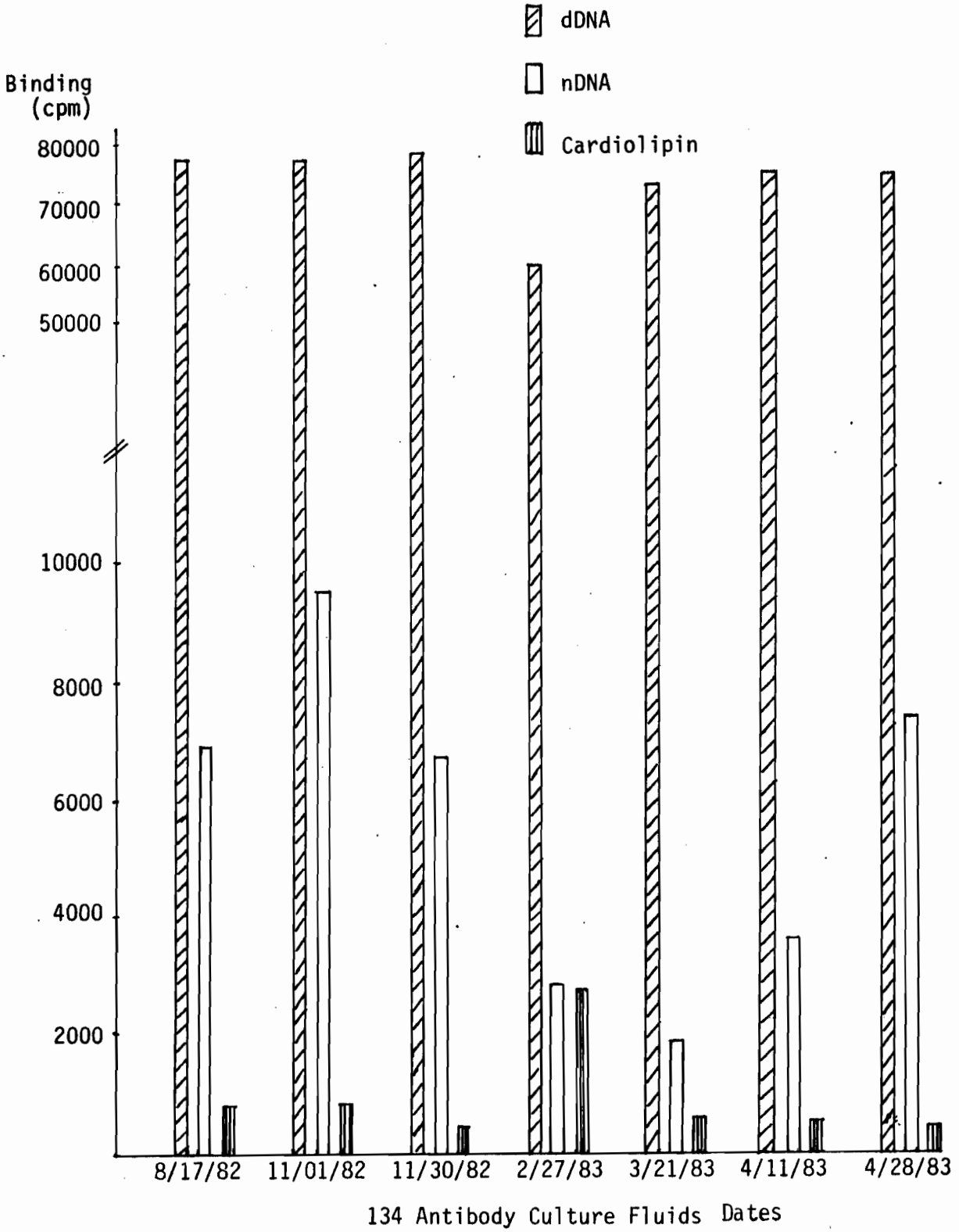


TABLE 12

dDNA BINDING, IgG, AND IgM CONTENT OF DIFFERENT CLONE CULTURE FLUIDS AT DIFFERENT PERIODS OF TIME OF TISSUE CULTURE

Clones	Date of Culture fluid	Amount in µg/ml IgM	IgG	Binding to dDNA (in cpm)
134-2	08/17/82	N.D.*	0.0	39691
	11/30/82	N.D.	0.0	34394
	12/22/82	1.29	0.12	17554
	01/10/83	0.24	0.16	14462
	02/27/83	0.0	0.13	962
	04/11/83	0.0	0.19	215
	06/09/83	0.0	0.17	126
103-1	07/21/82	1.94	0.0	42669
	01/07/83	0.01	0.18	175
	02/27/83	0.0	0.15	61
	03/21/83	0.0	0.14	228
	04/11/83	0.0	0.13	109
1311	02/27/83	2.58	0.0	401
	04/11/83	3.45	0.0	411
	05/16/83	2.58	0.0	103
	06/09/83	2.85	0.0	417
	07/13/83	2.90	0.0	197
	08/16/83	3.03	0.0	181
	10/14/83	3.20	0.0	183
GM 4672	04/11/83	0.0	0.25	98
1501	02/14/82	0.0	0.0	38
1908	03/03/83	0.0	0.0	0

* not determined

FIGURE 6. dDNA, nDNA, CARDIOLIPIN BINDING OF DIFFERENT ANTI-DNA ANTIBODY CULTURE FLUIDS DILUTED TO 2 ug OF IgM/ml

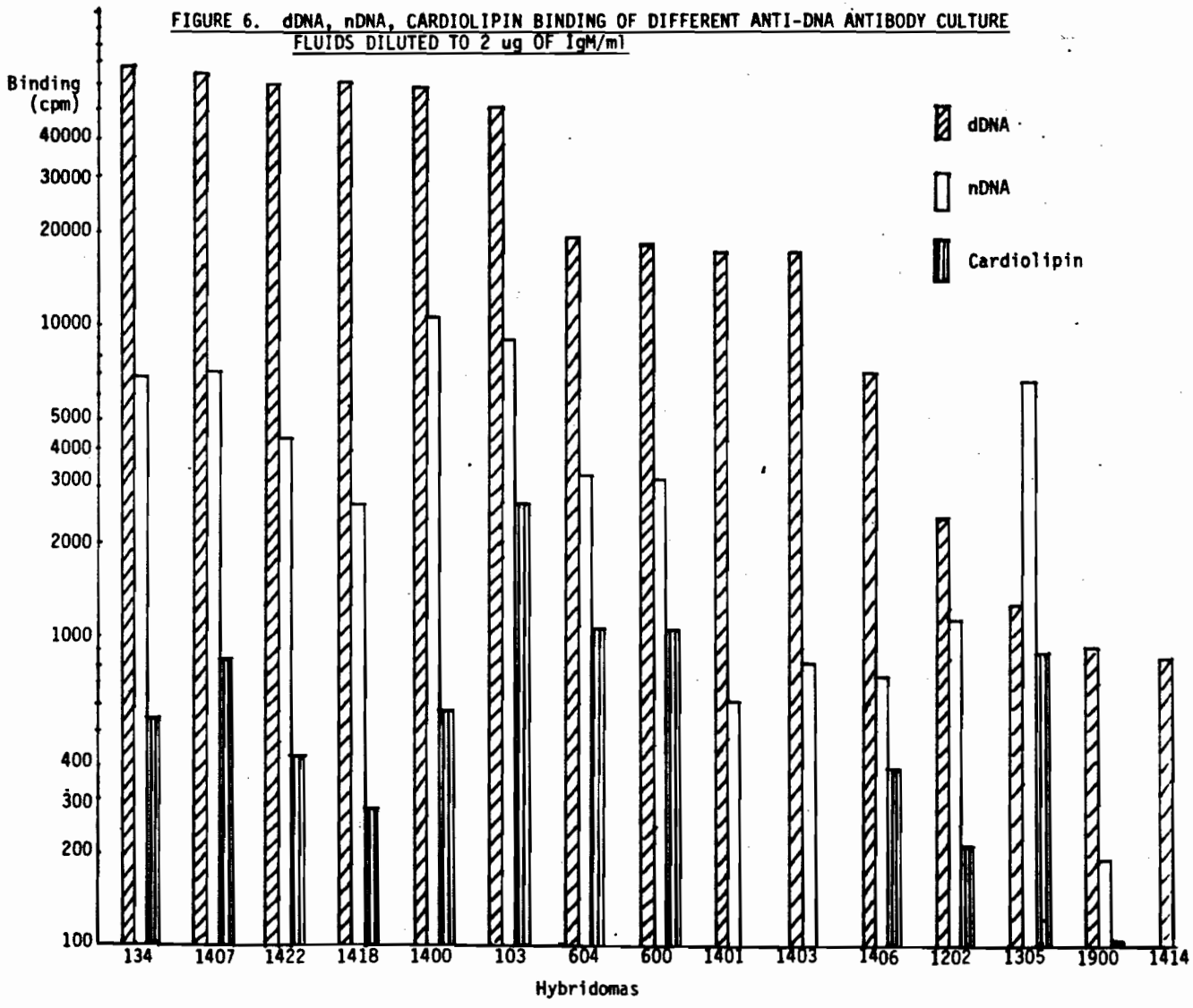


TABLE 13

QUANTITATION OF IgM PRODUCED BY 103 ANTI-DNA CLONE AND ITS SUBCLONES

		Amount of IgM
Clone	103	4.3
Subclones	103-1	13.1
	103-2	1.9
	103-3	4.9
	103-4	18.2
	103-5	5.1
	103-6	5.8
	103-7	4.7
	103-8	2.7

FIGURE 7. dDNA BINDING OF HYBRIDOMA ANTIBODIES PRODUCED BY CLONE 103 AND ITS SUBCLONES

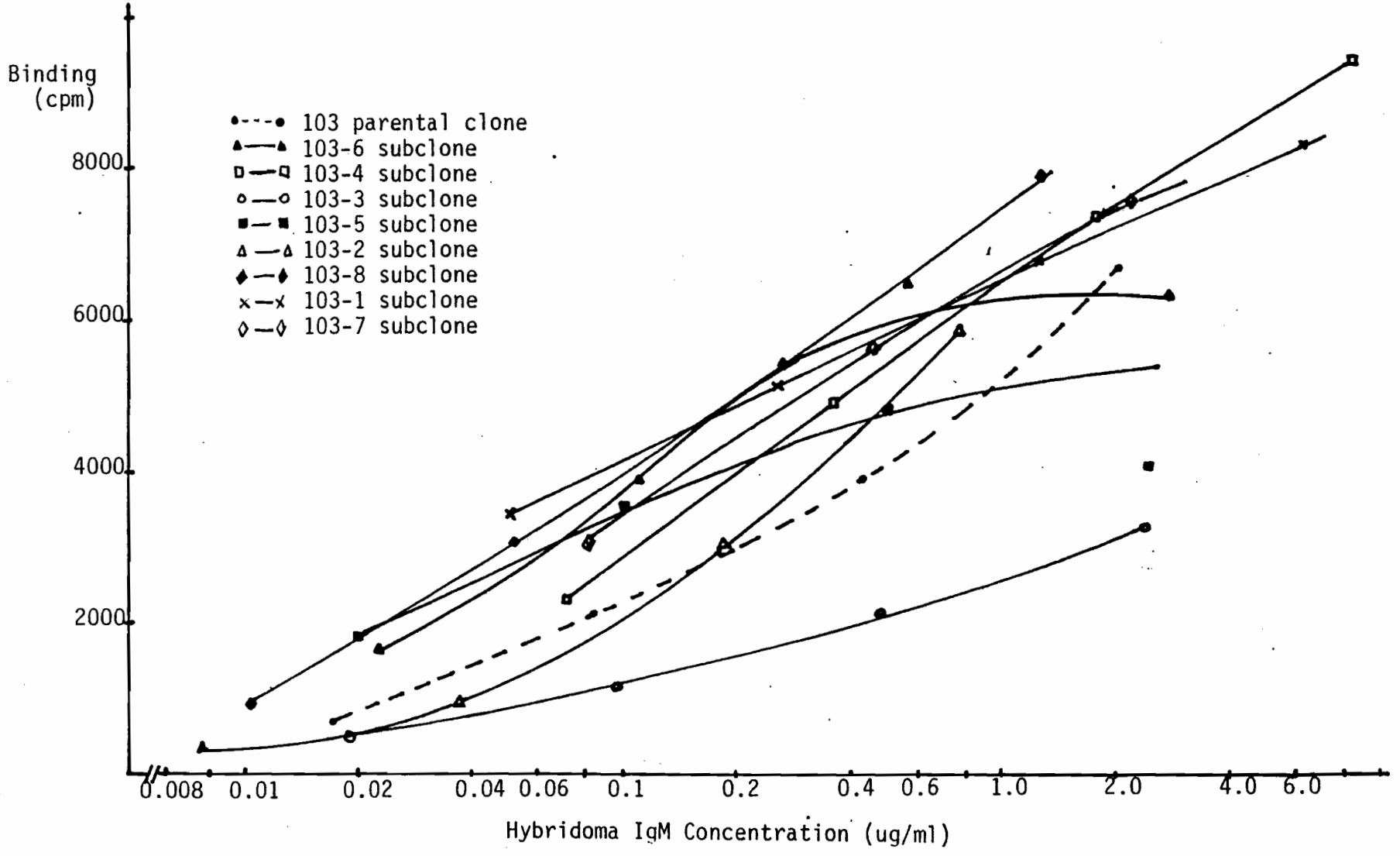


FIGURE 8. dDNA, nDNA, CARDIOLIPIN BINDING OF 103 CLONE AND SUBCLONE CULTURE FLUIDS DILUTED TO 2 ug OF IgM/ml

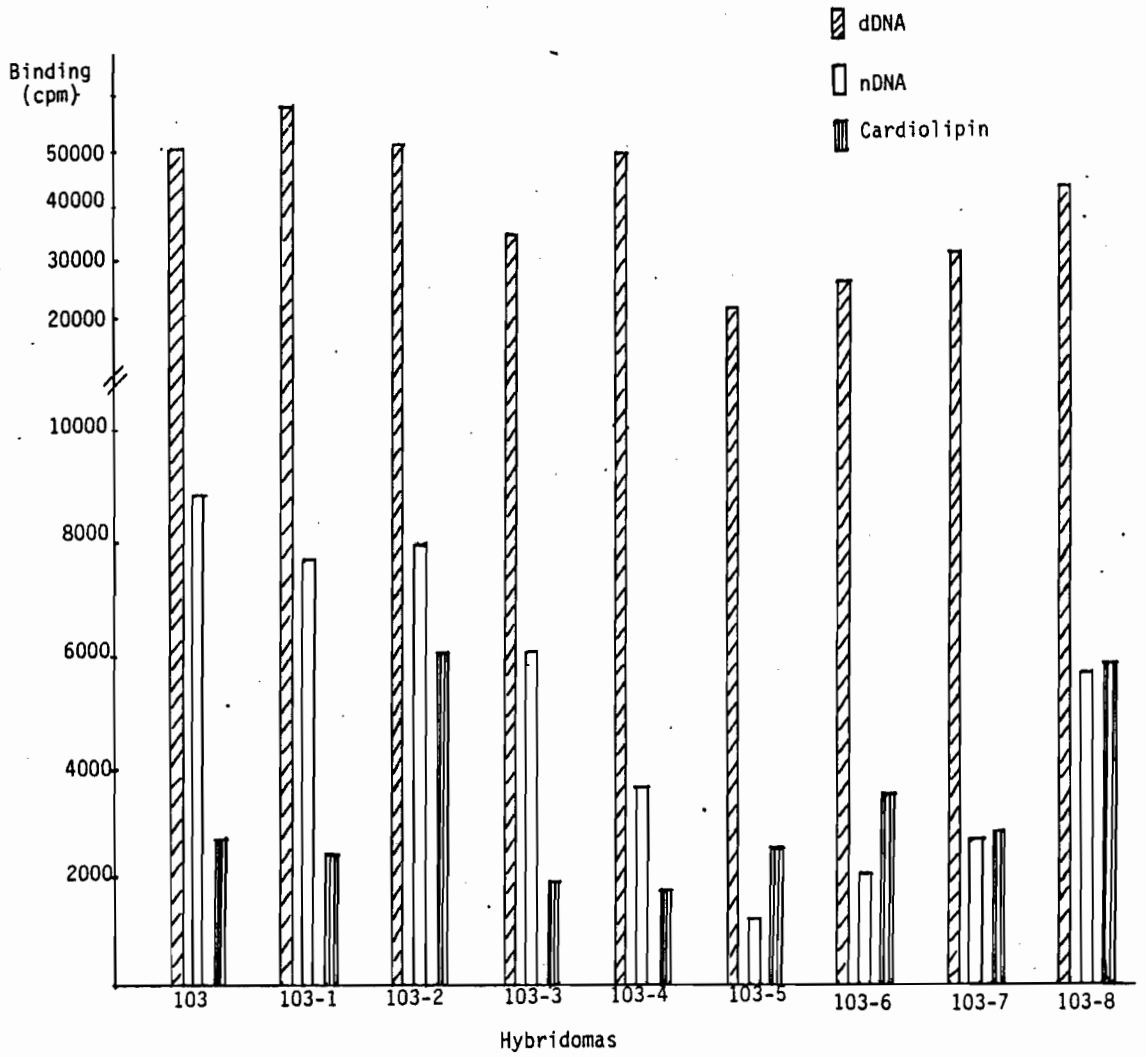
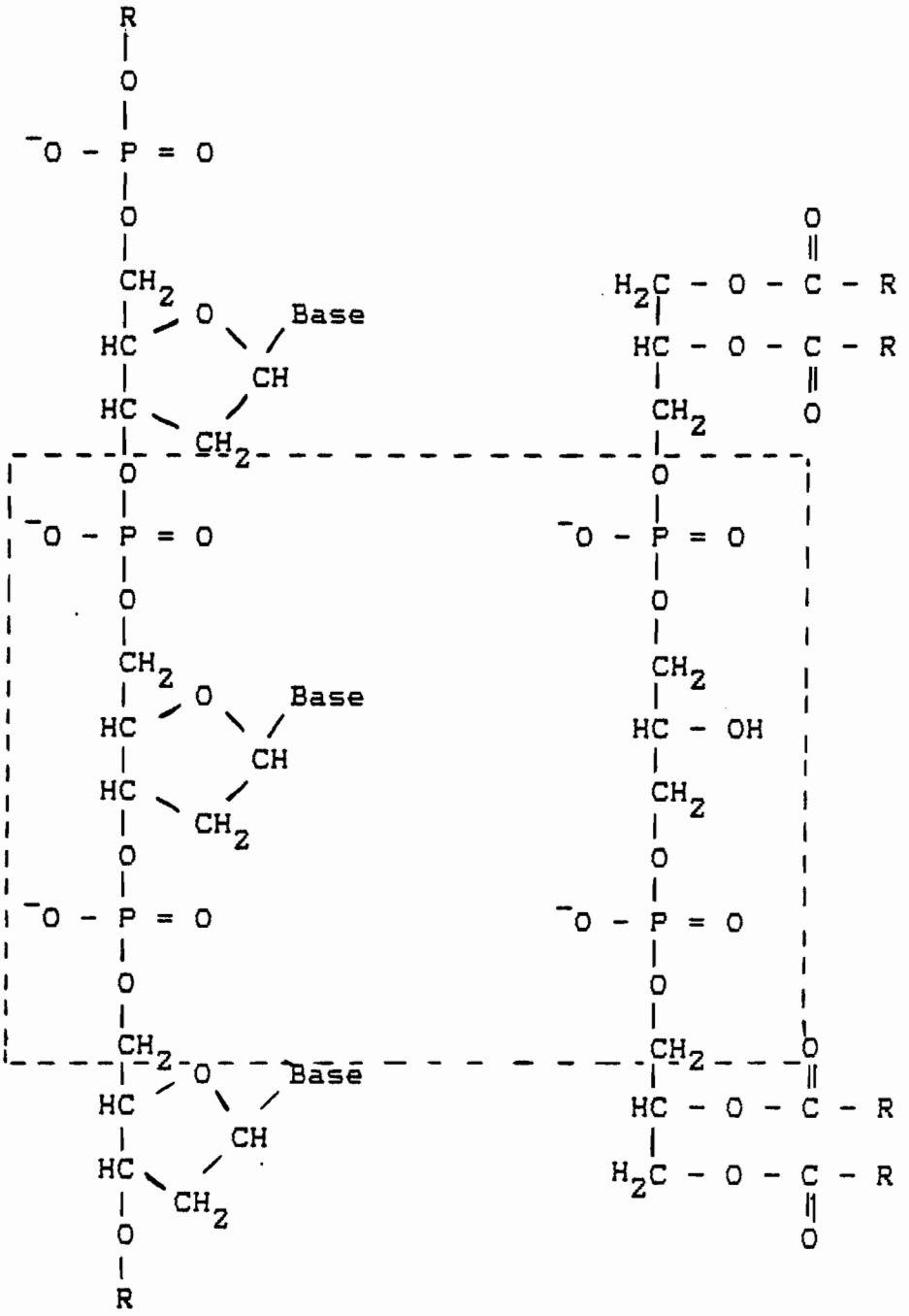


FIGURE 9

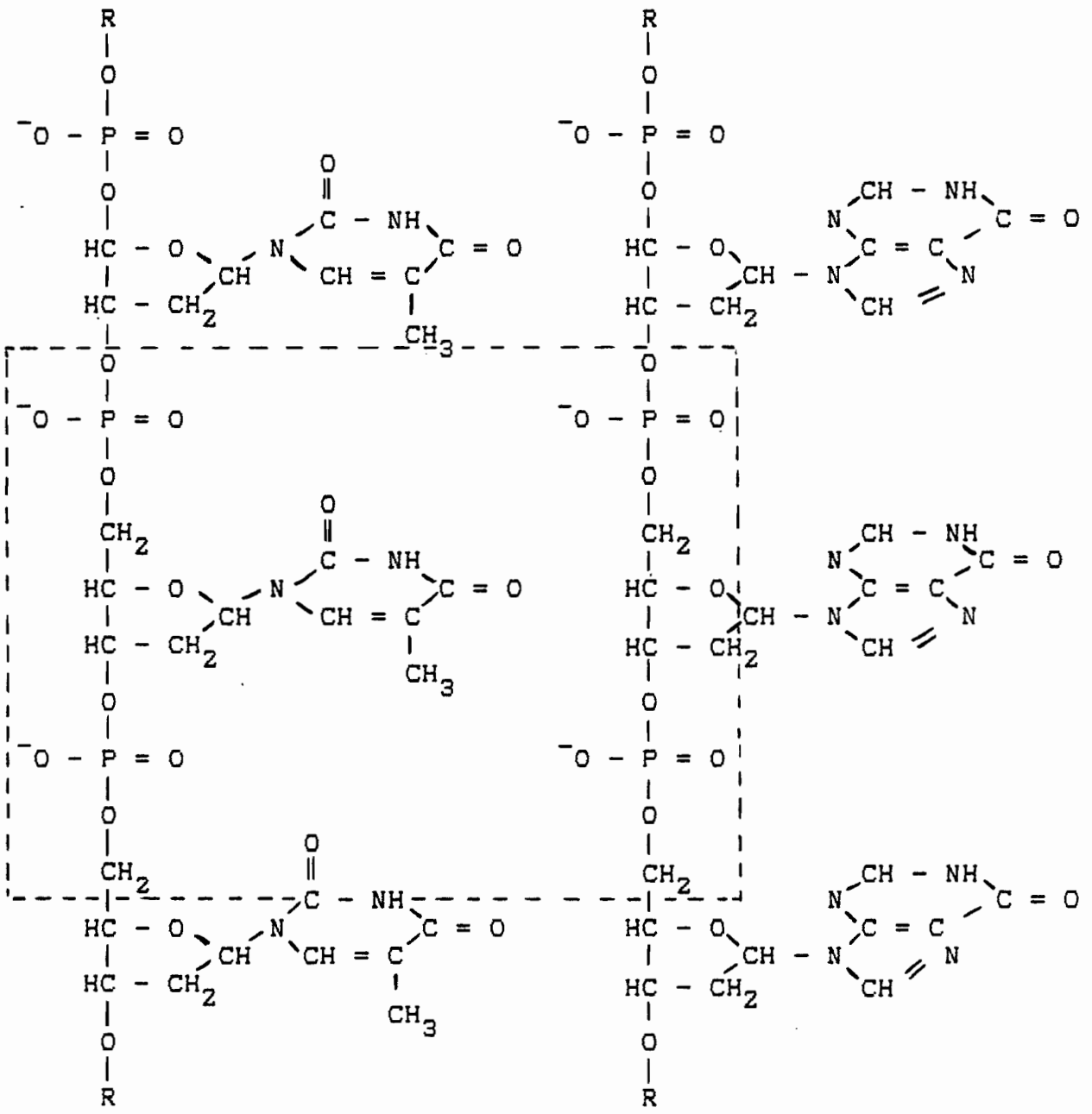
COMPARISON OF STRUCTURE OF DNA, CARDIOLIPIN, POLY (dT), AND POLY (I)



DNA

CARDIOLIPIN

FIGURE 9 (continued...)



POLY (dT)

POLY (I)

FIGURE 10. INHIBITION OF dDNA BINDING OF 103 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

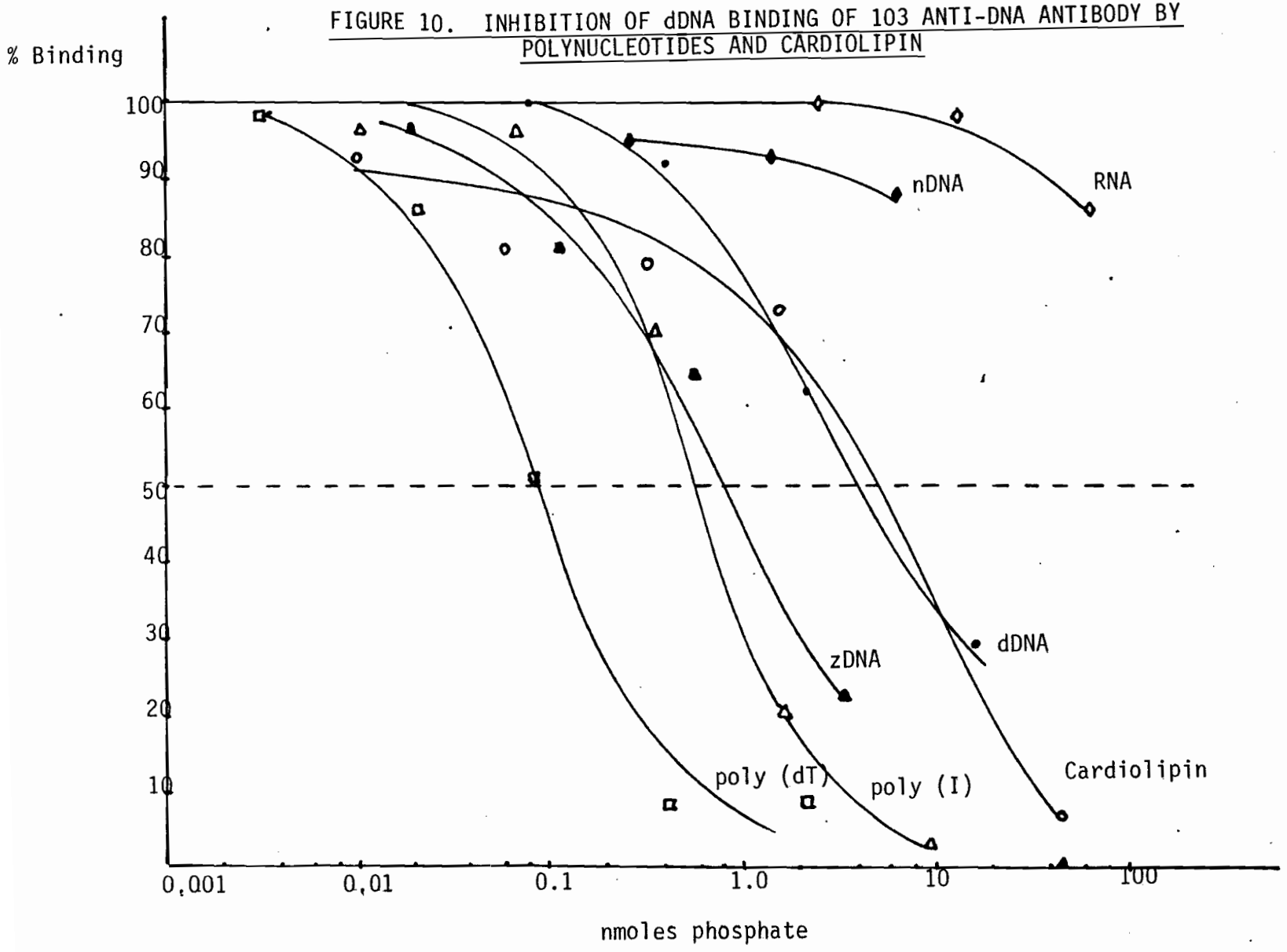


FIGURE 11. INHIBITION OF dDNA BINDING OF 1401 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

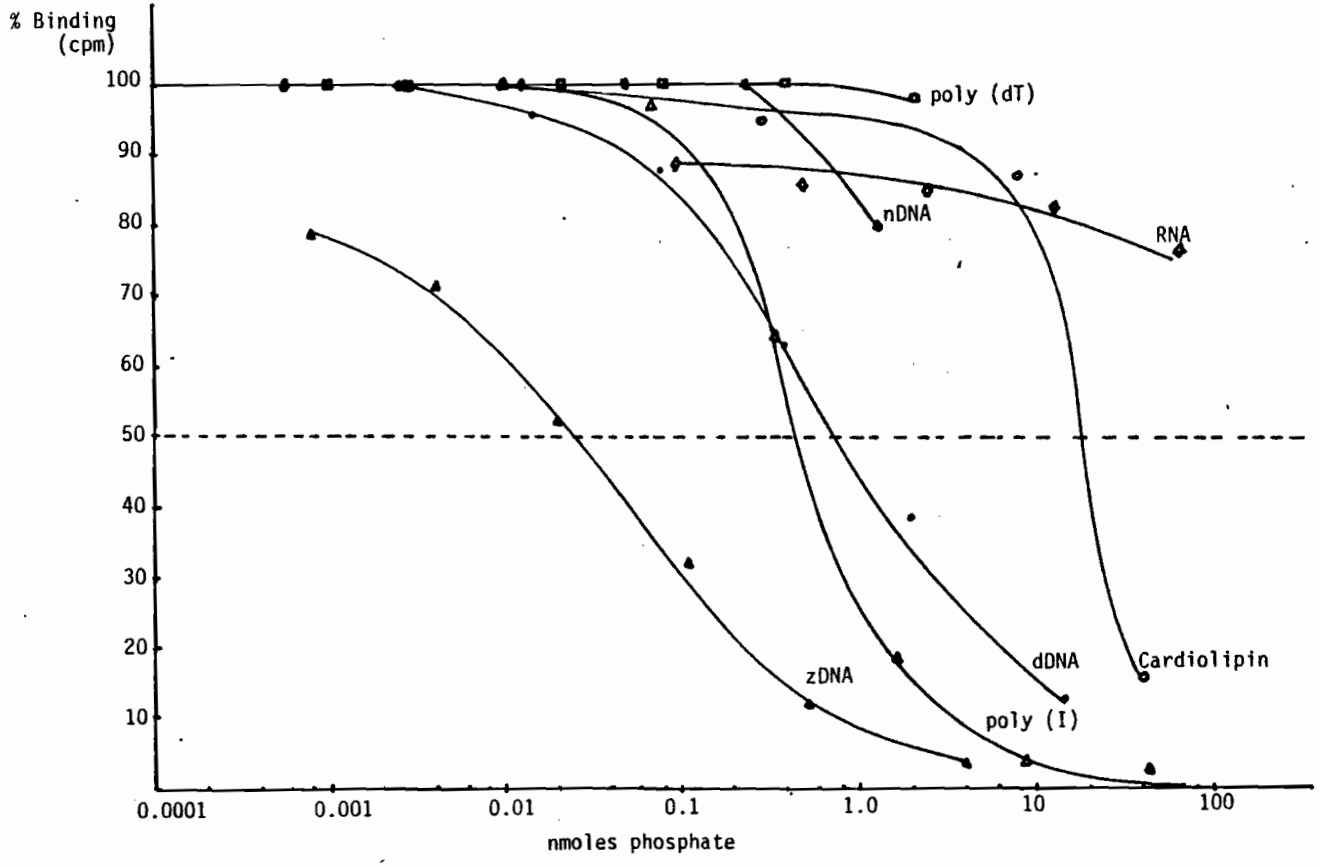


FIGURE 12. INHIBITION OF dDNA BINDING OF 1406 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

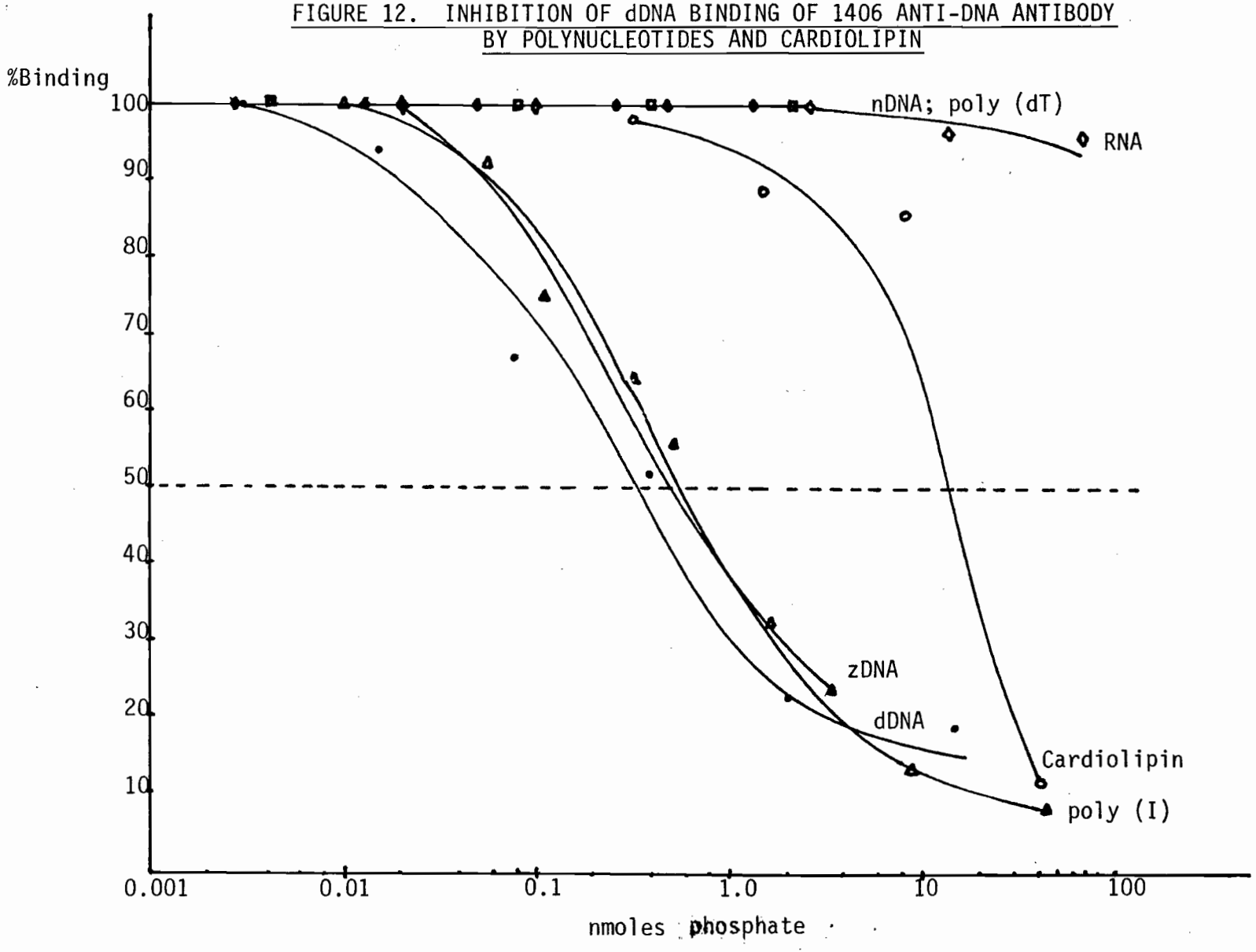


TABLE 14

NANOMOLES PHOSPHATE OF COMPETITOR REQUIRED TO ACHIEVE 50% INHIBITION OF dDNA BINDING

	dDNA	zDNA	nDNA	poly (I)	poly (dT)	Cardiolipin	RNA
1400	0.18	0.012	*	0.15	0.001	40.0	*
1406	0.27	0.5	*	0.6	*	19.0	*
1900	0.36	2.5	*	1.1	0.007	37.0	*
1418	0.44	0.08	*	0.45	0.0001	*	*
1407	0.58	0.05	*	1.2	0.058	40.0	*
1401	0.9	0.025	*	0.45	*	22.0	*
1422	0.9	0.062	*	0.38	*	8.0	*
121	0.9	0.0032	*	0.36	0.08	28.0	*
1206	0.99	0.07	*	0.34	0.0035	26.0	*
1403	1.4	0.0014	*	0.1	1.0	28.0	*
134	1.8	0.0001	*	0.028	0.68	7.5	*
1414	1.9	0.02	*	1.1	*	33.0	*
2607	2.3	3.2	*	30.0	0.0001	*	*
2608	2.6	1.9	*	3.6	0.9	*	*
103	4.2	0.95	*	0.68	0.07	4.2	*
1202	4.9	0.35	*	1.8	*	40.0	*
100	8.0	ND**	*	0.7	0.18	*	ND
600	8.0	2.8	*	3.2	0.18	12.0	*
604	18+	16+	*	2.6	0.20	21.0	*

* the highest quantities of competitors tested, in nanomoles phosphate, were 10.7 for dDNA, 3.2 for zDNA, 6.4 for nDNA, 45.2 for poly (I), 2.1 for poly (dT), 40.6 for cardiolipin, and 64.2 for RNA. The asterisk (*) indicates that less than 50% or no inhibition was achieved at the highest amount of competitor tested.

** not determined

\+ extrapolated values. At the highest amount tested (10.7 and 3.2 respectively for dDNA and zDNA), 58% inhibition of binding was attained.

TABLE 15

NANOMOLES PHOSPHATE OF COMPETITOR REQUIRED TO ACHIEVE 50% INHIBITION OF dDNA BINDING OF 103 AND ITS SUBCLONES

	dDNA	zDNA	nDNA	poly (I)	poly (dT)	cardiolipin	RNA
103	4.2	0.95	*	0.68	0.07	4.2	*
103-1	0.8	0.26	*	0.38	0.041	0.82	*
103-2	2.0	0.7	*	0.62	0.2	9.8	*
103-3	1.7	2.0	*	1.3	0.07	3.3	*
103-4	2.6	0.39	*	0.28	0.075	10.6	*
103-5	0.69	0.24	*	0.25	0.043	5.9	*
103-6	2.0	0.54	*	0.37	0.098	10.3	*
103-7	2.5	0.4	*	0.9	0.13	30.1	*
103-8	0.63	0.39	*	0.17	0.05	10.3	*

* the highest quantities of competitors tested, in nanomoles phosphate, were 10.7 for dDNA, 3.2 for zDNA, 6.4 for nDNA, 45.2 for poly (I), 2.1 for poly (dT), 40.6 for cardiolipin and 64.2 for RNA. The asterisk (*) indicates that less than 50% or no inhibition was achieved at the highest amount of competitor tested.

TABLE 16

NANOMOLES PHOSPHATE OF COMPETITOR REQUIRED TO ACHIEVE 50% INHIBITION OF nDNA BINDING

	dDNA	zDNA	nDNA	poly (I)	poly (dT)	Cardiolipin	RNA
1407	<0.001**	0.045	2.8	0.49	0.002	29	*
103-4	0.025	0.3	*	0.85	0.1	12	*

* the highest quantities of competitors tested in nanomoles phosphate were 10.7 for dDNA, 3.2 for zDNA, 6.4 for nDNA, 45.2 for poly (I), 2.1 for poly (dT), 40.6 for cardiolipin, and 64.2 for RNA. The asterisk (*) indicates that less than 50% or no inhibition was achieved at the highest amount of competitor tested.

** at the lowest quantities of competitor tested (0.003 nanomoles phosphate) 75% of inhibition of binding was still observed.

FIGURE 13. dDNA TITRATION CURVES OF PURIFIED HYBRIDOMA ANTIBODIES

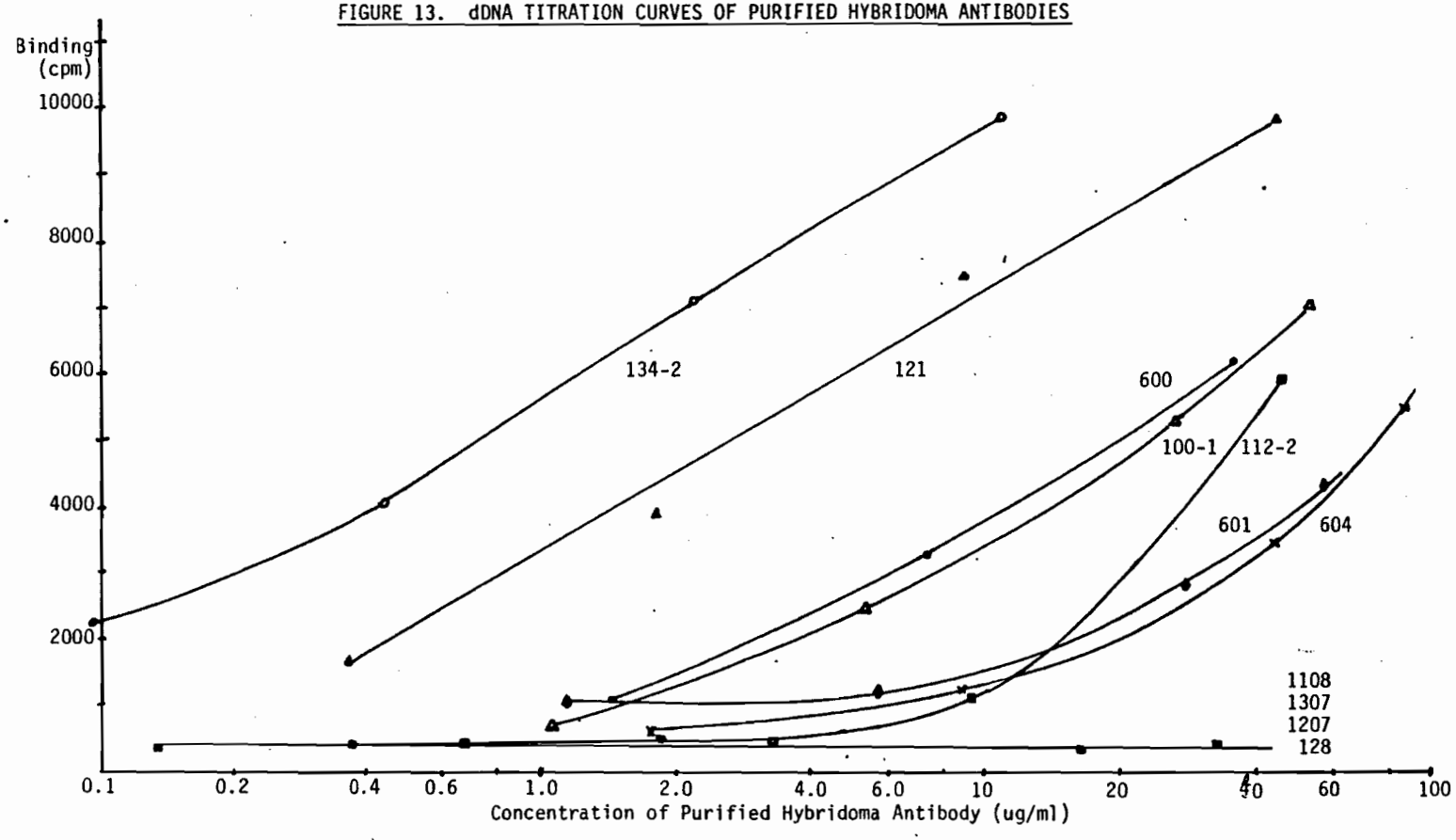


FIGURE 14. dDNA, nDNA, CARDIOLIPIN BINDING OF ANTI-DNA ANTIBODIES PURIFIED OVER A SEPHAROSE RABBIT ANTI-HUMAN IgM COLUMN DILUTED TO 9.8 ug/ml (OD_{280})

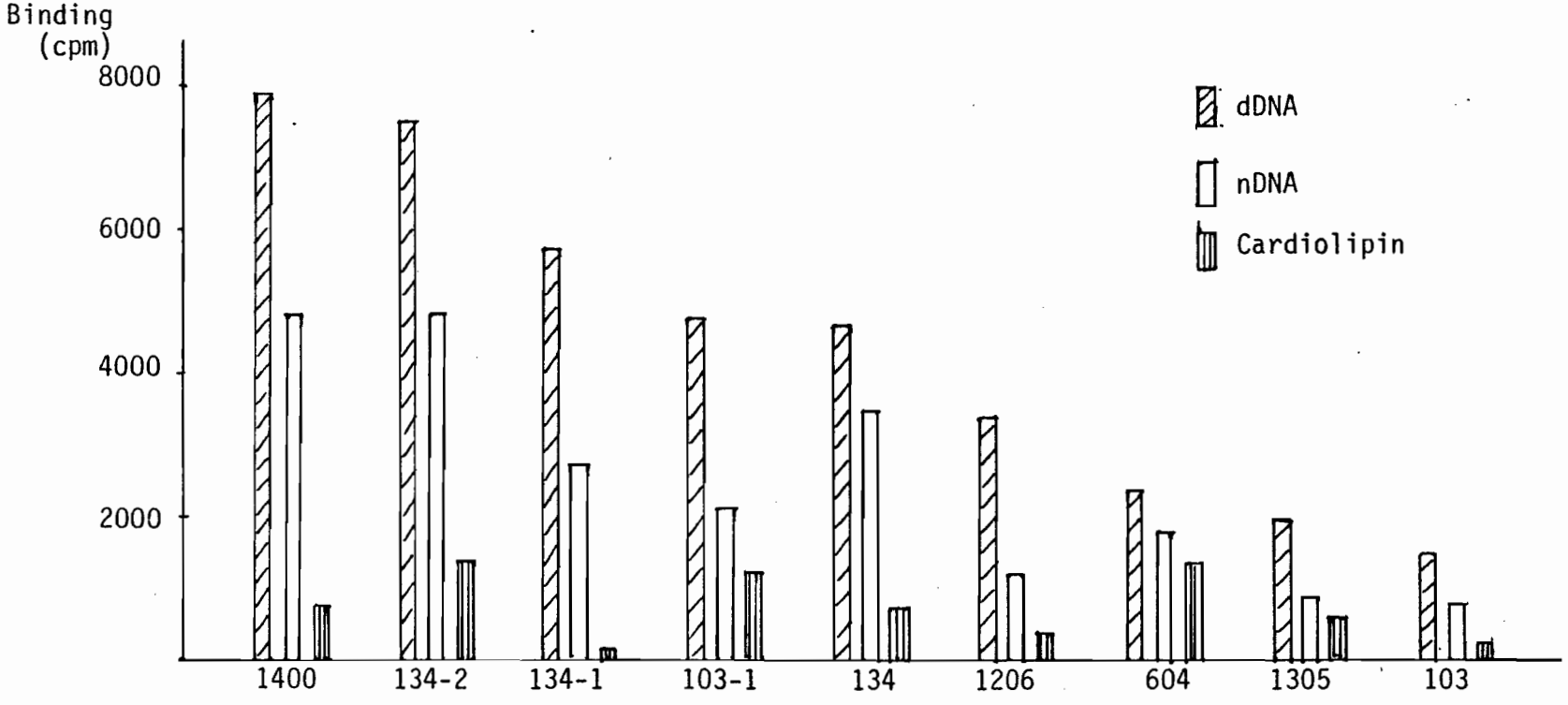


FIGURE 15. UREA FORMATE GEL ELECTROPHORESIS

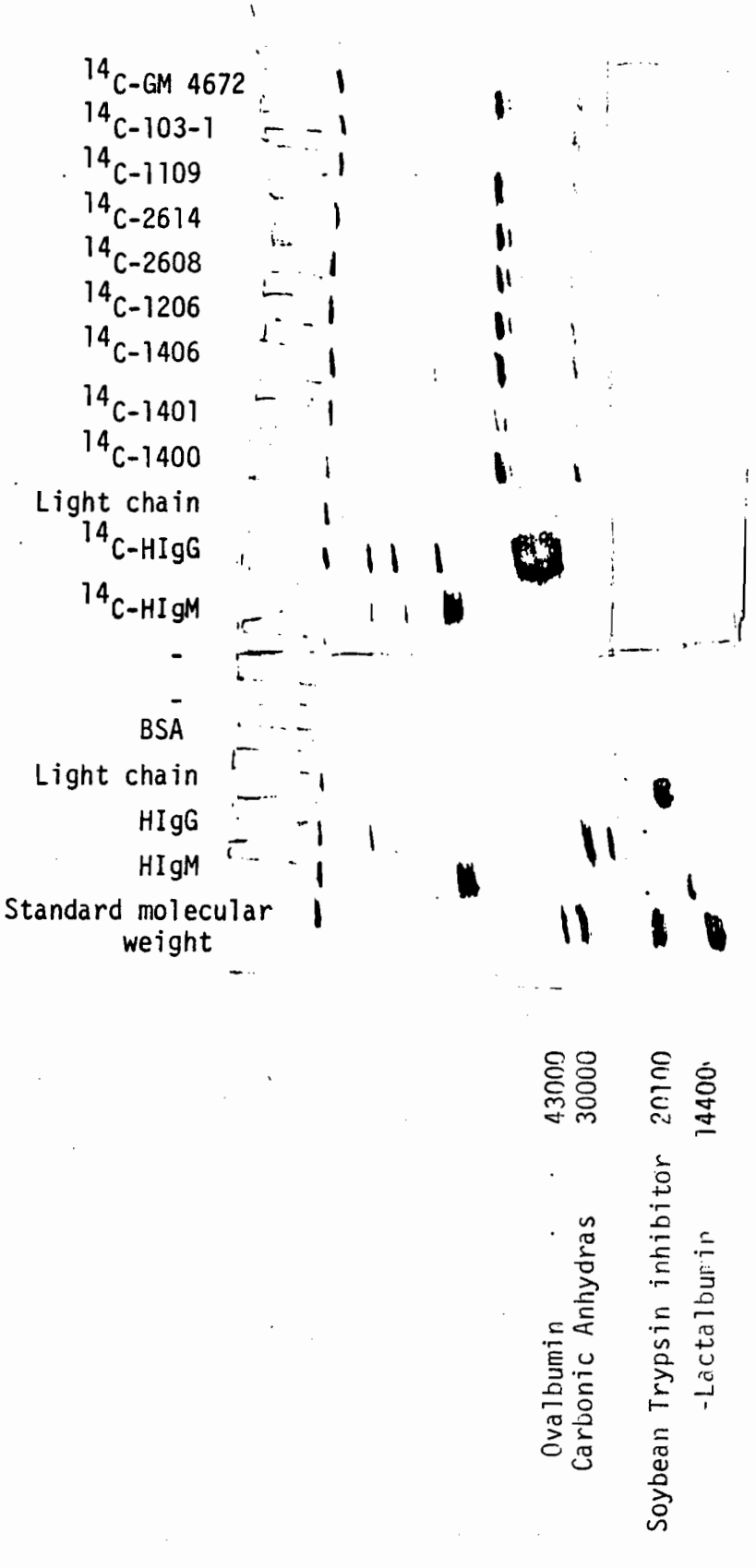
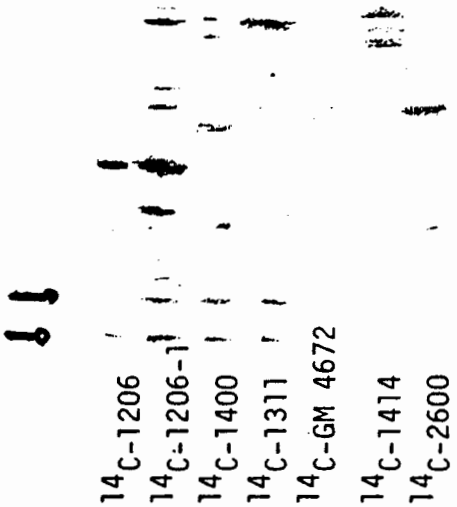
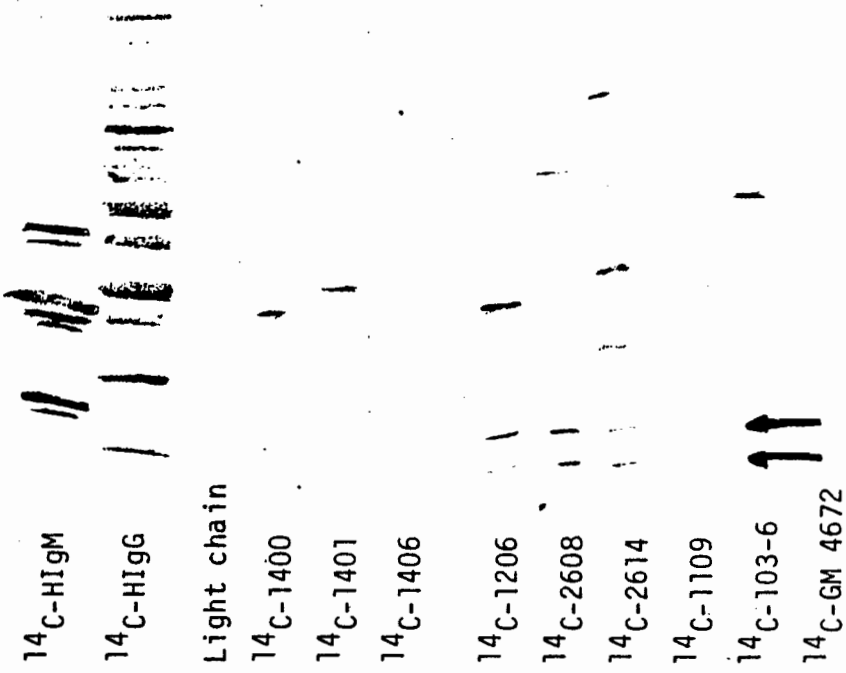


FIGURE 16. ISOELECTROFOCUSING GEL

A. Done in Sherbrooke



B. Done by the author



Chapter 12. CHARACTERIZATION OF RABBIT ANTI-IDIOTYPE ANTISERA

12.1 Screening of Rabbit Sera for Anti-Idiotype Activity

After each bleeding, the rabbit antisera were tested by direct binding to HIgM, HIgG and the immunogen to verify that the rabbits were producing antibodies to the immunogen (an anti-DNA IgM class antibody). Table 17 illustrates the results of a bleeding test assay. Two rabbit bleeds were tested, one immunized with 134 and the other immunized with 103-1. The binding of the antisera (in cpm) to HIgM, HIgG and their respective immunogen are given and compared to a normal rabbit serum. Each antisera bound to HIgG, HIgM and their respective idiotype coated tubes demonstrating that the rabbits were producing anti-HIgM, anti-HIgG antibodies and thus had responded to the immunogen.

12.2 Specificity of Antisera

All 8 rabbit antisera were absorbed on a Sepharose-human IgG or IgM column to render them anti-idiotype specific. For example, rabbit anti-103-1 antiserum was first absorbed three times on a Sepharose-human IgG column and then the specificity of the antiserum was tested by direct binding of the serum to HIgG and to 103-1 coated tubes. Figure 17 illustrates the binding of rabbit anti-103-1 antiserum to 103-1 and HIgG coated tubes before and after this absorption. It can be seen that the activity of anti-103-1 antiserum to HIgG (o——o) is completely removed after its absorption (o----o) and that the reactivity of this antiserum to its

idiotype, 103-1 (●——●) is diminished by only 13% after the absorption (●----●). The antiserum was then applied on a Sepharose-human IgM immunoadsorbent column and the remaining activity retested (Figure 18). Again the activity to HIgM (x——x) was completely removed (x----x) but the activity against the idiotype 103-1 (●——●), although diminished by 41% from its preabsorption value, still remained high after the absorption (●----●). These two figures show that the rabbit anti-103-1 antiserum, after absorptions on both IgG and IgM Sepharose columns, was anti-idiotype specific since it reacted only with 103-1 coated tubes and no longer with normal HIgM or HIgG.

The absorption of rabbit anti-134 antiserum was performed differently. The purpose was to see the effect when the antisera was first absorbed on a Sepharose-human IgM column and then on a Sepharose-human IgG column, which is opposite to the procedure followed for anti-103-1 antiserum. Figure 19 shows the binding of rabbit anti-134 antiserum to HIgM, HIgG and 134 coated tubes before and after three absorptions on Sepharose-HIgM columns and one absorption on Sepharose-HIgG columns. It can be seen that the binding to HIgM and HIgG (x——x, o——o) was completely removed after absorption (x----x, o----o), although the binding to 134 (●——●) remained high (●----●) with only a loss of 3% of binding. Again, this figure shows that anti-134 antiserum antiserum was now anti-idiotype specific. Moreover, it proves that the immnunoabsorbent procedure, whether done first on an HIgM

or on an HIgG column, has no importance. All the antisera were absorbed at least once on a Sepharose-human IgG column and at least twice on a Sepharose-human IgM column. The absorption procedure was stopped when the activity against HIgG and HIgM of absorbed sera was lower than the binding of normal rabbit serum. Table 18 shows the number of times that each antiserum was absorbed on each type of immunoadsorbent column in order to render it anti-idiotype specific.

12.3 Cross-Reactivity of Antisera by Direct Binding

Three antisera were chosen to test their binding activity to each of the other idiotypes. As shown in Figure 20, rabbit anti-134 antiserum was titrated on 134, 103-1, 101-1, IgM and IgG coated tubes. The anti-134 antiserum bound strongly to 134 antibody coated tubes, its homologous idiotype, but also bound highly to 100-1 antibody coated tubes. Binding to 103-1 antibody, IgM or IgG coated tubes was either minimal or completely absent. Therefore, anti-134 antiserum recognized an idiotype on 134 and 100-1 antibody. The rabbit anti-103-1 antiserum was tested in the same manner (Figure 21) and showed strong binding to 103-1 and 134 antibodies but not to 100-1 antibody, IgM or IgG coated tubes. Thus, an idiotype on 103-1 and 134 antibodies is recognized by anti-103-1 antiserum. The third serum tested for cross-reactivity by direct binding was rabbit anti-100-1 antiserum (Figure 22). It bound strongly to the three anti-DNA antibodies, 100-1, 103-1, and 134, but not to IgM or IgG coated tubes. The third antiserum therefore recognized an idiotype common to all three anti-DNA antibodies.

12.4 Direct Binding of the Anti-Idiotype Antisera to dDNA

This test was performed to assess direct binding of the anti-idiotype antisera to dDNA. The anti-idiotype antisera were diluted from 0.1 mg/ml to 0.0125 mg/ml, according to the OD_{280}, the upper range used in the inhibition of binding of idiotypes to dDNA by antisera (Section 12.6). The protein content of each absorbed antiserum determined by OD_{280} was 28.4 mg/ml for anti-100-1 antiserum; 21.06 mg/ml for anti-103-1; 28 mg/ml for anti-134 antiserum; 47.04 mg/ml for anti-1206 antiserum; 2.13 mg/ml for anti-600 antiserum; 10.42 mg/ml for anti-1305 antiserum and 0.27 mg/ml for anti-1400 antiserum. The protein content of the control normal-absorbed rabbit serum was 26.81 mg/ml.

The binding of the antisera to dDNA coated tubes was detected by a labelled goat anti-rabbit IgG. Normal rabbit serum was used for control. Figure 23 shows that, at 0.1 mg/ml all antisera and normal rabbit serum bound to dDNA, varying from less than 500 cpm to 3000 cpm. Three antisera showed high binding to dDNA (over 2500 cpm) when compared with normal rabbit serum (1500 cpm). However, the binding of anti-idiotype antiserum to dDNA was much lower than the binding of the same antiserum to its own idiotype (Section 12.2).

12.5 Anti-Idiotype Binding to dDNA Minimally Affects Anti-DNA Binding to dDNA

An assay was derived to study the effect of anti-idiotype antisera binding to dDNA (Section 12.4) on the ability of anti-DNA antibodies to combine with dDNA coated tubes. Only

idiotype 134 and its antiserum were used, since this antiserum had the highest binding to dDNA. Table 19 demonstrates that preincubation of dDNA coated tubes with normal rabbit serum or with anti-134 antiserum before the addition of 134 antibody did not significantly affect (5% less binding) the binding of 134 antibody to dDNA. When this experiment was performed in the reverse manner, by adding anti-DNA antibody to dDNA coated tubes followed by the anti-idiotype, the results obtained were slightly different. In this case, the binding of 134 to dDNA was 20% lower after the specific anti-idiotype antiserum was added. This 20% diminution in the second experiment compared with the 5% in the first experiment can be explained by taking into account the order of reagents used in each experiment. In the second case, anti-DNA antibody was added first and allowed to combine with the dDNA coated tubes. After three washes of the tubes to remove the free antibody, the anti-idiotype was added and allowed to combine with the anti-DNA antibody already fixed to the dDNA coated tubes. When the labelled rabbit anti-human IgM was added to detect the anti-DNA antibody it was unable (to a magnitude of 20%) to recognize all of the anti-DNA antibody as antigen since some of these molecules were then covered by the anti-idiotype antiserum.

12.6 Site of the Idiotype on the Anti-DNA Antibody

The purpose of this assay was to determine the location of the idiotype on the anti-DNA immunogen: that is, whether the idiotype was located within the binding site of the anti-DNA antibody molecule or outside the binding site of the

anti-DNA antibody molecule. This question was addressed by a series of competition assays where the binding of the anti-DNA antibody to dDNA was either inhibited or not affected in the presence of the specific rabbit antisera (Figure 24A, B, C, D, E). If the antiserum inhibited the binding of the anti-DNA antibody to its antigen (DNA) this indicated that the antiserum was directed against an idiotype in the binding site of the anti-DNA antibody. If this did not occur, then the anti-idiotype serum was directed to an idiotype located outside the binding site of the anti-DNA antibody.

Figure 24A illustrates the results of the inhibition of the binding of 134 anti-DNA antibody to dDNA in the presence of rabbit anti-134 antiserum and other antisera. The rabbit anti-134 antiserum inhibited the binding of 134 antibody to dDNA coated tubes. As the concentration of antiserum increased, from 0.0001 to 0.1 mg/ml, it became possible to completely block the binding of 134 antibody to DNA. The presence of normal rabbit serum or other anti-idiotype antisera, such as rabbit anti-1400, anti-600, and anti-604, did not affect the binding of 134 anti-DNA antibody to DNA. Since the rabbit 134 anti-idiotype antiserum affected the binding of the antibody 134 to the antigen DNA, this antiserum was directed against an idiotype located within the binding site of the 134 antibody. Figure 24A also demonstrates that rabbit anti-1305, anti-100-1, anti-103-1, and anti-1206 antisera were also capable of inhibiting the binding of 134 antibody to dDNA albeit to a lesser extent than anti-134, suggesting that anti-1206, anti-

103-1, anti-100-1, anti-1305, and the anti-134 antisera each recognized an idiotype structure expressed in the DNA-binding site of 134 antibody, but not necessarily the same one.

Figure 24B shows the results of the inhibition of binding of 103-1 anti-DNA antibody to dDNA in the presence of rabbit anti-103-1 antiserum and other anti-idiotype antisera. The anti-103-1 antiserum was directed against an idiotype located in the binding site of the 103-1 anti-DNA antibody, since the anti-103-1 antiserum blocked the binding of 103-1 antibody to dDNA, while normal rabbit serum, anti-1400, anti-1206, anti-600, anti-604, and anti-1305 antisera did not have any effect. Antisera anti-100-1 and anti-134 partially inhibited the binding of 103-1 to dDNA, indicating that they both recognized an idiotype on 103-1 antibody.

Figure 24C shows that rabbit anti-1400 antiserum was directed against an idiotype located in the binding site of 1400 antibody. An idiotype on 1400 antibody was also recognized by anti-134 and anti-1305 antisera, but not by normal rabbit serum or by any of the other antisera, as shown in Figure 24C.

Figure 24D shows that rabbit anti-604 antiserum, at a concentration of 0.1 mg/ml, reduced the binding of 604 antibody to dDNA to 73% of its original binding. An experiment repeated with higher amount of antiserum (from 0.1 to 2 mg/ml) showed that, in the presence of 2 mg/ml of antiserum, the binding of 604 antibody to dDNA was reduced to 11%. Thus, rabbit anti-604 antiserum was directed against an idiotype located close to or in the binding site of the 604 antibody molecule. An idiotype

on 604 antibody was also recognized by rabbit anti-134 and anti-101-1 antisera, and not by other anti-idiotype antisera or normal rabbit serum.

Finally, figure 24E shows that rabbit anti-100-1 antiserum was directed against an idiotype located in the binding site of the 100-1 antibody. An idiotype expressed on 100-1 antibody, however, was better recognized by the anti-134 antiserum (less binding observed in its presence). Also, all the other antisera recognized a determinant present on 100-1 antibody that was not recognized by normal rabbit serum.

The two other antisera, anti-600, and anti-1305, did not inhibit the binding of their idiotypes to dDNA even at high amounts (0.1 to 2 mg/ml), which suggests that they were directed against an idiotype located outside of the binding site of their respective immunogens. Moreover, none of the other antisera succeeded to inhibit the binding of the 600, 1305, and 1206 idiotypes, but in fact some enhancement of the binding of 600 and 1305 anti-DNA antibodies to dDNA was observed. 15% inhibition of binding of 1206 antibody to dDNA was observed in the presence of the anti-1206 antiserum. At higher amounts of anti-1206 antiserum (0.1 to 2 mg/ml), more inhibition was observed, but inhibition of binding by normal rabbit serum was also evident. The location of the idiotype on the 1206 anti-DNA antibody molecule seems to be close to the antigen binding site. Additional results are needed to confirm this (see Section 12.7).

All of the above results are summarized in Table 20. Five antisera were directed against an idiotype located in the antigen binding site of the antibody, one idiotype was located near the antigen binding site, while the other two were directed against an idiotype located outside of the antigen binding site. The idiotypes located in the antigen binding sites of these antibody molecules were recognized by at least two antisera other than the one raised against that antibody.

12.7 Inhibition of Binding of Idiotypes to Homologous Anti-Idiotype Antisera Coated Tubes by Polynucleotides, Cardiolipin and Anti-Idiotype Antisera

This competition assay was another indirect approach to assess whether the anti-idiotype antisera were directed against idiotypes located within or outside of the antigen combining sites of the antibody molecules. Each anti-DNA antibody (idiotype) was placed in the presence of polynucleotides, cardiolipin or their homologous anti-idiotype antisera (Section 5.6) prior to testing the binding of the mixture to anti-idiotype antisera coated tubes. If the idiotype was located within the antigen combining site of the antibody, then the binding of the competitor to the anti-DNA antibody would block the idiotype, preventing the anti-DNA from binding to homologous anti-idiotype coated tubes. However, if the idiotype on the anti-DNA molecule was located outside of the antigen combining site of the antibody molecule, then the binding of the competitor to the antibody would not affect the binding of the idiotype to its homologous anti-

idiotype coated tubes.

Figures 25 to 29 show the inhibition of binding of five anti-DNA antibodies (idiotypes) to their homologous anti-idiotype coated tubes by polynucleotides, cardiolipin and by anti-idiotype antisera. All five anti-DNA antibodies were inhibited by their homologous anti-idiotype antisera and not by normal rabbit serum. They were also inhibited by polynucleotides and by cardiolipin, depending upon the affinity of the individual anti-DNA antibodies for these competitors (Section 11.7, Table 14). In these experiments, the order of competitors for the inhibition of binding of 103-1 idiotype to its anti-103-1 antiserum coated tubes (Figure 25) was poly (dT), poly (I), dDNA, cardiolipin, nDNA which was similar to the order seen in the competition of the binding of 103-1 to dDNA (Section 11.8, Table 15). The order of competitors for the inhibition of binding of 1206 and 1400 idiotypes to their anti-idiotype antisera coated tubes (Figures 27, 28) is also similar to the order seen in Table 14. By contrast, the order of competitors for the inhibition of binding of 134 idiotype to its anti-134 antiserum coated tubes (Figure 26) was dDNA, poly (dT), cardiolipin, poly (I), nDNA, which is completely different from what was observed in Table 14 (poly (I), poly (dT), dDNA, cardiolipin, nDNA). In the case of the inhibition of binding of 604 idiotype to its anti-604 antiserum coated tubes (Figure 29), 50% inhibition was not reached at the highest amount of competitor used.

The inhibition of binding of 1305 idiotype to its

anti-1305 antiserum coated tubes was successful only at 20% inhibition in the presence of anti-1305 antiserum, but not by either normal rabbit serum, polynucleotides or cardiolipin. In fact, an enhancement of the binding of 1305 idiotype to its anti-1305 antiserum coated tubes was observed in the presence of the polynucleotides.

The inhibition of binding of 600 or 100-1 idiotypes to their anti-idiotype antisera coated tubes was not performed because it was impossible to obtain the binding of the idiotype to the coated tubes once it was diluted 1:2 with the competitors.

The above leads to the conclusion that the 134, 103-1, 1206, 1400, and 604 idiotypes are all located inside the antigen combining site of the anti-DNA antibody, since the binding to the anti-DNA antibody to the polynucleotides blocked the binding of the antibody to its homologous anti-idiotype antiserum coated tubes. The 1305 idiotype is located outside the antigen combining site. These results confirm previous data demonstrated in Section 12.6, Table 20.

12.8 Study of Two Antisera Raised Against the Same Idiotype by Two Different Rabbits

This section compares the response of two individual rabbits to the same immunogen. Each time an anti-idiotype antiserum was raised, two rabbits were immunized with the same purified idiotype (anti-DNA antibody). Both antisera shared the same name but one was called A and the other B, referring to the two different rabbits. For example, if the

idiotype used as immunogen was called 604, the antisera raised against it were called anti-604 antiserum A and anti-604 antiserum B. Each of the antisera raised were submitted to the same manipulations: absorption, test of specificity, location of the idiotype on the immunogen, and inhibition of binding of the anti-idiotype to its homologous idiotype coated tubes by purified hybridoma autoantibodies.

The number of times that both antisera A and B were absorbed on each Sepharose HIgM and HIgG columns differed, as shown for 5 anti-idiotype antisera in Table 21. Some antisera, for example anti-100-1B, needed more passages on one column (IgG) than its corresponding antiserum, anti-100-1A.

The direct binding reactivity of absorbed antisera A and B to the immunogen coated tubes for two different anti-idiotype antisera (anti-604 and anti-1305) is shown in Figures 30A and B. It can be seen that one rabbit (B in both cases) had a better immune response to the immunogen than the other. This difference was seen with all antisera A and B raised.

Antisera A and B of two other anti-idiotype antisera were further compared and characterized. Figure 31 shows the inhibition of binding of 103-1 idiotype to dDNA coated tubes by the presence of anti-103-1 antisera (A and B), anti-134 (A and B), and of normal rabbit serum. As mentioned in Section 12.6, this assay localizes the site of the idiotype on the immunogen molecule and may show the sharing of idiotype by different anti-DNA antibody molecules. It can be seen in Figure 31 that both anti-103-1 antisera (A and B) are directed

against an idiotype located in the antigen-combining site of 103-1 anti-DNA antibody, since both antisera completely blocked the binding of 103-1 antibody to dDNA coated tubes. Less rabbit anti-103-1 antiserum B than anti-103-1 antiserum A was needed to achieve 100% inhibition of binding of 103-1 antibody. Rabbit anti-134 antiserum B also partially inhibited the binding of 103-1 idiotype to dDNA coated tubes, thus recognizing an idiotype located in the antigen-combining site of 103-1 antibody molecule. Rabbit anti-134 antiserum A and normal rabbit serum did not have any effect on the binding of 103-1 antibody to dDNA coated tubes.

Figure 32 shows the inhibition of binding of 134 anti-DNA antibody to dDNA coated tubes in the presence of the same sera as above. Both anti-134 antisera, A and B, completely blocked the binding of 134 idiotype to dDNA coated tubes, thus recognizing an idiotype located in the antigen-combining site of the anti-DNA antibody molecule. Again, less quantity of one antiserum (A) compared to the other (B) was required to achieve 100% inhibition of binding of 134 idiotype. Rabbit anti-103-1 antisera, A and B, and normal rabbit serum had no effect on the binding of 134 idiotypes to dDNA coated tubes.

Figures 33 and 34 show the inhibition of binding of anti-103-1 antiserum B and anti-134 antiserum B, respectively, to their homologous idiotype coated tubes by 12 purified hybridoma anti-DNA antibodies, one non-DNA binding antibody, and normal IgM and IgG (the latter purified from the culture fluid of GM 4672 cell line). A similar experiment was

performed with anti-103-1 antiserum A and anti-134 antiserum A and the data is presented in Figures 35 and 36 of the following chapter. It can be seen that the binding of both anti-idiotype antisera A and B of the anti-103-1 and anti-134 antisera was inhibited by the same anti-DNA antibodies with the same strength.

In this study, no major differences were noted between antisera A and B raised against the same idiotype but in different animals. Therefore, only those antisera (A or B) having the highest direct binding reactivity to their idiotype coated tubes were used in the Sections 12.2 to 12.6, and in Chapter 13. Thus, the antiserum A was used for anti-100-1, anti-103-1, anti-134, and anti-1206 and the antiserum B for the other anti-idiotype antisera (anti-600, anti-604, anti-1305, and anti-1400).

TABLE 17

BINDING OF NORMAL RABBIT SERUM AND OF UNABSORBED RABBIT ANTISERA TO IgM, IgG, AND THE IDIOTYPE USED AS IMMUNOGEN

	Dilution of serum used	Binding to (in cpm) HIgG	HIgM	134	103-1
Normal rabbit serum	1/100	1341	1841	941	767
	1/200	936	1136	323	426
	1/400	913	1198	323	287
Rabbit anti-134 antiserum	1/100	6659	55226	50383	N.D.*
	1/200	7011	54418	50887	N.D.
	1/400	5589	52097	48300	N.D.
Rabbit anti-103-1 antiserum	1/100	8550	55831	N.D.	45472
	1/200	8328	53781	N.D.	43985
	1/400	7007	54639	N.D.	42143

* not determined

FIGURE 17. REACTIVITY OF ANTI-103-1 ANTISERUM BEFORE AND AFTER ABSORPTION ON A SEPHAROSE-HUMAN IgG COLUMN

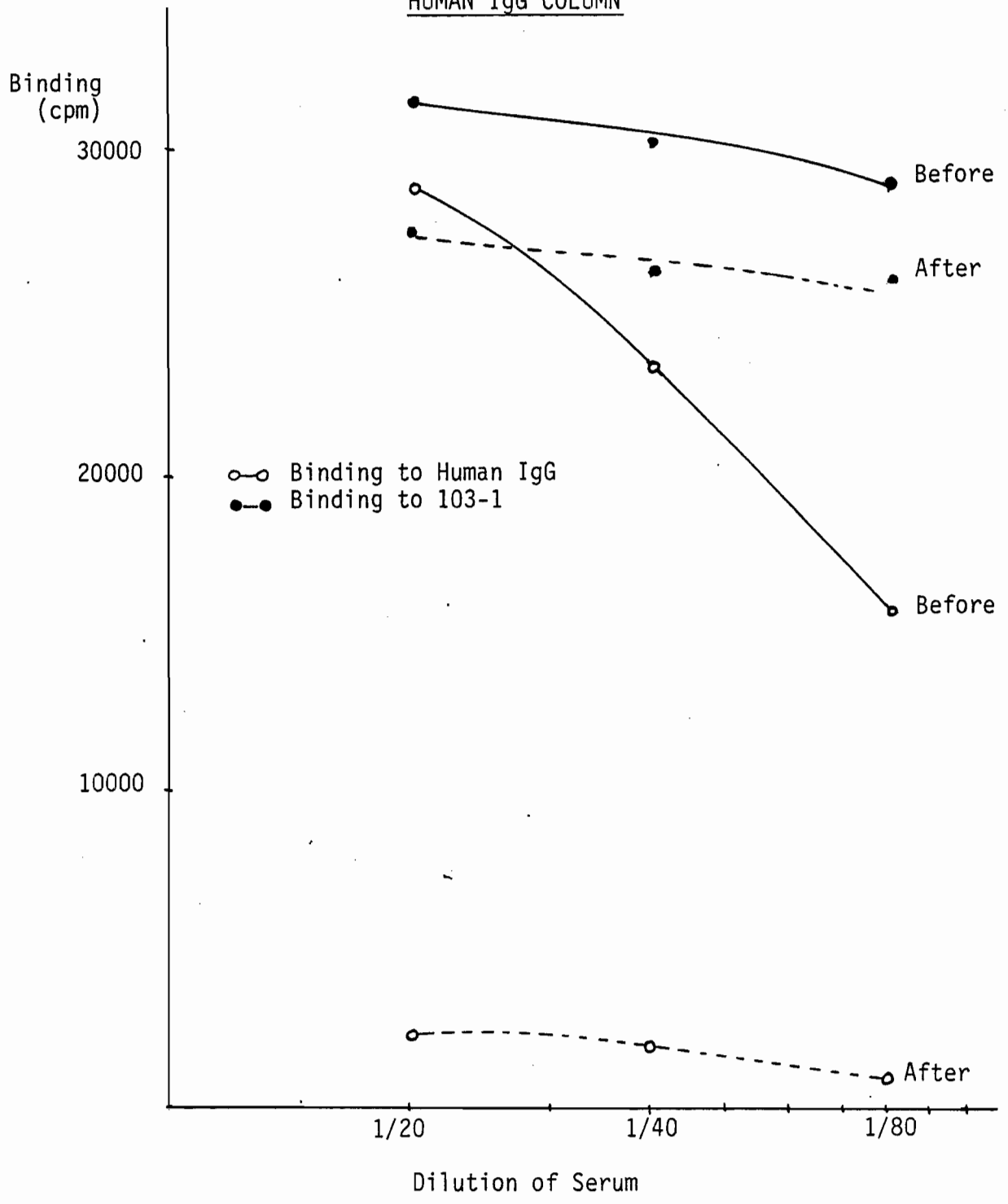


FIGURE 18. REACTIVITY OF ANTI-103-1 ANTISERUM BEFORE AND AFTER ABSORPTION ON A SEPHAROSE-HUMAN IgM COLUMN

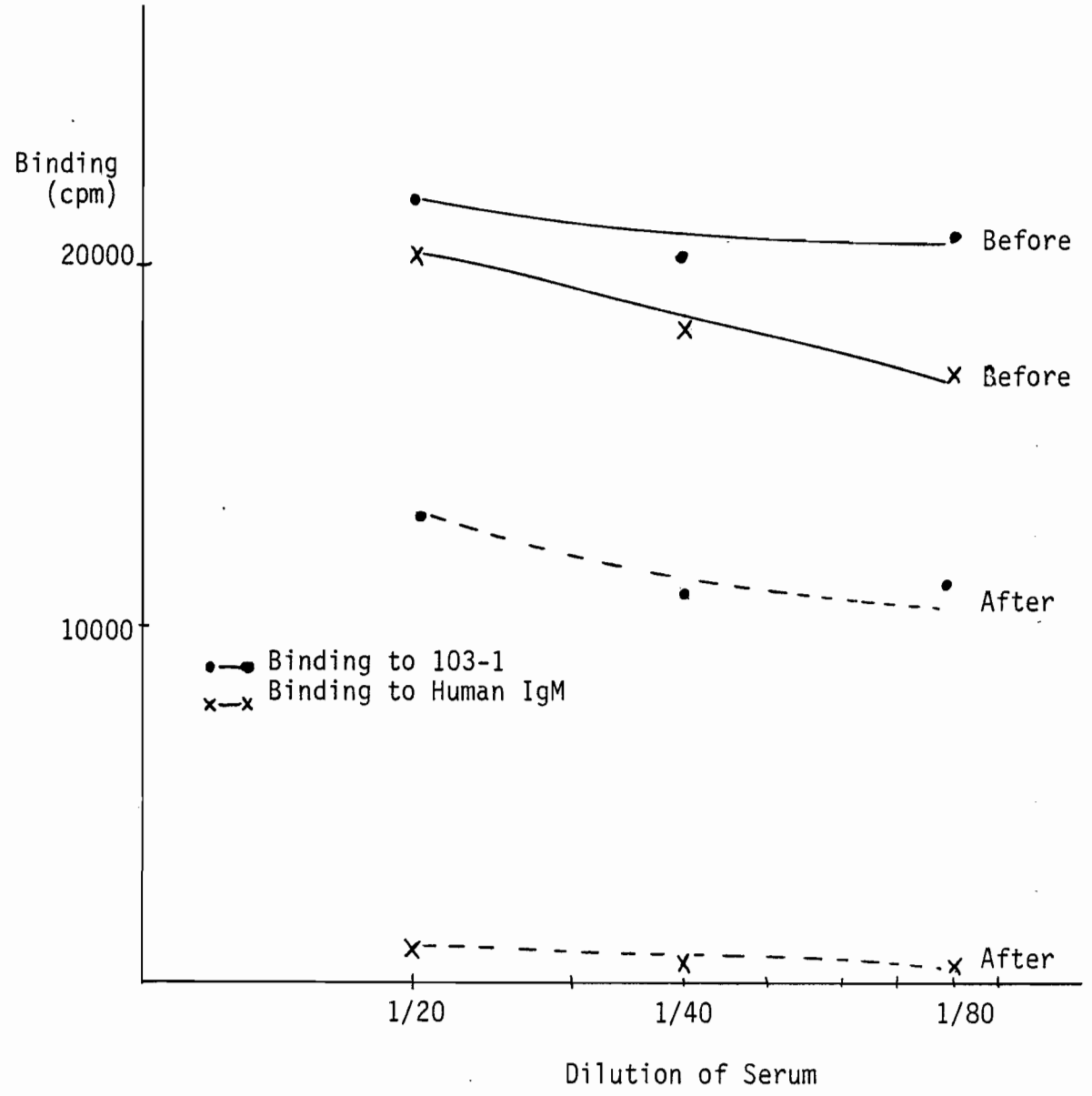


FIGURE 19. REACTIVITY OF ANTI-134 ANTISERUM BEFORE AND AFTER ABSORPTION ON SEPHAROSE-HUMAN IgM AND IgG COLUMNS

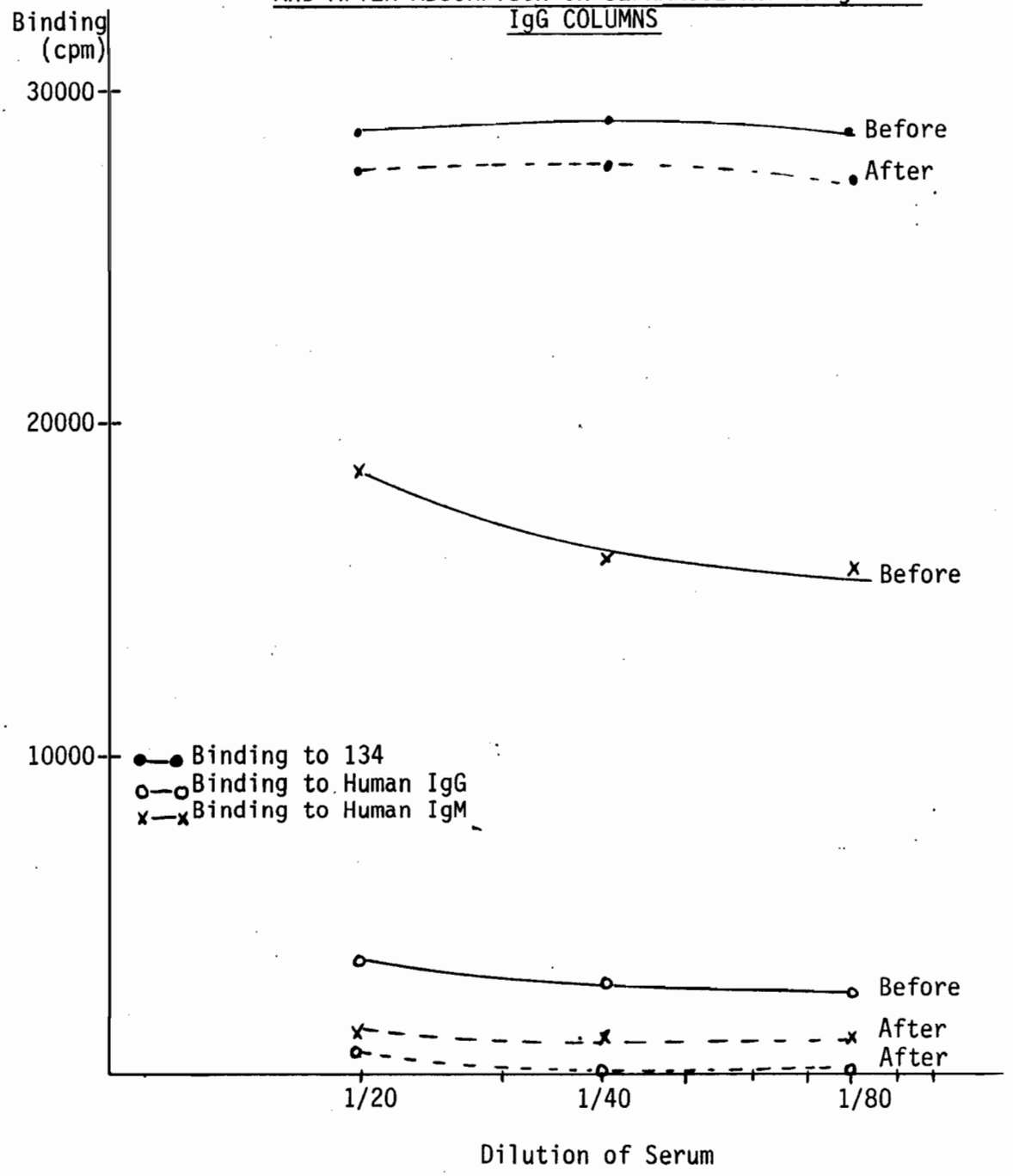


Table 18

NUMBER OF TIMES THAT EACH ANTISERUM WAS PASSED OVER EACH HIgM AND HIgG COLUMN

Antisera	Number of times passed through HIgM	HIgG
anti-100-1	2	2
anti-103-1	3	3
anti-134	3	3
anti-600	1	1
anti-604	3	2
anti-1206	2	1
anti-1305	2	1
anti-1400	9	1

TABLE 19

dDNA BINDING OF 134 ANTI-DNA ANTIBODY AFTER PREINCUBATION OF THE TUBES WITH NORMAL RABBIT SERUM OR WITH RABBIT ANTI-134 ANTISERUM OR VICE VERSA

Preincubation with	Incubation with	Binding (in cpm)
Tween 0.01%	134	9144
Normal rabbit serum		
0.1 mg/ml	134	8667
0.06 mg/ml	134	8941
Rabbit anti-134 antiserum		
0.1 mg/ml	134	8994
0.06 mg/ml	134	9336
0.03 mg/ml	134	9346
0.015 mg/ml	134	9110
	Rabbit anti-134 antiserum	
134	0.1 mg/ml	7470
134	0.06 mg/ml	7805
134	0.03 mg/ml	7795
134	0.015 mg/ml	8234
134	0.007 mg/ml	8755

FIGURE 20. REACTIVITY OF RABBIT ANTI-134 ANTISERUM WITH DIFFERENT HYBRIDOMA ANTI-DNA ANTIBODIES

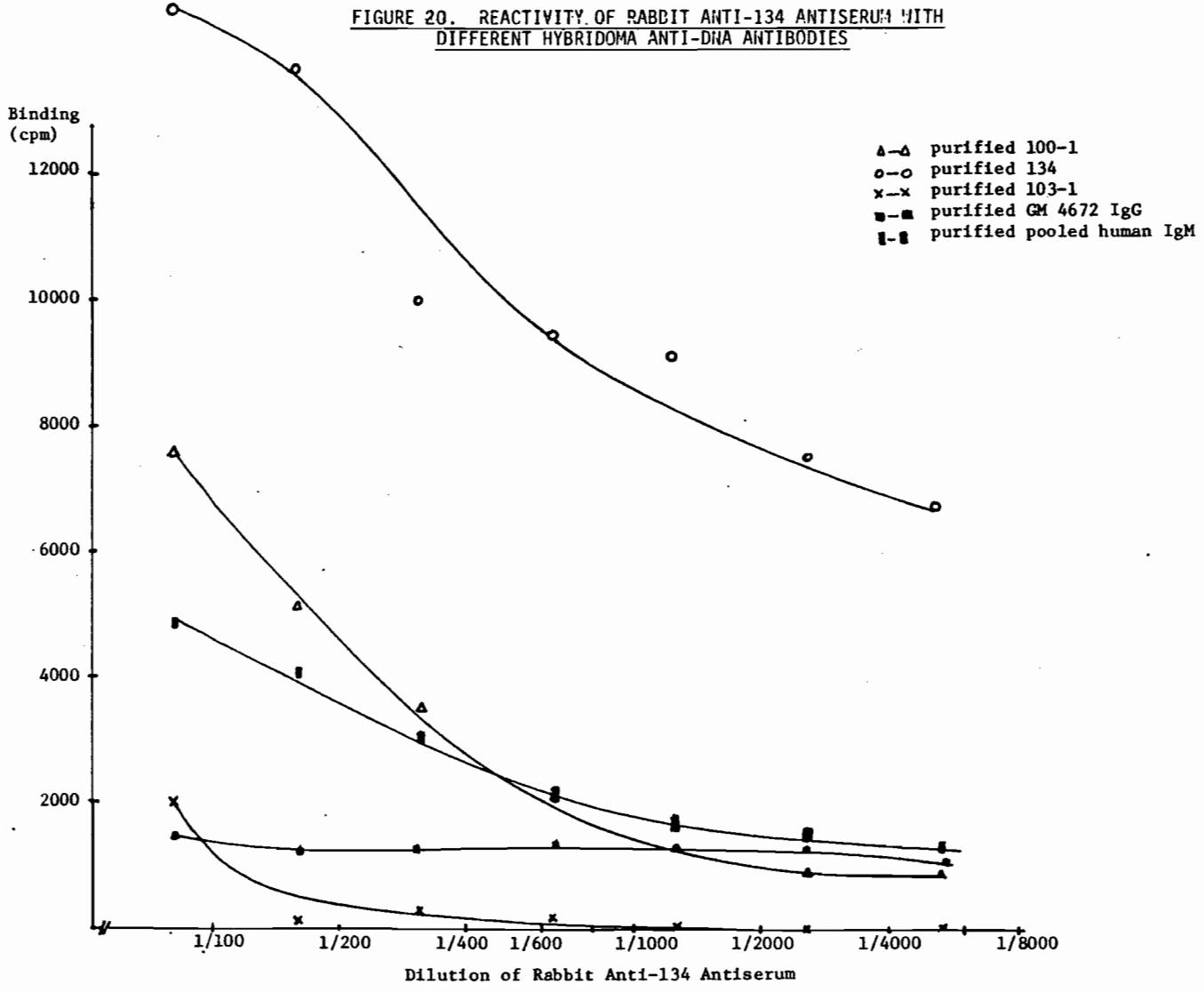


FIGURE 21. REACTIVITY OF RABBIT ANTI-103-1 ANTISERUM WITH DIFFERENT HYBRIDOMA ANTI-DNA ANTIBODIES

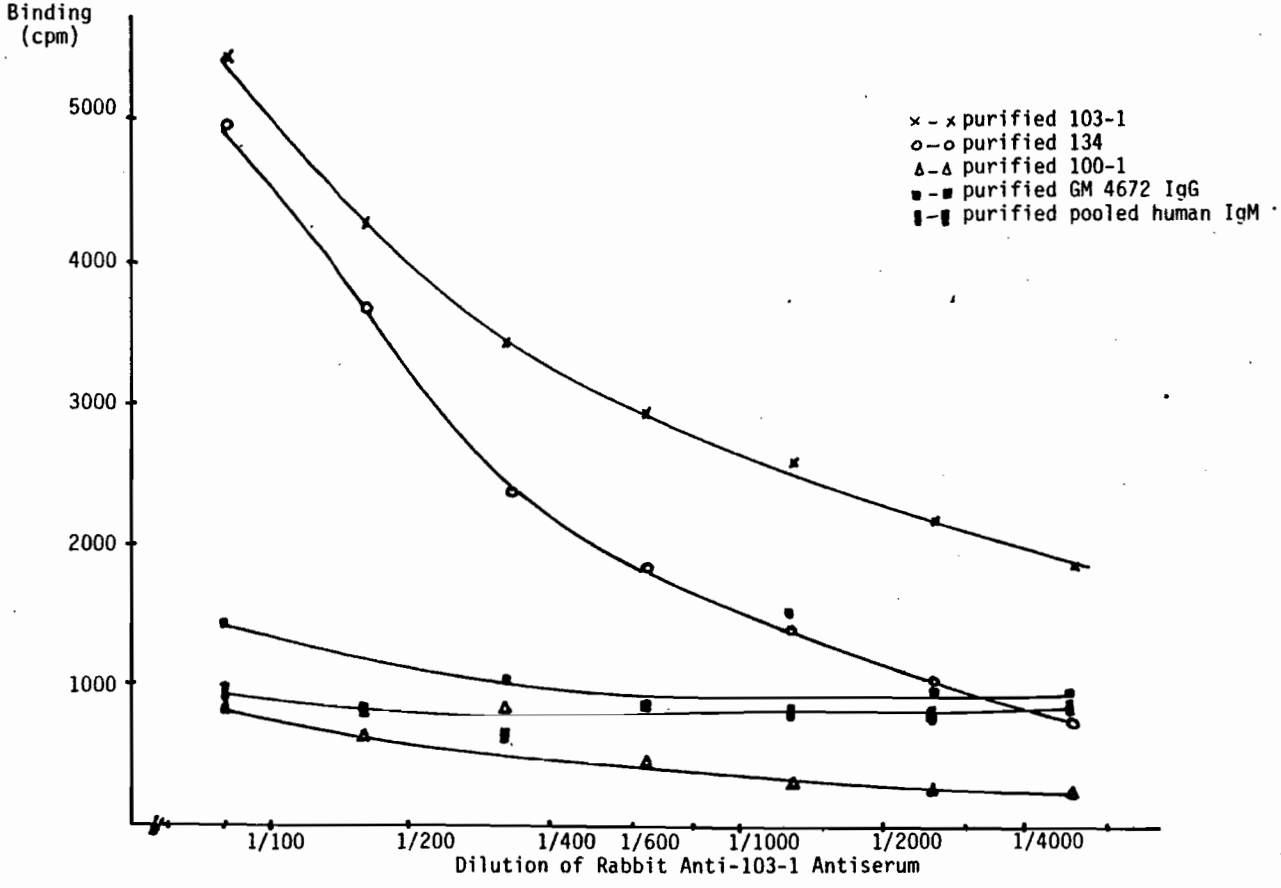


FIGURE 22. REACTIVITY OF RABBIT ANTI-100-1 ANTISERUM WITH DIFFERENT HYBRIDOMA ANTI-DNA ANTIBODIES

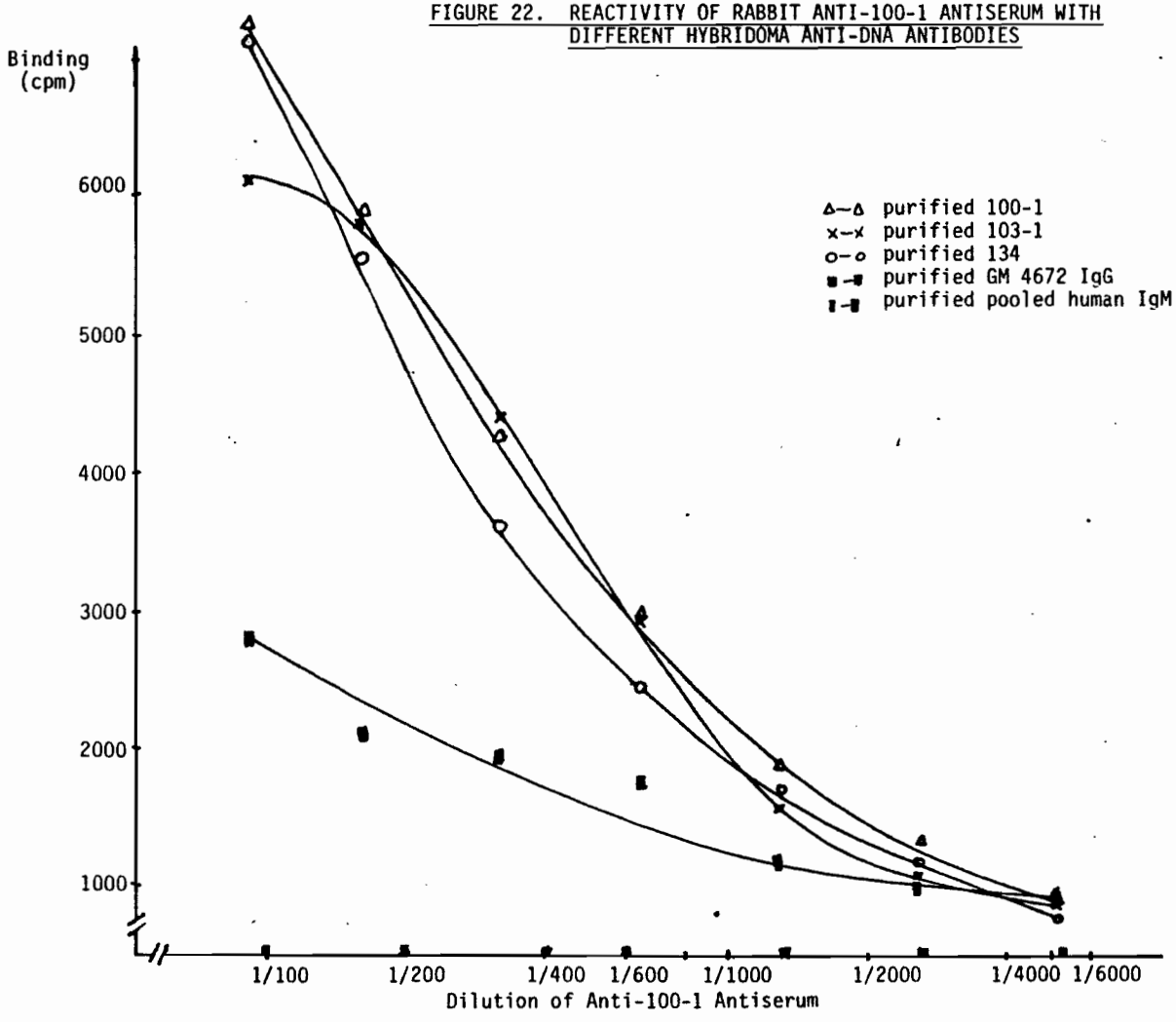


FIGURE 23. dDNA BINDING OF RABBIT ANTI-IDIOTYPE ANTISERA

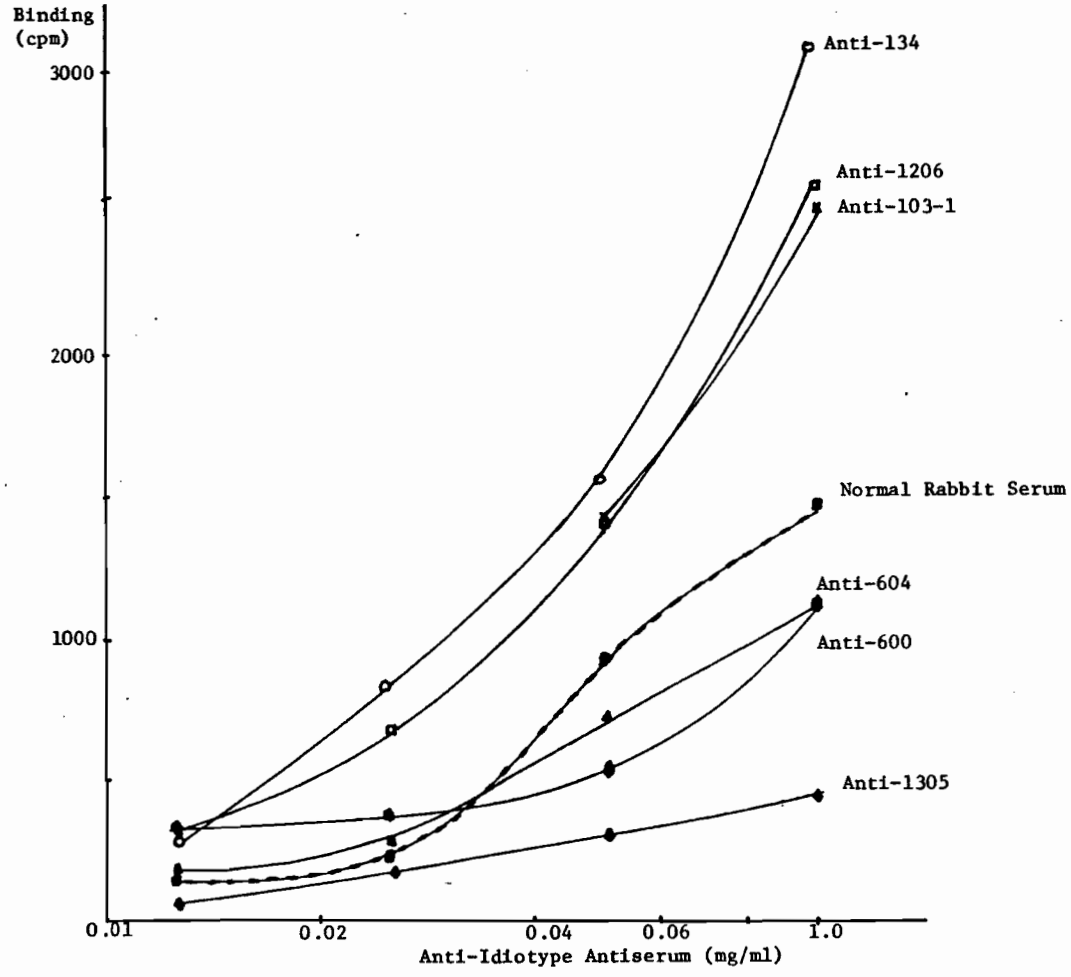


FIGURE 24. INHIBITION OF dDNA BINDING OF HYBRIDOMÄ ANTI-DNA ANTIBODIES BY ANTI-IDIOTYPE ANTISERA

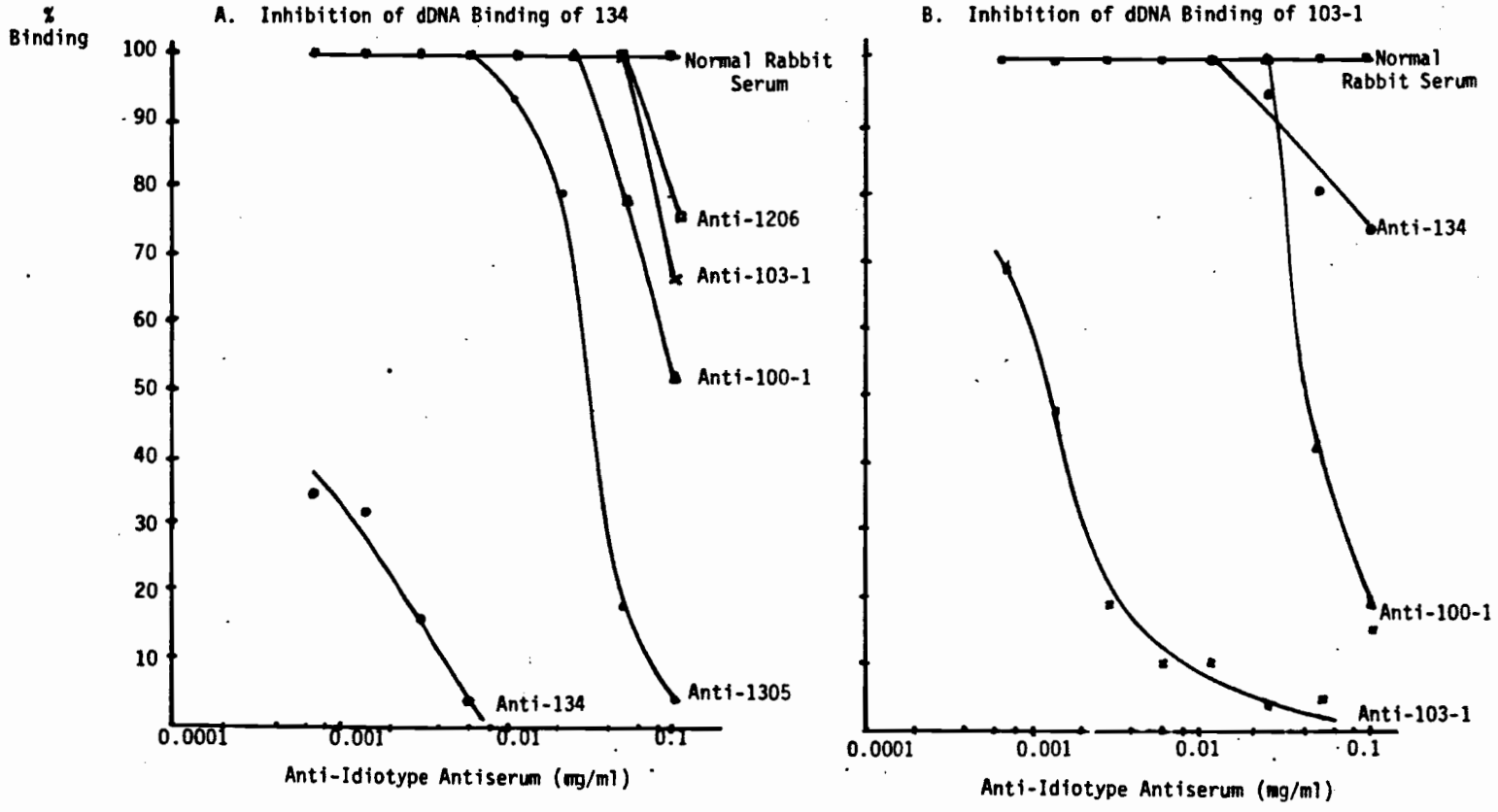


FIGURE 24. (continued)

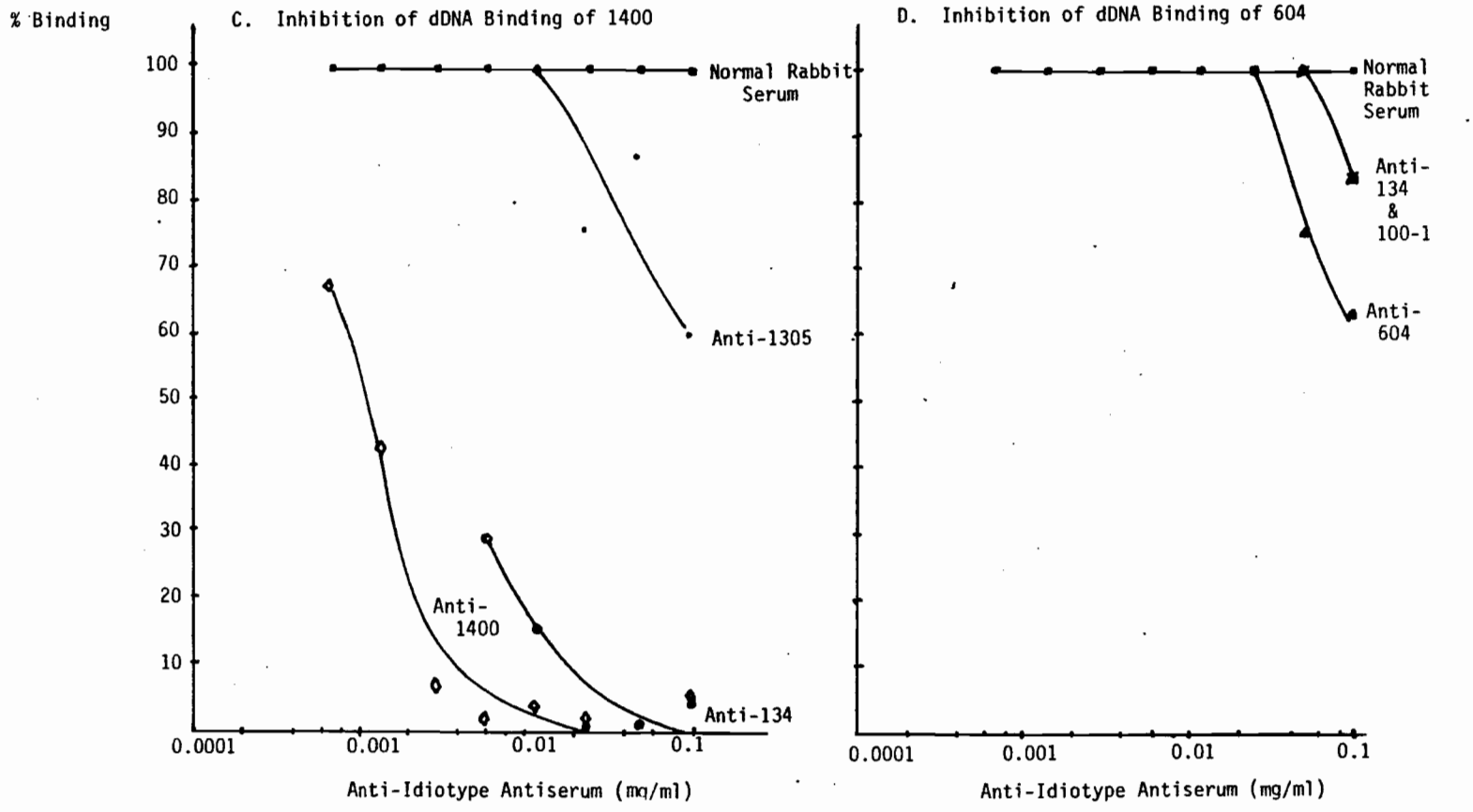


FIGURE 24. (continued)

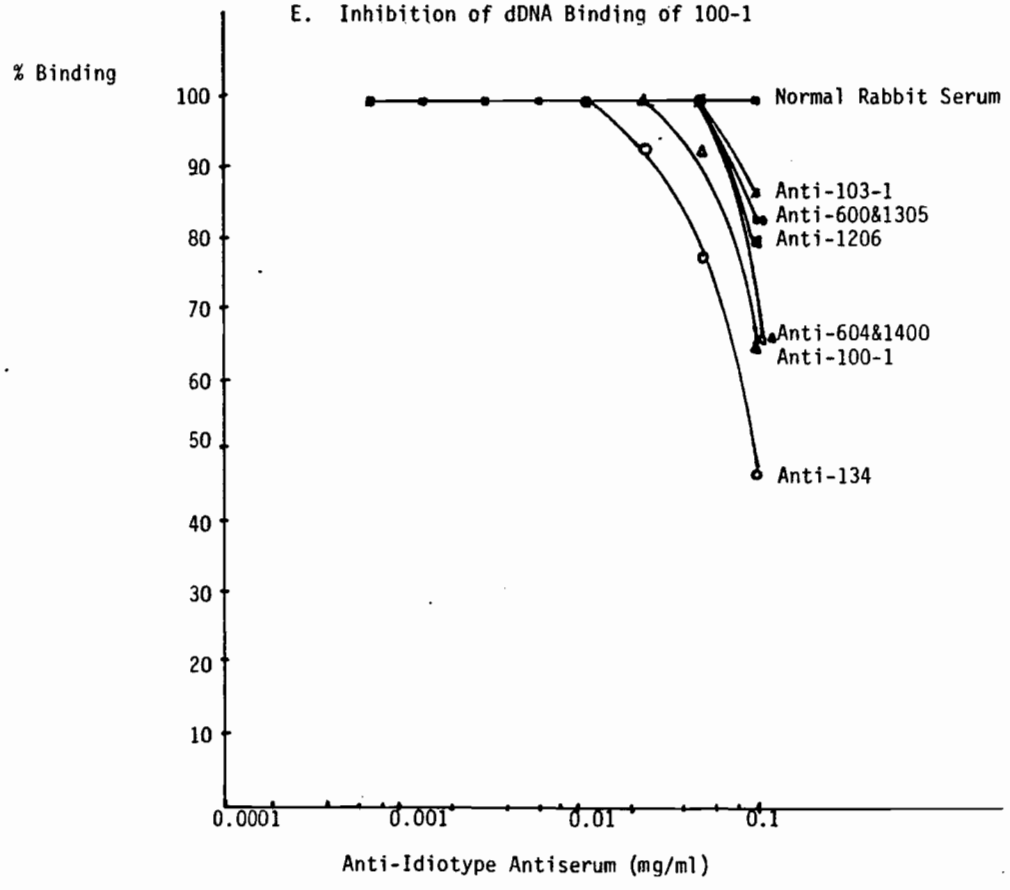


TABLE 20

LIST OF ANTISERA THAT INHIBIT THE dDNA BINDING OF EACH ANTI-DNA ANTIBODY USED AS IMMUNOGEN AND LOCALIZATION OF THE IDIOTYPE ON THAT ANTI-DNA ANTIBODY

	Idiotype (anti-DNA antibody)							
Rabbit Anti-idiotype	100-1	103-1	134	604	1400	1206	600	1305
Anti-100-1	+*	+	+	+	-	-	-	-
Anti-103-1	+	+	+	-	-	-	-	-
Anti-134	+	+	+	+	+	-	-	-
Anti-604	+	- **	-	+	-	-	-	-
Anti-1400	+	-	-	-	+	-	-	-
Anti-600	+	-	-	-	-	-	-	-
Anti-1206	+	-	+	-	-	-	-	-
Anti-1305	+	-	+	-	+	-	-	-
Site of the idiotype on the antibody molecule	inside					near	outside	
	of the antigen binding site							

* (+) indicates that the antiserum inhibited the binding of the idiotype to dDNA coated tubes

** (-) signifies that the antiserum did not have any effect on the binding of the idiotype to dDNA coated tubes

FIGURE 25. INHIBITION OF BINDING OF 103-1 ANTI-DNA ANTIBODY TO RABBIT ANTI-103-1 ANTISERUM BY POLYNUCLEOTIDES, CARDIOLIPIN, AND RABBIT ANTI-103-1 ANTISERUM

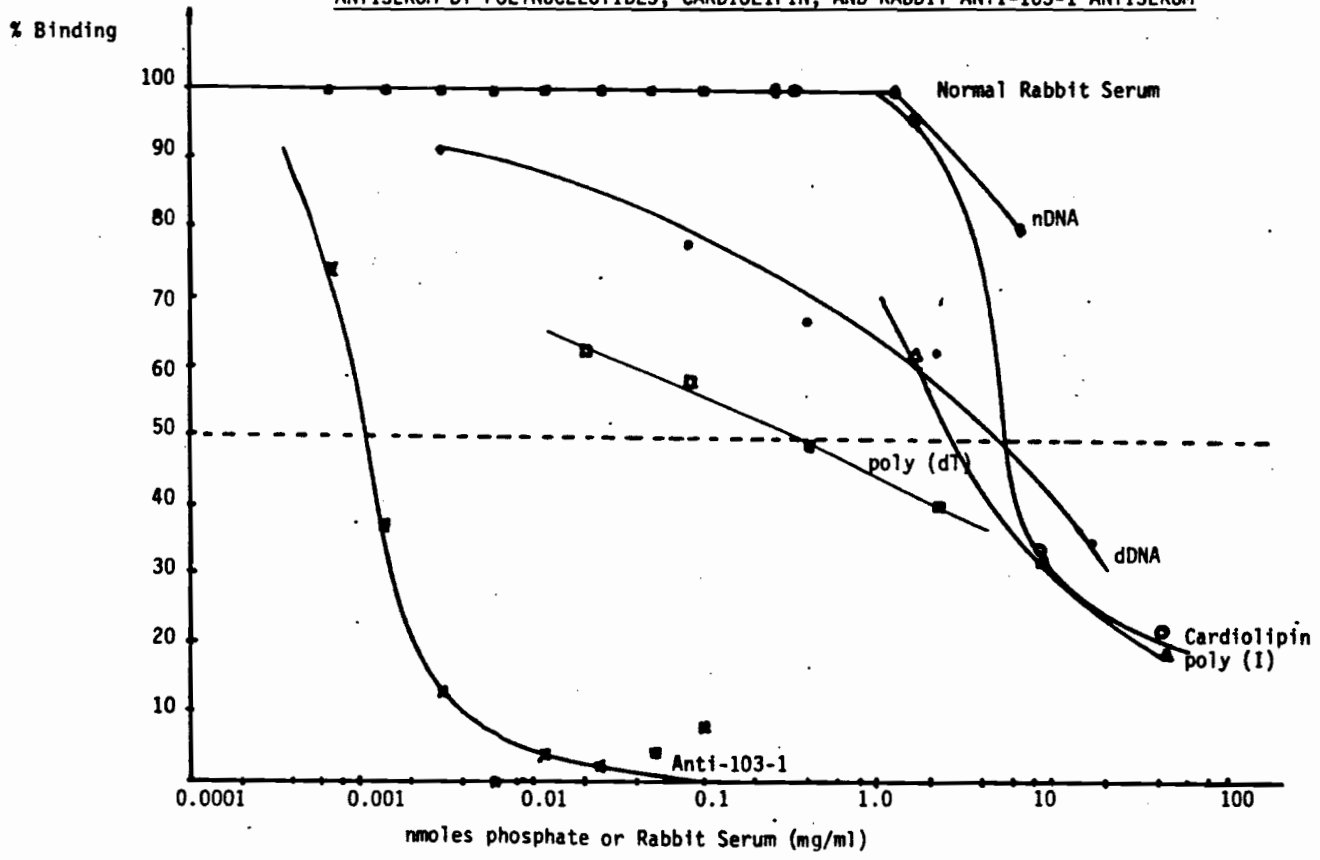


FIGURE 26. INHIBITION OF BINDING OF 134 ANTI-DNA ANTIBODY TO RABBIT ANTI-134 ANTISERUM BY POLYNUCLEOTIDES, CARDIOLIPIN, AND RABBIT ANTI-134 ANTISERUM

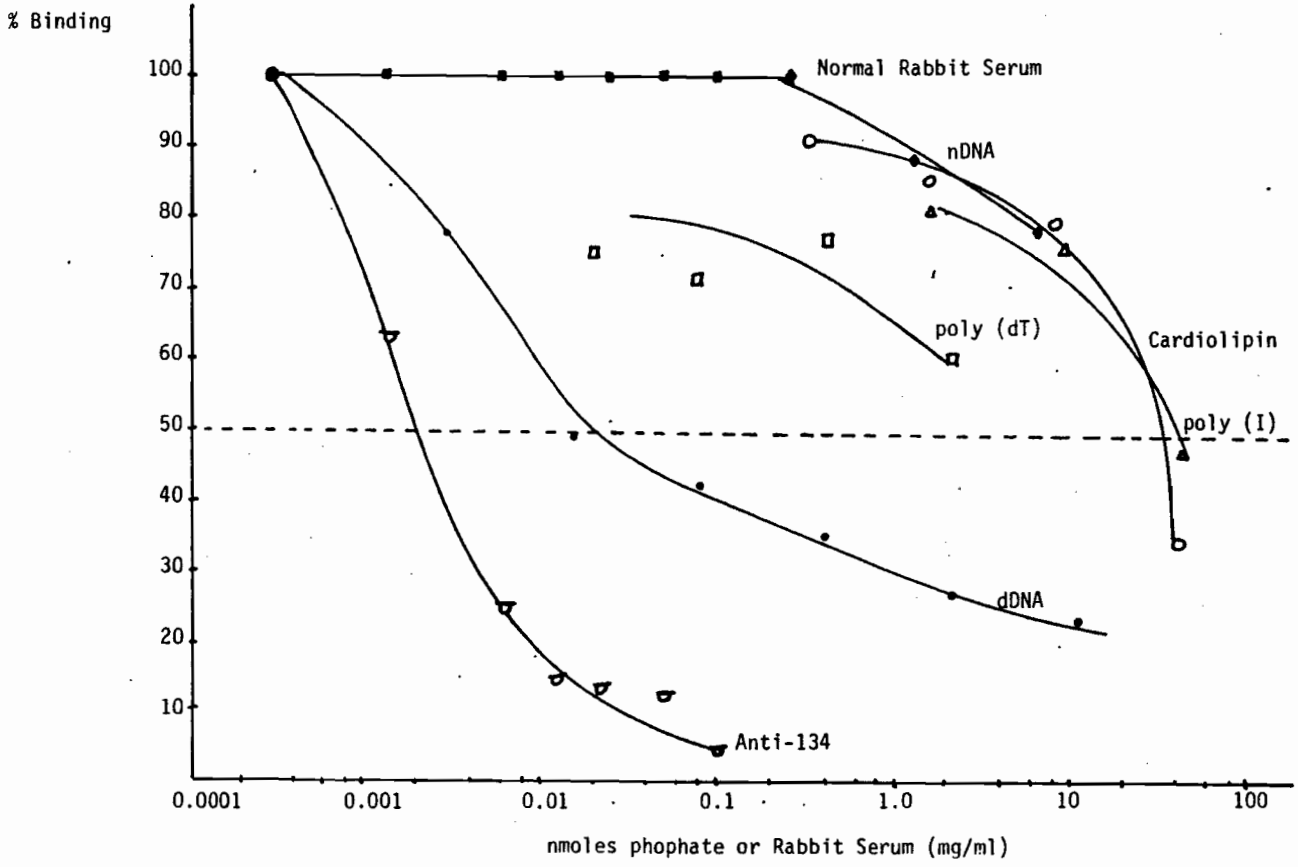


FIGURE 27. INHIBITION OF BINDING OF 1206 ANTI-DNA ANTIBODY TO RABBIT ANTI-1206 ANTISERUM BY POLYNUCLEOTIDES, CARDIOLIPIN, AND RABBIT ANTI-1206 ANTISERUM

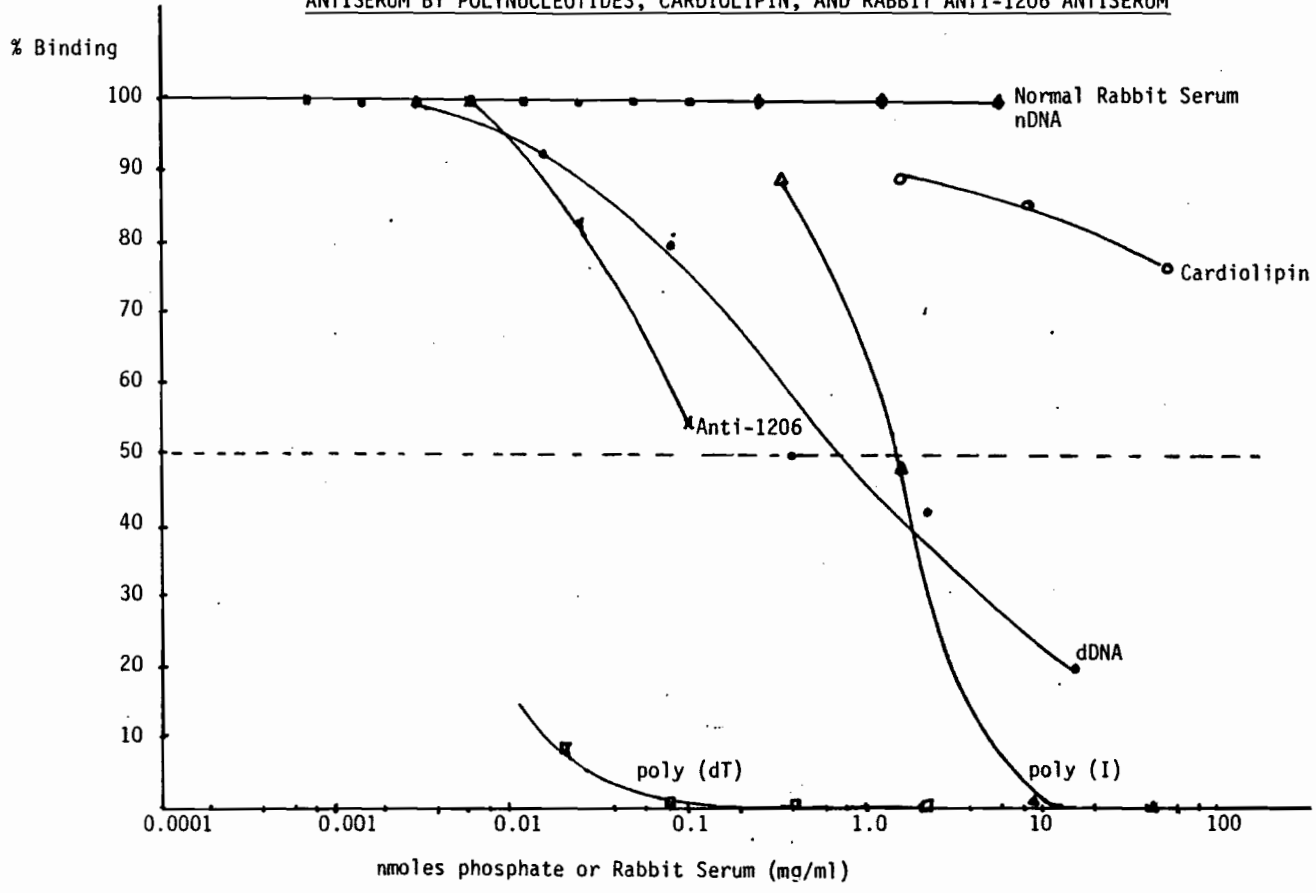


FIGURE 28. INHIBITION OF BINDING OF 1400 ANTI-DNA ANTIBODY TO RABBIT ANTI-1400 ANTISERUM BY POLYNUCLEOTIDES, CARDIOLIPIN, AND RABBIT ANTI-1400 ANTISERUM

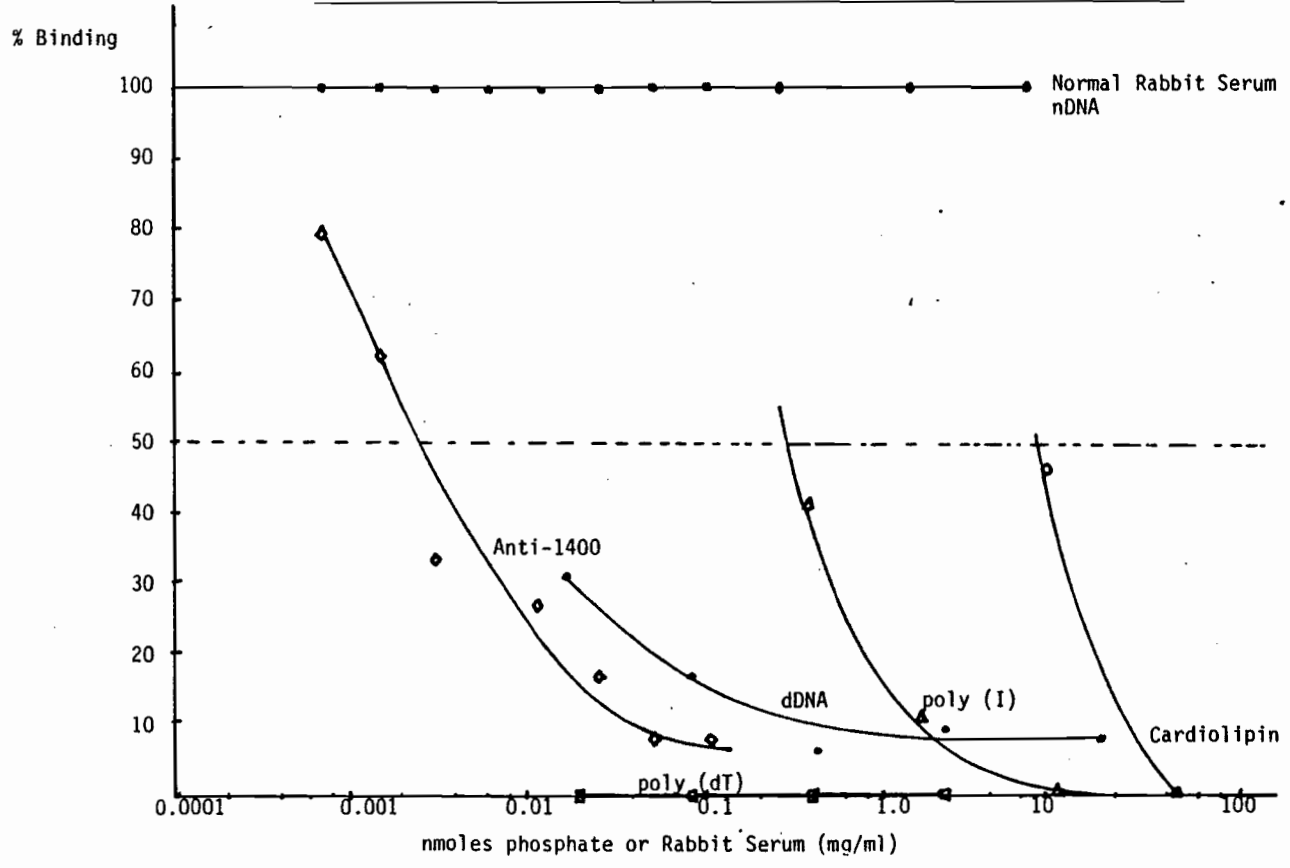


FIGURE 29. INHIBITION OF BINDING OF 604 ANTI-DNA ANTIBODY TO RABBIT ANTI-604 ANTISERUM BY POLYNUCLEOTIDES, CARDIOLIPIN, AND RABBIT ANTI-604 ANTISERUM

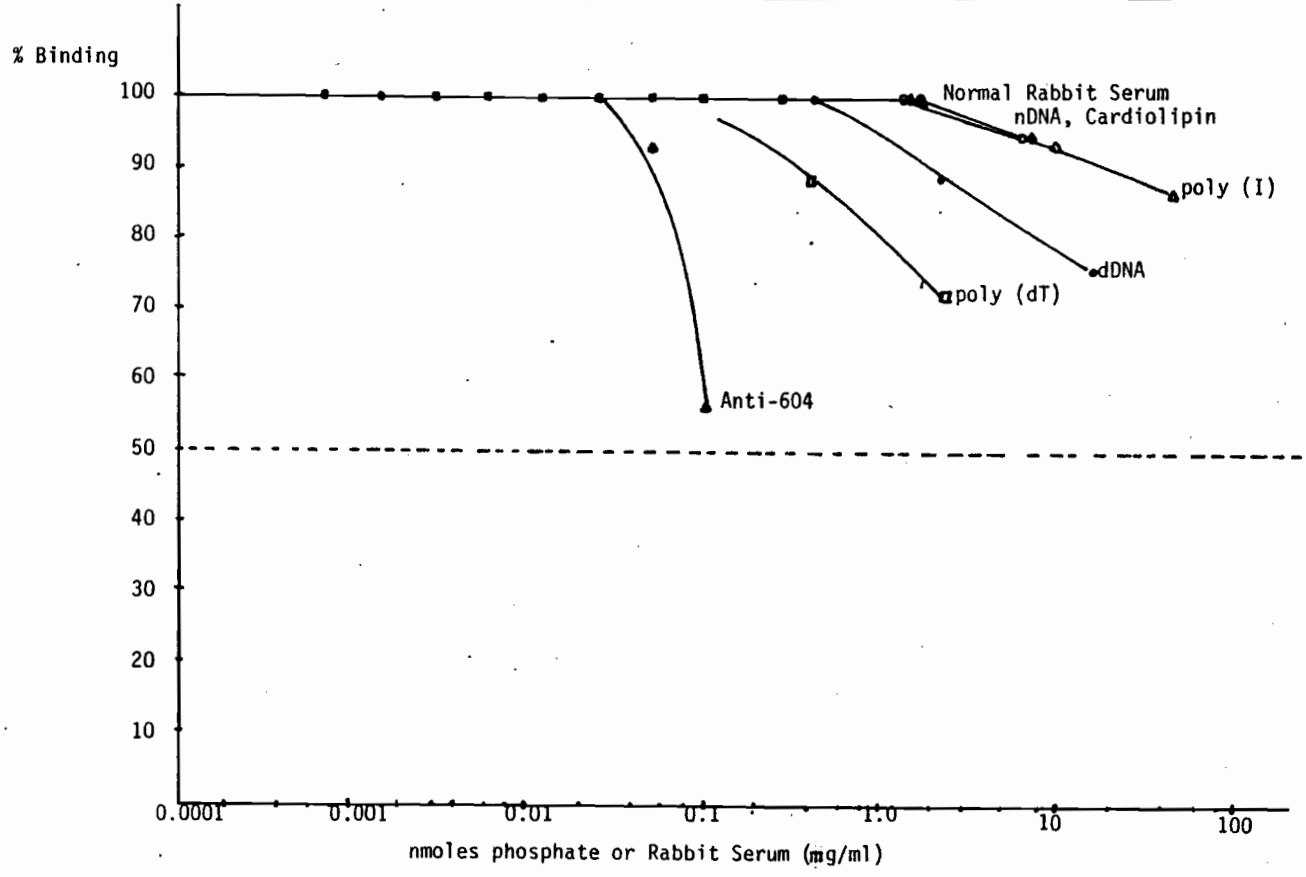


TABLE 21

COMPARISON OF NUMBER OF TIMES THAT EACH ANTISERUM A AND B* WAS PASSED OVER EACH HIgM AND HIgG COLUMN

	Antisera	Number of times passed through HIgM	HIgG
+	Anti-100-1A	2	2
	Anti-100-1B	2	3
+	Anti-103-1A	3	3
	Anti-103-1B	2	2
+	Anti-134A	3	3
	Anti-134B	3	2
+	Anti-604A	3	2
	Anti-604B	3	2
+	Anti-1305A	1	2
	Anti-1305B	2	1

* refers to the antisera raised against the same idiotype but made into two rabbits - A and B

\+ antisera used to complete Table 18 and the following data

FIGURE 30. DIRECT BINDING COMPARISON OF 2 ANTI-IDIOTYPE ANTISERA RAISED AGAINST THE SAME IDIOTYPE INTO TWO DIFFERENT ANIMALS

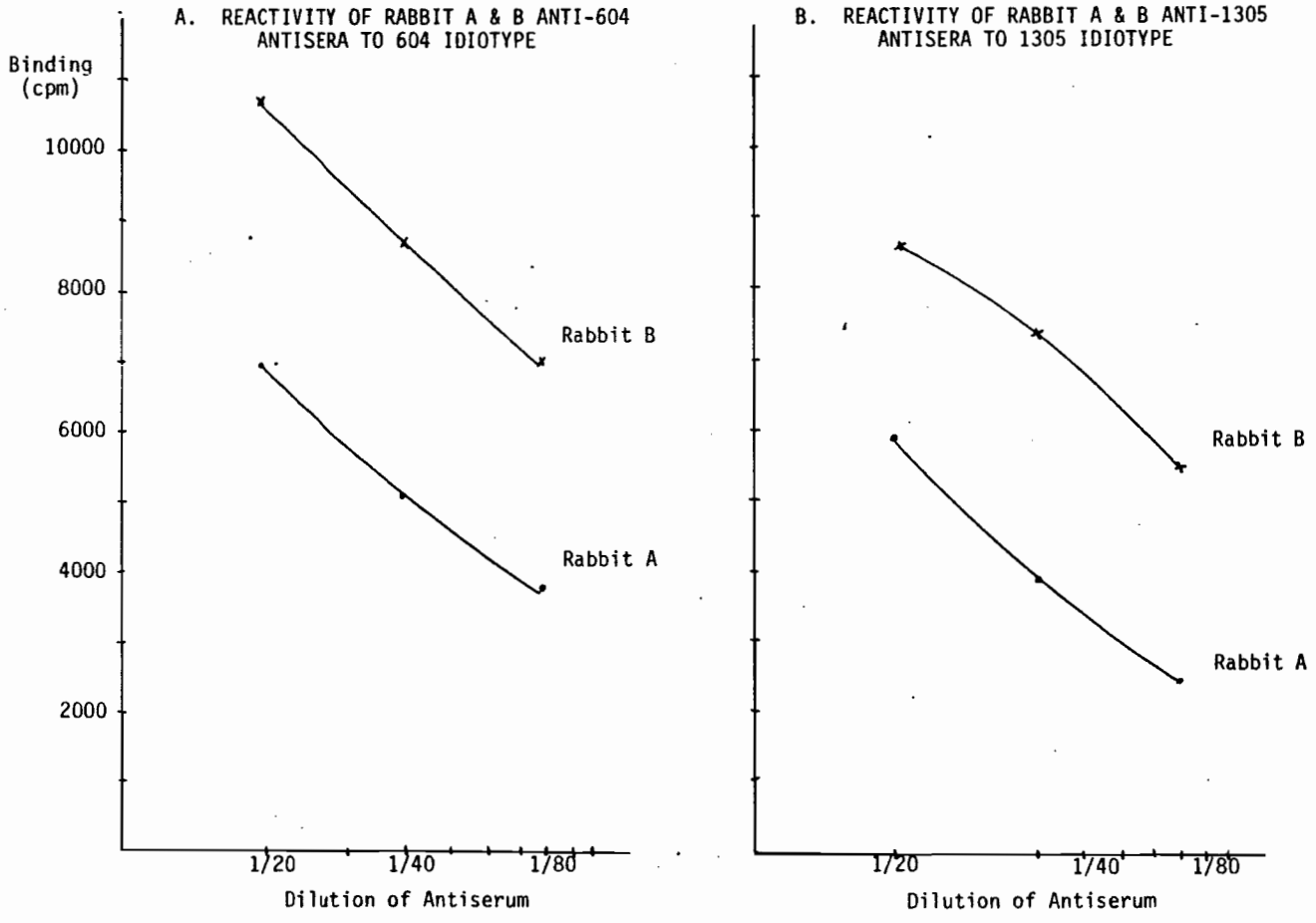


FIGURE 31. INHIBITION OF dDNA BINDING OF 103-1 ANTIBODY BY ANTI-IDIOTYPE ANTISERA

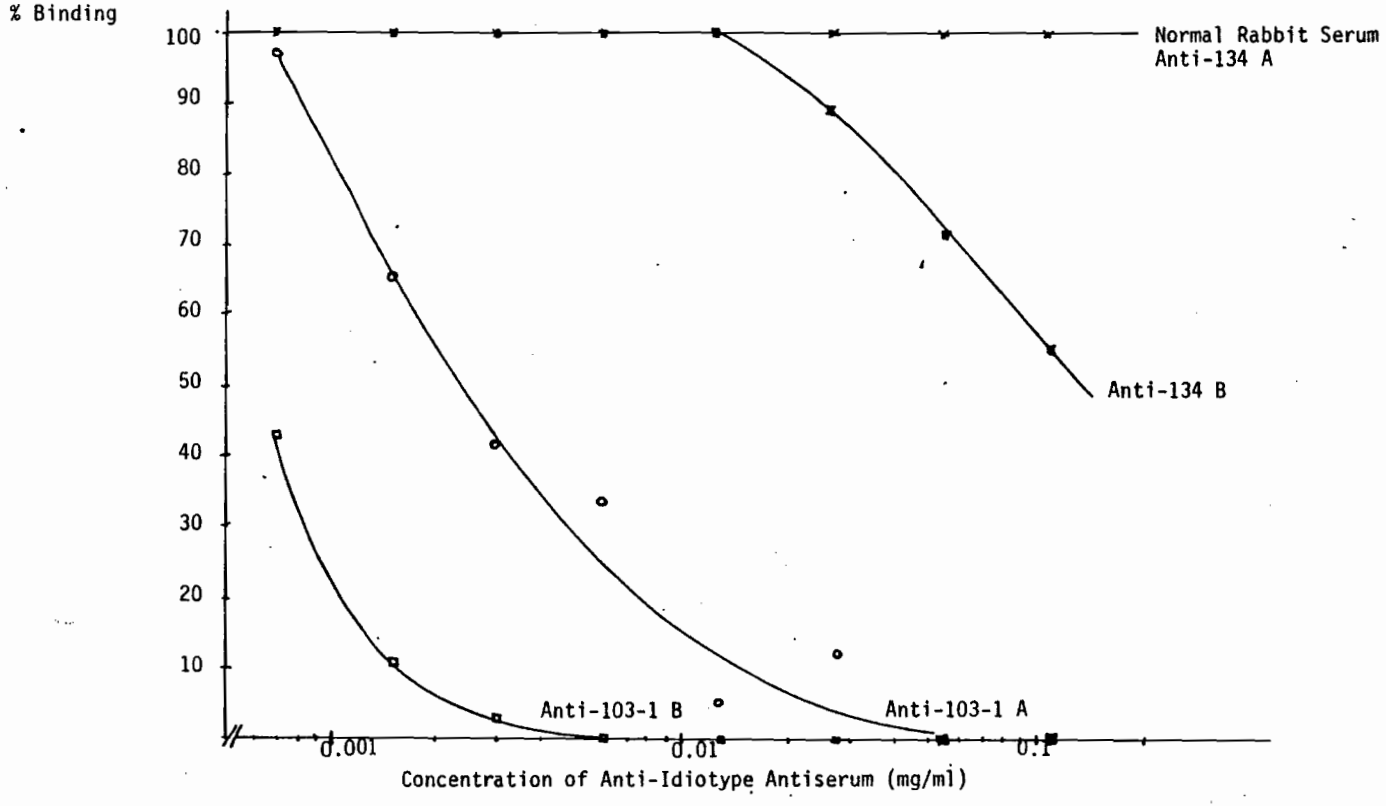


FIGURE 32. INHIBITION OF dDNA BINDING OF 134 ANTIBODY BY ANTI-IDIOTYPE ANTISERA

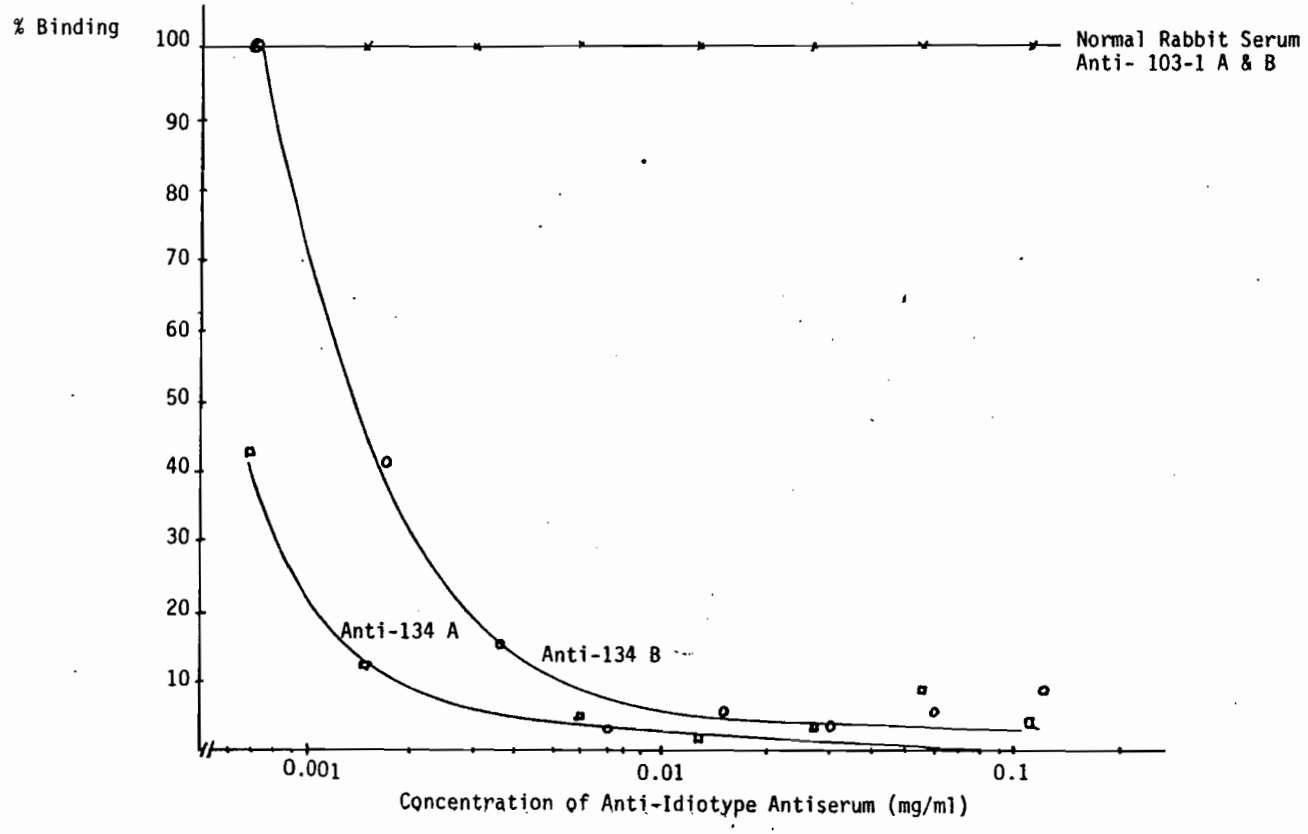


FIGURE 33. INHIBITION OF BINDING OF RABBIT ANTI-103-1 ANTISERUM B TO 103-1 BY PURIFIED HYBRIDOMA ANTIBODIES

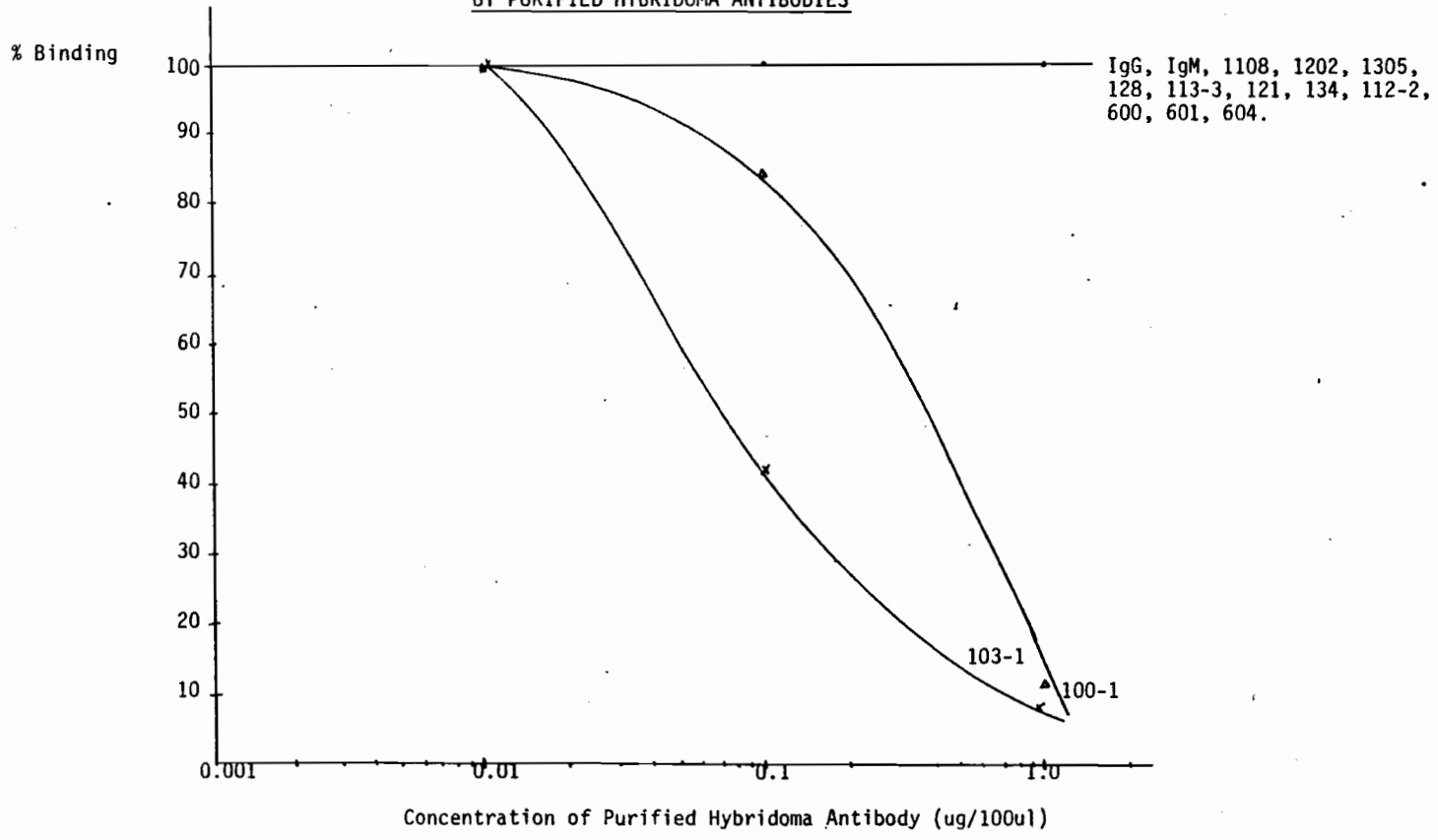
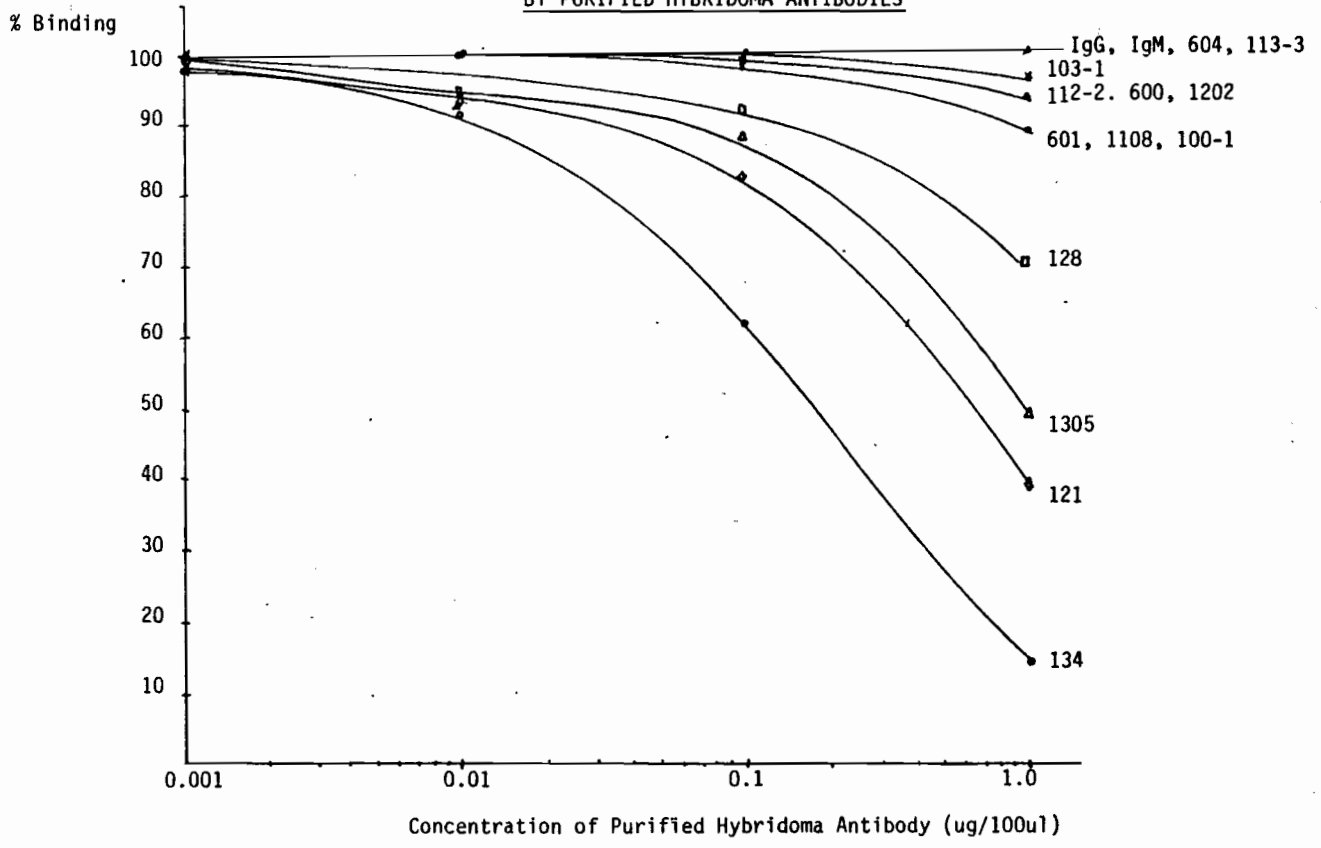


FIGURE 34. INHIBITION OF BINDING OF RABBIT ANTI-134 ANTISERUM B TO 134 BY PURIFIED HYBRIDOMA ANTIBODIES



Chapter 13. ANALYSIS FOR DOMINANT CROSS-REACTIVE IDIOTYPES

13.1 Best Idiotype Coating Concentration

The previous tests (Sections 12.1 to 12.3) were performed on tubes coated with 0.5 µg of anti-DNA antibody. To determine the optimal amount of antibody for use in further tests, the binding of a single dilution of anti-idiotype antiserum was tested on tubes coated with different amounts of idiotype as described in the first paragraph of Section 6.1. Two criteria were used to establish the optimal test conditions. First, the background counts of the control tubes (buffer and culture fluid of GM 4672 cell line) had to be low, and second the binding of anti-idiotype antisera had to remain high. The results for one system, idiotype 1305 and its anti-idiotype antiserum, anti-1305, are given in Table 22. The best results were obtained with tubes coated with 0.03 µg of 1305 purified antibody. The same experiment was repeated for each idiotype anti-idiotype system and it can be concluded that the optimal concentration for coating 103-1, 100-1, 600, and 604 antibodies was 0.1 µg/tube; for 134, it was 0.05 µg/tube and for 1206, it was 0.5 µg/tube.

13.2 Analysis for Cross-Reactive Idiotypes by Inhibition Study

A competition assay was established to determine whether an anti-idiotype antiserum raised against a single hybridoma anti-DNA antibody reacted with other hybridoma anti-DNA antibodies. In this assay, the binding of the anti-idiotype antiserum to its homologous idiotype was

competed by the addition of other purified anti-DNA idiotype antibodies (Section 6.1). If the added anti-DNA antibody shared determinants with the idiotype expressed on the anti-DNA antibody molecule against which the anti-idiotype antiserum was raised, it would bind and possibly saturate the anti-idiotype antiserum. This would prevent the anti-idiotype antiserum from reacting with the immunizing anti-DNA antibody coated tubes. Rabbit anti-103-1 antiserum was incubated in the presence of its idiotype, normal pooled IgM, IgG (from the GM 4672 cell line), and 12 purified antibodies, prior to testing the residual binding to its homologous 103-1 idiotype coated tubes. The results (Figure 35) show that the binding of rabbit anti-103-1 antiserum to 103-1 idiotype coated tubes was inhibited by the presence of its idiotype 103-1 antibody (best inhibition) or by 100-1 antibody. Thus, 100-1 and 103-1 antibodies seem to have a similar idiotype. The IgM, IgG, and 10 other anti-DNA antibodies (1108, 1202, 1305, 128, 121, 134, 112-2, 600, 601, and 604) or the non-DNA binder, 113-3, had no effect on the binding of anti-103-1 antiserum to its idiotype 103-1 antibody coated tubes.

The results of another idiotype/anti-idiotype system is shown in Figure 36. The rabbit anti-134 antiserum was exposed to the same series of antibodies noted above, prior to testing its residual binding to its homologous idiotype (134 antibody coated tubes). The binding of anti-134 antiserum to its 134 idiotype coated tubes was best inhibited by the homologous idiotype, 134 antibody, followed by 121, 1305, and 128

antibodies (an inhibition of binding of less the 10% was not considered positive). All these antibodies thus share a similar idiotype to the one found on the 134 antibody molecule. Pooled normal IgM, IgG, five other anti-DNA antibodies (103-1, 600, 601, 604, 1202) and the non-DNA binder (113-3) did not have an effect on the binding of rabbit anti-134 antiserum to its 134 idiotype coated tubes.

Table 23 shows all the clones tested, which had an effect on the binding of each anti-idiotype antiserum to its homologous idiotype. It also shows the total number of patients from whom reactive hybridoma antibodies were derived. Rabit anti-100-1 antisera recognized an idiotype found on 7 anti-DNA antibodies (100-1, 103-1, 134, 112-2, 1108, 1305, and 1202) of which four (including the one against which the antisera were raised) were obtained from the same patient, while three anti-DNA antibodies were derived from three other patients. Rabbit anti-103-1 antiserum recognized an idiotype found on only 2 anti-DNA antibodies, on 103-1 (against which it was raised) and 100-1 antibodies, both originating from the same patient. Rabbit anti-134 antiserum recognized 5 anti-DNA antibodies (121, 128, 134, 1206, 1305) derived from three patients. Rabbit anti-604 antiserum recognized 7 anti-DNA antibodies (100-1, 600, 601, 604, 1206, 1311, 1400) derived from five patients. Rabbit anti-1206 antiserum recognized only the immunizing its antibody 1206. Finally, rabbit anti-1305 antiserum recognized 4 anti-DNA antibodies (604, 1305, 1311, 1108) and one non-DNA binding antibody (1207), derived from four

different patients.

In another experiment (Figure 37), the rabbit anti-134 antiserum was incubated in the presence of 1206, 1305, 134 and the subclones of 134, 134-1, and 134-2 antibodies. All of these clones inhibited the binding of rabbit anti-134 antiserum to 134 idiotype coated tubes. However, better inhibition was observed with the two subclones. Rabbit anti-103-1 antiserum which recognized two subclones, 103-1 and 100-1 antibodies, was also tested in presence of the parental clones, 103 and 100 anti-DNA antibodies (data not shown). Again best inhibition occurred with the subclones rather than with the parental clones Inhibition of binding was also observed in all cases with homologous idiotype culture fluid, indicating that the purified idiotype was the same as the one in culture fluid, and that there was no alteration of the major idiotype in the purification step.

13.3 Best Anti-Idiotype Coating Concentration

Tubes were coated with different amounts of anti-idiotype antiserum (Section 5.6) to select the optimal coating concentration required for establishing a direct binding assay of idiotype to anti-idiotype antisera coated tubes. The background on each set of antisera coated tubes was always below 400 cpm. Figure 38 shows that the binding of the idiotypes 134 or 103-1 to their respective anti-idiotype antisera coated tubes increased, reached a maximum, and then decreased as the antisera were diluted. The dilution which gave the maximum binding of the idiotype was chosen as the ideal

amount for coating anti-idiotype antiserum tubes. Thus, the dilution used to coat anti-134 and anti-103-1 antisera was 1:4000 (1.4 and 1.06 μg/tube respectively). From similar experiments with each of the other anti-idiotype antisera, the dilution used to coat anti-600 antiserum was 1:4000 (2.35 μg/tube), anti-604 antiserum 1:2700 (0.79 μg/tube), anti-1206 antiserum 1:24300 (0.12 μg/tube), anti-1305 antiserum 1:2700 (0.78 μg/tube), anti-100-1 antiserum 1:4000 (0.35 μg/tube) and anti-1400 antiserum 1:900 (0.3 μg/tube). For control, normal rabbit serum was coated at a dilution of 1:4200 (1.32 μg/tube).

13.4 Direct Binding Study For Cross-Reactive Idiotypes

The neat culture fluids of 47 IgM hybridoma antibodies (26 anti-DNA binding and 21 non-DNA binding antibodies) were each tested three times in a direct binding assay on each of the eight anti-idiotype antisera and normal rabbit serum coated tubes. Each time, the results were treated as follows: a) the buffer background and the non-specific binding to normal rabbit serum coated tubes were subtracted from the experimental observation, b) the results were recorded as + for binding of 500 to 1000 cpm, ++ for binding of 1000 to 2000 cpm, or +++ for binding greater than 2000 cpm. A subtracted binding value lower than 500 cpm was not recorded as positive because this was considered to be within the range of error due to the gamma counter and experimental manipulations. The results of all three experiments are summarized in Table 24. Of the 26 anti-DNA antibody producing clones, 17 (65%) bound to one or

more of the 8 anti-idiotype antisera coated tubes. Only 3 (14%) non-DNA binding clones bound to these tubes, and they each reacted with anti-134. Eleven (42%) anti-DNA antibody producing clones (134, 100-1, 600, 604, 1202, 1400, 1404, 1406, 1407, 2607, 2608) bound to the anti-134 antiserum coated tubes, suggesting a common idiotype similar to 134 on each of these anti-DNA clones. Two anti-DNA antibody producing clones (103-1, 1406) bound to anti-103-1, two (1400, 1401) bound to anti-1400, five (604, 134, 600, 1305, 2607) bound to anti-604, four (1305, 100-1, 134, 1403) bound to anti-1305, six (100-1, 103-1, 1108, 1305, 1206, 1407) bound to anti-100-1, and, finally, five (103-1, 600, 604, 1305, 100-1) bound to anti-1206 antiserum coated tubes. In contrast to the other idiotype/anti-idiotype antisera system, the idiotype 1206 did not bind to its homologous anti-idiotype antisera coated tubes, anti-1206. However, some of the other anti-DNA antibodies did bind weakly to this antiserum. In addition, neither the homologous antibody 600 nor any of the other antibodies showed binding on anti-600 antisera coated tubes.

The neat culture fluid of eight IgG hybridoma antibody producing clones (134-1, 134-2, 1607, 824, 1414, 1417, 103-1, 1203), of which four culture fluids originated from anti-DNA producing clones which had lost their anti-DNA activity and had switched class, were tested on these anti-idiotype antisera coated tubes. Their binding was detected by a rabbit anti-human IgG label and the results were calculated as described above. No positive binding was recorded.

The neat culture fluid of the subclones of 103 and 134 clones (103-2,..., 103-8, 134-1, 134-2) were also tested on each set of tubes (antisera and normal rabbit serum coated tubes). The clones and subclones 103, 103-2, 103-3, 103-4, 103-5, 103-6, 103-7, 103-8 bound +++ only with rabbit anti-103-1 antiserum coated tubes. No binding was recorded on any of the other antisera coated tubes, except for 103-8 which bound + with rabbit anti-100-1 antisera coated tubes. Similarly, the subclones 134-1 and 134-2 bound +++ to only their anti-134 antisera coated tubes, and no binding was recorded on any of the other antisera coated tubes.

13.5 Titration of Direct Binding of Idiotypes on Cross-Reactive Anti-Idiotype Coated Tubes

It was shown in Section 13.2, Table 24, that some anti-DNA antibodies cross-reacted on several anti-idiotype antiserum coated tubes. For example, the 103-1 idiotype bound +++ to anti-134, anti-100-1, anti-1305, and anti-103-1 antisera coated tubes; the 100-1 idiotype bound +++ to anti-100-1 and anti-134 antisera coated tubes (Table 24). These two idiotypes (100-1, 103-1) were titrated here on each anti-idiotype antiserum coated tube to which they bound. The raw binding of these titrations (after the subtraction of the binding value of the buffer tube) is given in Table 25, along with the titration of two other idiotypes (134 and 1305) on their homologous anti-idiotype antisera coated tubes (anti-134 and anti-1305), and the non-specific binding of all four idiotypes (100-1, 103-1, 134, and 1305) on normal rabbit serum

coated tubes. Table 25 shows, as previously seen, that the neat culture fluid of each four anti-DNA antibodies (100-1, 103-1, 134, and 1305) bound +++ to each cross-reactive anti-idiotype antiserum coated tube (binding values were greater than 2000 cpm over the non-specific binding on normal rabbit serum). The binding of 134 and 103-1 idiotypes, two strong anti-DNA binders, to their homologous anti-idiotype antisera coated tubes remained positive (+++) upon major dilution (1:250). The binding of 103-1 idiotype to the cross-reactive anti-idiotype antisera coated tubes dropped drastically even upon a 1:2 dilution (it is + at 1:10 on anti-100-1 and 1:2 on anti-134, and negative at 1:2 on anti-1305 antisera coated tubes). The binding of two weak anti-DNA binders, 100-1 and 1305 idiotypes to their homologous anti-idiotype antisera coated tubes differed upon dilution: one (1305) could be titrated to + at 1:50 while the other (100-1) was no longer positive at 1:2. The binding of 100-1 idiotype could only be titrated to + at 1:10 on the cross-reactive anti-idiotype antisera coated tubes, anti-134.

Only the neat culture fluid of 103-1 idiotype showed high non-specific binding on normal rabbit serum coated tubes, which dropped 7-fold and became negative upon a 1:2 dilution. The specific binding of 103-1 on the cross-reactive anti-idiotype antisera coated tubes also decreased a fraction (1/7 - 1/18) of that seen using the neat culture fluid.

TABLE 22

BINDING OF RABBIT ANTI-1305 ANTISERUM TO DIFFERENT AMOUNTS OF 1305 IDIOTYPE COATED TUBES

	Binding (in cpm) to 1305 idiotype tubes coated at the concentration of antibody of				
SAMPLES	0.5 µg/tube	0.25 µg/tube	0.12 µg/tube	0.06 µg/tube	0.03 µg/tube
0.05% Tween buffer	2348	1058	595	401	273
Culture fluid of GM 4672	3188	1391	619	415	326
Rabbit anti-1305 antiserum 1/100	27099	26076	23671	20947	13105

FIGURE 35. INHIBITION OF BINDING OF RABBIT ANTI-103-1 ANTISERUM TO 103-1 BY PURIFIED HYBRIDOMA ANTIBODIES

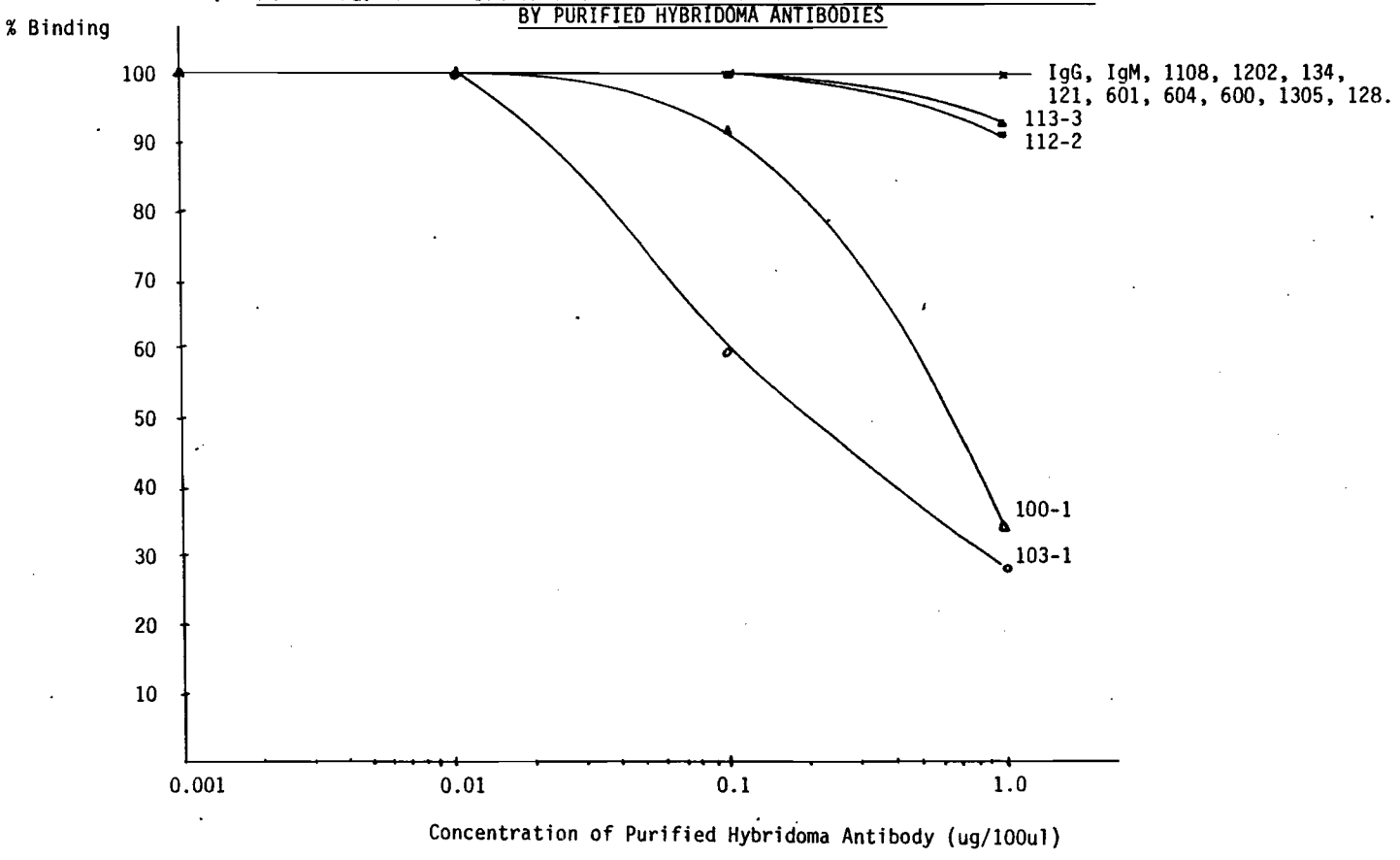


FIGURE 36. INHIBITION OF BINDING OF RABBIT ANTI-134 ANTISERUM TO 134 BY PURIFIED HYBRIDOMA ANTIBODIES

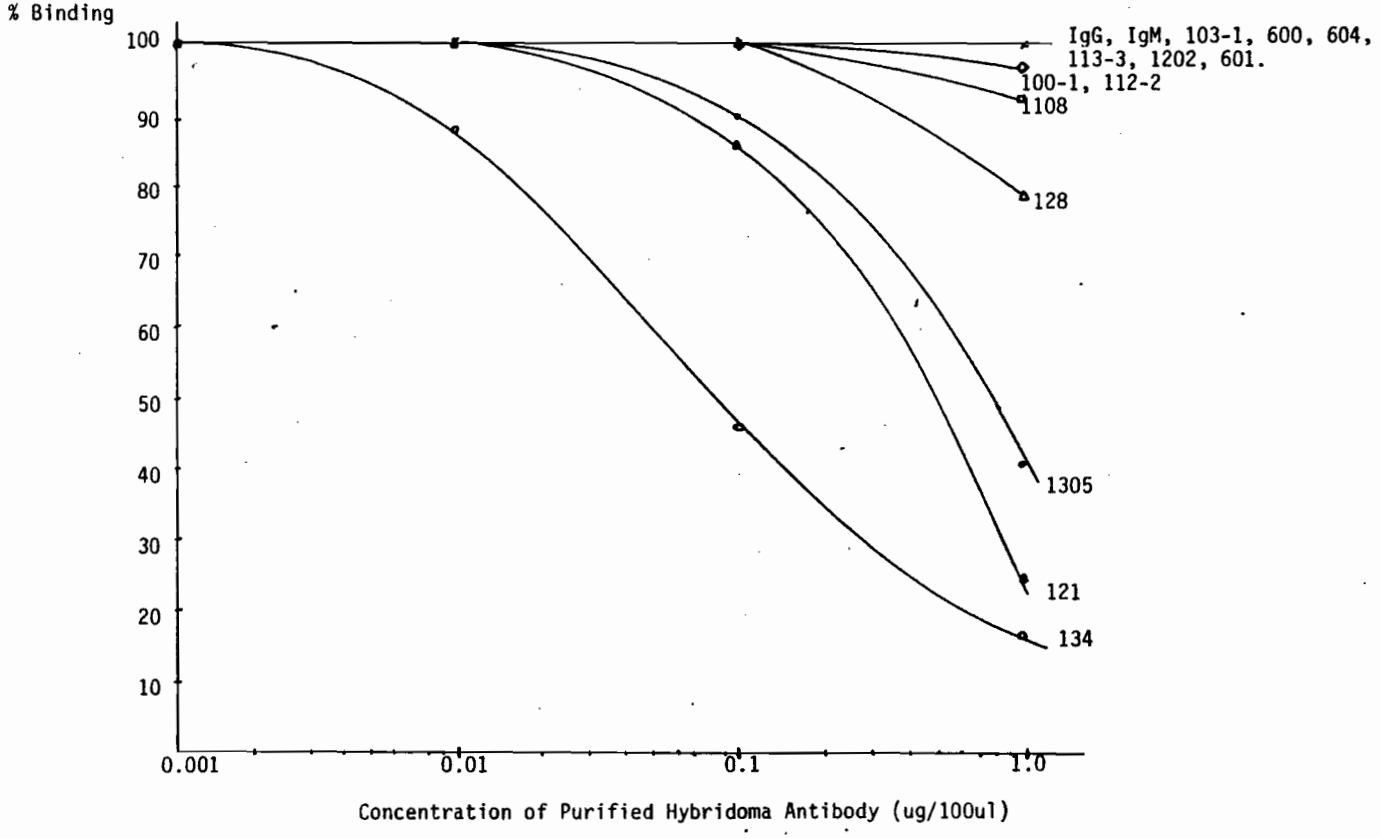


TABLE 23

HYBRIDOMA ANTI-DNA ANTIBODIES DETECTED BY ANTI-IDIOTYPE ANTISERA BY INHIBITION STUDY

Nomenclature of all purified antibodies tested	Anti-100-1	Anti-103-1	Anti-134	Anti-604	Anti-1206	Anti-1305
Anti-DNA Antibodies						
100-1	+	+	-	+	-	-
103-1	+	+	-	-	-	-
134	+	-	+	-	-	-
600	-	-	-	+	-	-
604	-	-	-	+	-	+
1202	+	-	-	-	-	-
1206	N.D.	N.D.	+	+	+	-
1305	+	-	+	-	-	+
1400	N.D.	N.D.	N.D.	+	-	-
112-2	+	-	-	-	-	-
1311	N.D.	-	-	+	-	+
601	-	-	-	+	-	-
121	-	-	+	-	-	-
128	-	-	+	-	-	-
1108	+	-	-	-	-	+
Non-Anti-DNA Binders						
IgG (GM 4672)	-	-	-	-	-	-
Pooled normal IgM	-	-	-	-	-	-
824	N.D.	-	-	-	-	-
113-3	-	-	-	N.D.	N.D.	N.D.
1207	N.D.	-	-	-	-	+
Hybridoma Antibodies Reactive with Anti-Idiotype Antiserum	7/13	2/16	5/17	7/17	1/17	5/17
Number of Patients From Whom Reactive Hybridomas Were Derived	4	1	3	5	1	4

\+ indicates that the anti-DNA antibody inhibited the binding of anti-idiotype antiserum to its idiotype

\- indicates that the anti-DNA antibody did not have any effect on the binding of the anti-idiotype antiserum to its idiotype

N.D. not determined

FIGURE 37. INHIBITION OF BINDING OF RABBIT ANTI-134 ANTISERUM TO 134 BY PURIFIED HYBRIDOMA ANTIBODIES

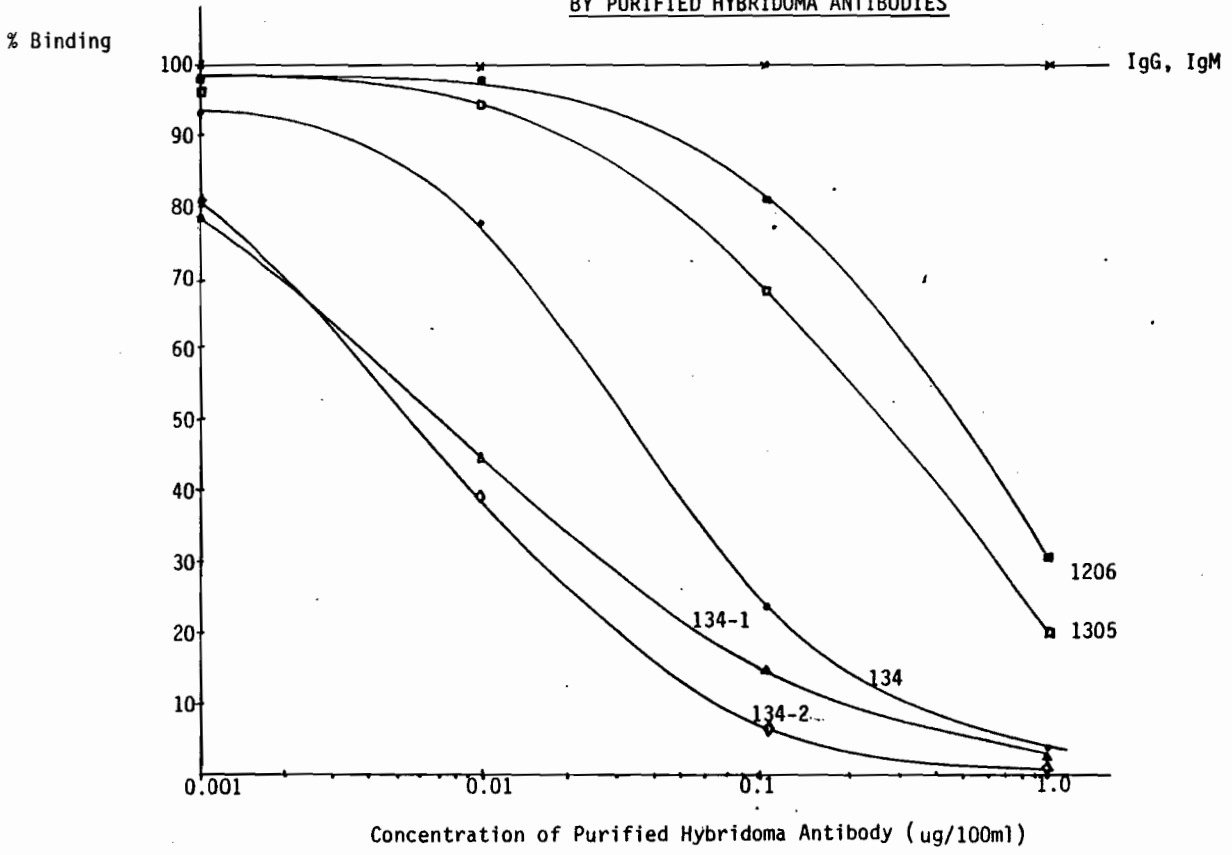


FIGURE 38. BINDING OF IDIOTYPE TO DIFFERENT ANTI-IDIOTYPE COATED TUBES

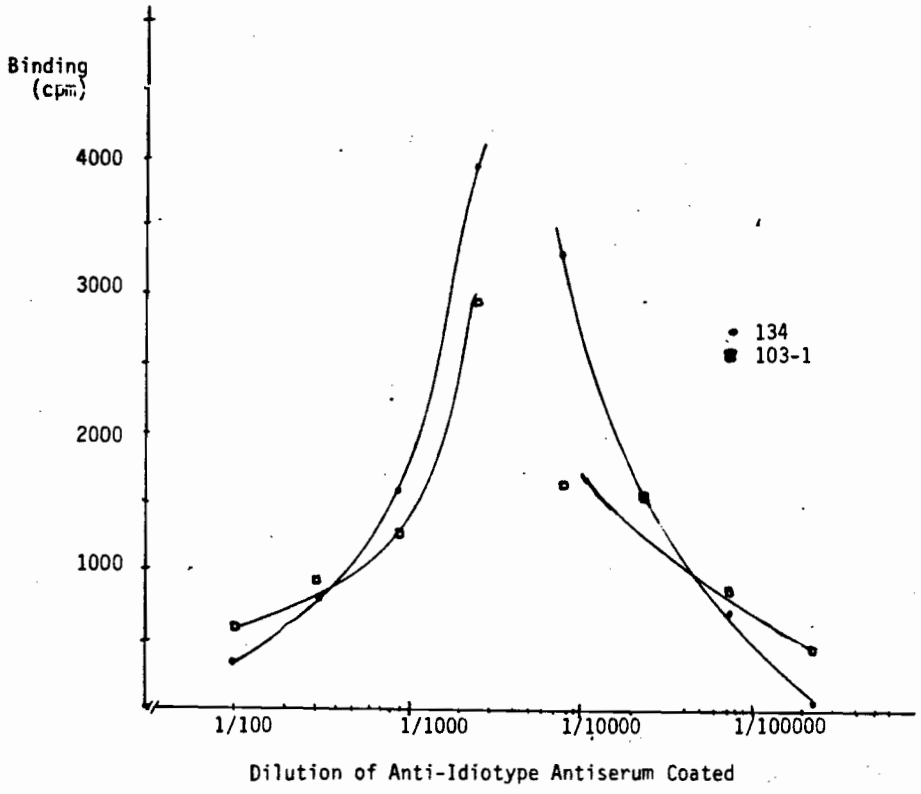


TABLE 24. REACTIVITY OF ANTI-DNA AND NON-DNA BINDING HYBRIDOMA ANTIBODIES WITH RABBIT ANTI-IDIOTYPE ANTISERA

Clones	Anti-100-1	Anti-103-1	Anti-134	Anti-604	Anti-1305	Anti-1400	Anti-1206
*							
100-1	+++		+++				+
103-1	++	+++			++		+
121-1							
134			+++	++	+		
600			++	+			+
604			++	++			+
1101							
1108	+		+				
1202							
1204							
1206	+						
1305	++			+	++		+
1400			+++			+++	
1401						+	
1402							
1403					+		
1404			+				
1406		+	+++				
1407	+		+				
1414							
1418							
1422							
1900							
1905							
2607			+	+			
2608			+++				
**							
801							
1003							
1104							
1105							
1109							
1201			++				
1302							
1410							
1411							
1420							
1500							
1520							
1600							
2300							
2301							
2600			+				
2602							
2610							
2611							
2613			+				
2614							

*---> anti-DNA antibodies **---> non-anti-DNA antibodies

TABLE 25

COMPARISON OF TITRATION OF DIRECT BINDING OF CROSS-REACTIVE HYBRIDOMA ANTI-DNA ANTIBODIES TO ANTI-IDIOTYPE ANTISERUM COATED TUBES

Anti-DNA Antibody Idiotype	Dilutions of Idiotype Used	Binding in cpm to **Normal Rabbit Serum	antiserum anti-134	anti-100-1	anti-103-1	anti-1305
103-1	Neat	7095	15020	15555	28149	18829
	1/2	966	1335	2156	20057	1010
	1/10	439	687	1011	20017	562
	1/50	233	332	671	17184	260
	1/250	136	184	468	13212	194
100-1	Neat	1279	8214	6247	*	*
	1/2	308	2872	457	*	*
	1/10	198	904	162	*	*
134	Neat	1532	21207	*	*	*
	1/2	298	19402	*	*	*
	1/10	297	14133	*	*	*
	1/50	149	9668	*	*	*
	1/250	171	4480	*	*	*
1305	Neat	598	*	*	*	4949
	1/2	255	*	*	*	1578
	1/10	134	*	*	*	1148
	1/50	261	*	*	*	703

* not determined

** normal rabbit serum coated tubes are used as control for non-specific binding of the anti-DNA antibody

Chapter 14. CHARACTERIZATION OF MOUSE ANTI-IDIOTYPE HYBRIDOMAS

14.1 Fusion Results

Three attempts were made to produce mouse anti-idiotype hybridomas. The procedure used and the results of these three attempts are summarized in Table 26.

Experiment I involved 8 female mice, divided into groups of 2, each group receiving an idiotype mixed either with Staph A or CFA at first injection. Only 4 mice survived the immunization period. The fused splenic cells of these 4 mice became contaminated before hybridoma clones were observed.

Experiment II involved 6 female mice of which 3 survived and whose splenic cells were fused. Due to a lack of Sp2/0 plasmacytoma cells, only the spleen cells of a 134 injected mouse and half the splenic cells of two 103-1 injected mice were fused with the Sp2/0 cell line, while the remainder of the 103-1 sensitized splenic cells were fused with MPC-11 cells. Only 3 clones arose from the fusion of 134 sensitized splenic cells, while 135 clones arose from the fusion of 103-1 sensitized splenic cells with MPC-11 cells. 24 clones arose from the 103-1 sensitized splenic cell fusion with Sp2/0 cells.

Experiment III involved 9 male mice of which 5 survived. No clones were observed after fusion of their splenic cells with Sp2/0. This result was attributed to a loss in the efficiency of the Sp2/0 line to produce hybridomas. This was concluded when a repeated fusion

experiment was performed using the same Sp2/0 cells and freshly thawed Sp2/0 cells. Hybridomas were obtained with the fresh preparation, while the same splenic cells fused with the old Sp2/0 cells failed to produce hybridomas.

14.2 Screening of Mouse Sera for Anti-Idiotype Activity

The mouse antisera collected by phlebotomy were tested by direct binding to HIgM, HIgG and the immunogen in order to verify that the mice were producing antibodies to the immunogen (an anti-DNA IgM class antibody). Table 27 shows the results of a bleeding test assay. Two BALB/C mice were injected with 103-1 antibody and a third one with 134 antibody. Their respective antisera were called mouse anti-103-1 A, B and mouse anti-134. The high binding of mouse anti-134 antiserum to HIgM and to 134 antibody as well as HIgG coated tubes (Table 27) indicated that the mouse injected with 134 antibody had responded to the immunogen and produced anti-HIgM and anti-idiotype antibodies. Similarly, the binding of the mouse anti-103-1 B antiserum to HIgM, 103-1 antibody and HIgG coated tubes indicated that the 103-1 immunized mouse B also produced anti-HIgM and anti-idiotype antibodies. In contrast, the 103-1 immunized mouse A produced mostly anti-HIgM antibody with some trace of anti-103-1 idiotype antibody, since its antiserum bound to HIgM but very poorly to 103-1 coated tubes. The normal BALB/C serum did not bind to these antigen coated tubes (background binding), indicating that there were no anti-HIgM, anti-HIgG, anti-134 or anti-103-1 antibodies present in these sera.

Table 28 gives the results of another mouse bleeding

assay. In that test, the immune antibody response of a mouse injected with an antigen bound to Staph A was compared to that of another mouse injected with the same antigen, but mixed with CFA. The immunogen used was 103-1 anti-DNA antibody. The antiserum obtained from the mouse injected with 103-1 attached to Staph A (called mouse anti-103-1 Staph antiserum) bound equally to HIgM, HIgG, and 103-1 antibody coated tubes, while the antiserum of the mouse which received 103-1 mixed with CFA (mouse anti-103-1 antiserum) bound equally to HIgM and 103-1, but not to HIgG coated tubes. Both mice responded to the immunogen and thus produced anti-HIgM and anti-103-1 antibodies. It seems, however, that the immune response of the mouse injected with 103-1 antibody mixed with CFA was more specific to the immunogen than that of the mouse injected with the same idiotype, but mixed with Staph A, because of the high binding to HIgG coated tubes observed with the latter and not with the former.

14.3 Screening for Hybridoma Anti-Idiotype Activity

A total of 162 hybridomas were screened on HIgM, HIgG and 103-1 or 134 coated tubes to isolate a hybridoma producing anti-idiotype antibody. The hybridomas were named after their parental idiotype, followed by a period and a number of identification. For example, the first hybridoma arising from the mouse injected with 103-1 antibody, was called anti-103-1.1. The following hybridomas arising from the same fusion were called anti-103-1.2, anti-103-1.3, etc. A representative screening test is shown in Table 29.

That table shows the binding of eight hybridoma culture fluids from the 103-1 sensitized splenic cells fused with either MPC-11 or Sp2/0 cells. The negative control included MPC-11 and Sp2/0 cluture fluids. It had turned out that MPC-11 immunoglobulin showed anti-HIgM, anti-HIgG, and anti-103-1 activity which could not be explained. Of the eight culture fluids tested, 2 demonstrated anti-103-1 activity but also anti-HIgG and anti-HIgM activities (anti-103-1.13, anti-103-1.29). One of the remaining hybridomas (anti-103-1.106) demonstrated elevated anti-HIgG, compared to the activity observed with MPC-11 antibody, which was considered non-specific binding. Of all 162 clones screened, none showed activity against the idiotype only. The one which had anti-idiotype activity also demonstrated anti-HIgM or anti-HIgG activity.

Two attempts were made to isolate the anti-idiotype activity of two clones by limiting dilution: anti-103-1.106 and anti-103-1.29. A total of 10 and 1 subclones, respectively, arose from the subcloning procedure of these clones. The subclones were named as the parental cell, with a hyphen and a number was added. Table 30 shows the results of a screening assay of these 11 subclones. Again, non-specific binding was observed on each antigen coated tube with MPC-11 culture fluid, while no binding was recorded with Sp2/0 culture fluid. None of the subclones demonstrated only anti-idiotype activity. As a result, all mouse anti-idiotype clones were put aside and their studies stopped.

TABLE 26

RESUME OF MOUSE FUSION EXPERIMENTS

Experiment	Idiotype Injected	Number and * Sex of Mouse Injected	Method Used**	Number of Spleen Fused	Number of Clones Arose
I	103-1	2 ♀	Staph A	1	0
		2 ♀	CFA	1	0
	100-1	2 ♀	Staph A	0	0
		2 ♀	CFA	2	0
II	103-1	3 ♀	CFA	2	159
	134	3 ♀	CFA	1	3
III	1400	3 ♂	CFA	1	0
	1206	3 ♂	CFA	2	0
	1305	3 ♂	CFA	2	0

* Male: ♂ , female: ♀

** the idiotype was mixed with Staph A or CFA for the first injection

TABLE 27

BINDING OF NORMAL BALB/c SERUM AND OF BALB/c ANTISERA TO HIgM, HIgG, AND TO THE IDIOTYPE USED AS IMMUNOGEN

	Dilution of sera used	Binding in cpm to HIgG	HIgM	103-1	134
Normal Balb/c serum	1/20	370	190	0	788
	1/40	182	405	0	246
	1/80	201	212	0	156
Mouse Anti-103-1A antiserum	1/20	791	13060	1175	N.D.*
	1/40	1021	10196	379	N.D.
	1/80	699	6309	0	N.D.
Mouse anti-103-1B antiserum	1/20	2506	36529	8813	N.D.
	1/40	2293	35932	8847	N.D.
	1/80	1589	32795	6070	N.D.
Mouse anti-134 antiserum	1/20	2674	20988	N.D.	20716
	1/40	2155	20704	N.D.	22646
	1/80	1442	17707	N.D.	17698

* not determined

TABLE 28

COMPARISON OF DIRECT BINDING TO HIgM, HIgG, AND 103-1 OF TWO ANTISERA DIRECTED AGAINST THE SAME IDIOTYPE BUT OBTAINED DIFFERENTLY

	Dilution of Sera Used	Binding in cpm to HIgG	HIgM	103-1
Normal BALB/c serum	1/40	1879	4313	3509
	1/80	2372	4859	2650
Mouse anti-103-1 Staph * antiserum	1/40	16738	13548	16956
	1/80	13188	13645	15553
Mouse anti-103-1 antiserum	1/40	3643	20701	21689
	1/80	2616	26837	22086

* means that the immunogen 103-1 was bound to Staph A particles instead of to CFA as the other anti-103-1 antiserum

TABLE 29

DIRECT BINDING OF MOUSE ANTI-IDIOTYPE HYBRIDOMA ANTIBODIES TO HIgG, HIgM, AND 103-1 ANTIBODY COATED TUBES

Culture Fluids (Neat)	Binding to (in cpm) HIgG	HIgM	103-1
MPC-11	7086	8174	5797
Sp2/0	236	93	84
anti-103-1.13	15855	16598	23728
anti-103-1.29	12137	14128	11182
anti-103-1.87	9141	7476	7482
anti-103-1.93	5932	4926	5147
anti-103-1.105	7902	5013	6592
anti-103-1.106	11673	8618	7680
anti-103-1.121	8375	3618	4573
anti-103-1.128	N.D.	1867	4650

N.D. not determined

TABLE 30

DIRECT BINDING OF MOUSE ANTI-IDIOTYPE HYBRIDOMA SUBCLONES TO HIgG, HIgM, AND 103-1 ANTIBODY COATED TUBES

Culture Fluids (Neat)	Binding to (in cpm) HIgG	HIgM	103-1
MPC-11	4303	4949	3198
Sp2/0	155	96	120
anti-103-1.106-1	7573	6731	7657
anti-103-1.106-2	5918	7214	7681
anti-103-1.106-3	7081	7825	79
anti-103-1.106-4	5783	7308	7407
anti-103-1.106-5	N.D.*	1511	935
anti-103-1.106-6	6577	7380	8082
anti-103-1.106-7	6727	8331	8587
anti-103-1.106-8	6988	8082	8948
anti-103-1.106-9	N.D.	6649	7173
anti-103-1.29-1	14721	11393	4373

* not determined

Chapter 15. OTHER HUMAN HYBRIDOMA ANTIBODY ACTIVITIES

15.1 Prolonged Partial Thromboplastin Time

The presence of an anticoagulant hybridoma antibody was tested by the prolonged partial thromboplastin time assay (done by a co-worker). A total of 67 clones from 13 fusions were screened. Ten demonstrated a prolonged partial thromboplastin time. Table 31 lists the clotting time and the IgM concentration of nine of these positive clones. No correlation between the amount of IgM and the prolongation of the clotting time was observed. The clotting time of the IgG from GM 4672 was chosen as the referred time.

15.2 Rheumatoid Factor Activity

The presence of rheumatoid factor activity in the culture fluid of 30 clones from 13 fusions was assessed (by another co-worker). Preliminary screening was done using HIgG coated tubes (Table 32). True rheumatoid factor activity was then confirmed by screening the positive clone on Fc and Fab coated tubes (Table 33). Reactivity to Fc coated tubes only indicated that the hybridoma antibody had true rheumatoid factor activity. Reactivity to Fc, Fab, and BSA indicated non-specific binding, and a hybridoma antibody having this kind of reactivity was not considered a true rheumatoid factor. Sixteen clones were confirmed to be true rheumatoid factors: they were 100, 801, 1104, 1109, 1400, 1401, 1402, 1412, 1414, 1420, 1423, 2300, 2610, 2611, 2613 and 2614.

TABLE 31

CLOTTING TIME OF HYBRIDOMA SHOWING ANTICOAGULANT ACTIVITY

Supernatant	IgM (µg/ml)	Clotting time (min.)
GM 4672	0.0	1.03
824	0.09	1.14
801	16	1.13
600	0.7	1.14
1105	9.5	1.14
1403	23	1.21
1420	9.2	1.15
1401	30	1.22
1104	9.0	1.15
1109	N.D.*	1.15

* not determined

TABLE 32

DIRECT BINDING OF HUMAN HYBRIDOMA ANTIBODIES TO HIgG AND BSA COATED TUBES

Clones	Binding in cpm of hybridoma antibodies to tubes coated with: HIgG	BSA
1400	2403	828
1900	604	630
2607	4149	694
2608	703	324
2610	3501	298
2611	4943	237
2613	2552	338
2614	1577	234
Controls		
Buffer	425	243
GM 4672*	501	182
MRF**	8035	395

* the cell supernatant of GM 4672, the lymphoblastoid line used for fusion and the buffer were used as negative control. An antibody was considered to be positive for binding to HIgG when the cpm bound exceeded the mean + 3 standard deviations (S.D.) of the controls (buffer, GM 4672). The mean + 3 S.D. were 624 for HIgG and 342 for BSA.

** monoclonal rheumatoid factor used as a positive control for HIgG coated tubes.

TABLE 33

DIRECT BINDING OF HUMAN HYBRIDOMA ANTIBODIES TO Fc, Fab, AND BSA COATED TUBES

Clones	Binding in cpm of hybridoma antibodies to tubes coated with: Fc	Fab	BSA
1400	12868	1257	612
1900	1912	634	571
2607	9538	3256	1322
2608	1993	901	736
2611	2054	1251	637
2614	932	742	372
Controls			
Buffer	398	379	529
GM 4672*	910	605	612
MRF**	9966	806	551

* the cell supernatant of GM 4672, the lymphoblastoid line used for fusion and the buffer were used as negative control. An antibody was considered to be positive for binding to HIgG when the cpm bound exceeded the mean + 3 standard deviation (S.D.) of the controls (buffer, GM 4672). The mean + 3 S.D. were 1740 for Fc, 971 for Fab, and 746 for BSA.

** monoclonal rheumatoid factor used as a positive control.

Chapter 16. SLE PATIENTS

16.1 Patients' Clinical History

The clinical status of the patients fused and whose fusion had led to hybridoma growth is given in Table 34. The table lists the SLE manifestations and clinical test data (done routinely) for each patient, along with his or her age, sex and duration of disease. The age of patients varied from 21 to 61 years, with a mean of 35 years, while the duration of their disease ranged from 1 to 20 years with a mean of 6.5 years. Of the fourteen patients fused, only three were male (fusions 14, 19, and 26). However, while the mean duration of the disease for men (7 years) was not different from the mean duration for women (6.3 years) the men's mean age was younger (29 years) than the women's (36.9 years).

Of fourteen patients, 12 had arthritis, 6 had butterfly rash, 4 had alopecia, 2 had psychiatric manifestations, 1 had neurological manifestations, 2 had vasculitis and 8 had nephritis. Thus, the common features most often seen in those patients were arthritis and nephritis. At the moment of fusion, none of these patients were untreated and thus were on medication. They were receiving corticosteroids in doses from 5-20 mg daily to 10 mg on alternate days. However, all patients were ANA positive although only 9 (fusions 6, 7, 10, 11, 12, 13, 16, and 26) had detectable serum anti-dsDNA antibodies, as determined by the hospital clinical laboratory, when they were fused. Seven patients were hypocomplementemic, 3 (fusions 8, 15, and 26) had an anticoagulant activity, and 3 (fusions 6, 12,

and 15) had a false positive VDRL test.

16.2 Serum RIA Activities

Patients' sera were tested for anti-dDNA, anti-nDNA, anti-cardiolipin and rheumatoid factor activities by RIA. The sera of a total of 6 normal individuals, 18 SLE patients and 12 RA patient were tested. These sera were from patients whose lymphocytes were fused with GM 4672 cell line. These 36 sera, tested in duplicate at 3 different dilutions, were separated into 3 sets of experiments. These 3 sets were done on three consecutive days to minimize fluctuations due to the aging of the radiolabelled second antibody. Each experiment set included a pool of 3 normal sera and a high anti-dDNA SLE serum as controls. Fresh dilutions of these 2 control sera were done each day with the use of fresh coated tubes. Although variation in the binding of these 2 sera was expected from day to day, the binding variation never varied more than 25%.

Figures 39 to 42 show the RIA binding of normal, SLE and RA sera to dDNA, nDNA, cardiolipin and HIgG coated tubes respectively. In all cases (except for normal patients' serum 39, because of its high RF activity) normal sera had low binding activities against the antigens studied. The highest binding of normal sera at 1/100 dilution on each antigen coated tubes was used as a reference point to determine the binding positivity of SLE and RA sera. A serum was thus recorded to have positive binding activity to an antigen when its binding activity, at 1/100 dilution, was higher than the normal serum

pool. Table 35 summarizes the positive RIA binding activities of each SLE and RA serum. It can be seen that no anti-DNA, anti-cardiolipin, PTT and rheumatoid factor activities were detected in the sera of fusions 14 and 19. These results correlate with a negative clinical DNA binding and anticoagulant activity, although both patient had a positive ANA test. In contrast, the serum of fusion 16, which had a positive clinical DNA binding, had no detectable RIA anti-DNA activity but had RIA rheumatoid factor activity. The clinical RF value of these patients was not available, thus no correlation of RIA RF activity with clinical activity was done. Fusions 6, 8, 15, and 23 showed RIA anti-DNA activity in their sera but not in the clinical laboratory. All the other fusions showed anti-DNA activity in RIA which correlated with the clinical values. Similarly, fusions 2, 3, and 5, which had positive clinical DNA binding values, also showed an RIA anti-DNA activity. Again, this contrasts with what was observed with fusion 4, which did have a RIA anti-DNA activity although it had a negative clinical DNA binding value (clinical results are not shown for fusions 2, 3, 4, and 5). There is, thus, no correlation between a positive clinical DNA binding of a serum and its RIA binding activity.

It can be seen in Table 35 that 61% SLE sera (11 out of 18) showed binding activity to both nDNA and dDNA. However, 2 sera (11, 13) demonstrated activity only against dDNA while 2 others (6, 12) demonstrated activity only against nDNA. In addition to their binding activity against DNA (dDNA and/or

nDNA), 5 sera (4, 8, 23, 6, 12) had activity against cardiolipin. One SLE serum (16) had only RF activity, while 5 others (4, 2, 10, 26, and 6) had RF activity with anti-DNA activity (dDNA and/or nDNA), either with or without anti-cardiolipin activity.

In contrast, a study of the reactivity of 12 RA sera showed that none of these sera demonstrated activity against dDNA, while they all (except 20) had RF activity. In addition, 7 of 12 RA sera also demonstrated other activities. They bound to both nDNA and cardiolipin or to either nDNA or cardiolipin, alone.

Thus, Table 35 shows that SLE sera as well as RA sera demonstrated polyreactivity. However, it was not determined whether the polyreactivity was due to the same binding population or to different antibody populations.

16.3 Hybridoma Antibody Activities

The activities of all 261 hybridomas obtained from 14 fusions are summarized in a Venn's diagram in Table 36. Activity against 3 antigens (dDNA, nDNA, and cardiolipin) and 2 serological activities (PTT, RF) were analyzed among the hybridoma antibodies. Eighteen percent (46 out of 261) of the hybridoma antibodies had one or a combination of these activities. Twelve groups of reactivity could be defined, as detailed in Table 37. Eight groups of hybridoma antibodies showed reactivity to dDNA, in addition to reactivity to nDNA and/or RF, PTT, and cardiolipin. One group of antibodies had both RF and PTT activity without activity against DNA and

cardiolipin. Finally, three groups of antibodies were monospecific, they had either a PTT, RF, or anti-DNA activity. None of the hybridoma antibodies had specificity for only nDNA or cardiolipin. These kinds of antibodies were seldom seen in screening assays but, subsequently developed other reactivities (always against dDNA).

Thus, among 46 reactive hybridoma antibodies, 14 (30.4%) showed monospecificity while 70.6% showed polyspecificity by direct binding analysis. Only 28% (13/46) of the reactive hybridoma antibodies showed no reactivity to DNA.

16.4 Correlation of Serum and Hybridoma Autoantibody Reactivities

Four SLE fusions (7, 10, 15, and 16) did not give rise to hybridoma antibodies of any known reactivity, despite the fact that the sera of these patients showed high RIA DNA binding activity or RF activity. On the other hand, fusions 14 and 19, whose sera did not show any reactivity, gave rise to a number of hybridoma antibodies with a wide range of reactivities, in the first case, and to 2 hybridoma antibodies reactive with both dDNA and nDNA, in the second case. Among fusions 8, 15, and 26, from patients with a clinical history of anticoagulant activity, only fusion 8 gave rise to hybridoma antibodies with PTT activity. In contrast, fusions 11 and 14 also gave rise to hybridoma antibodies with PTT activity, an activity not detected in the sera of either patient. Only 8 of 11 SLE patients

who showed anti-DNA activity in their sera gave rise to hybridoma anti-DNA antibodies, while 2 of 3 patients without demonstrable anti-DNA activity in their sera produced hybridoma anti-DNA antibodies.

Thus, the reactivity found in sera of SLE patients did not always correlate with the activity of hybridoma antibodies obtained from the fusion of their lymphocytes. This was also true of the results obtained from the fusion of normal and RA individuals (Rauch 1985).

16.5 Preliminary Data of Idiotype Activities in Sera

A preliminary screening for the presence of serum idiotype by the 8 anti-idiotype antisera was performed on 3 normal, 2 RA, and 4 SLE sera of patients whose lymphocytes were used to produce hybridomas. For SLE, the sera of fusions 1, 2, 8, and 13 were selected at random and tested. For RA, the sera of fusions 17 and 18 were used. Finally, a pool of 3 normal individual sera (N), as well as the sera of fusions 44 and 45 were used as normal serum controls. All sera were diluted at 1/100 and tested for direct binding to anti-idiotype antisera. The data shown in Figure 43 were derived from the subtraction of buffer control tubes and the non-specific binding of sera to normal rabbit serum-coated tubes from each individual specific binding values. Because of the few number of sera tested on each anti-idiotype antiserum, it can only be suggested that there may be a tendency for the expression of different idiotypes in SLE and RA. However,

binding values could be titrated (data not shown) and repeated twice. The amount of bound radioactivity found on each set of tubes was assumed to reflect the amount of Ig (idiotype) present in the sera. Thus, the higher the amount of cpm bound, the greater the experssion of idiotype in the serum. However, the detection of the presence of an idiotype in a serum also depends on the sensitivity of the assay and on the affinity of the anti-idiotype antibody for its homologous and cross-reactive idiotypes.

Two idiotypes were detected specifically but with low binding values (less than 3000 cpm), each in one type of serum: 1206 in SLE sera and 600 in normal sera. The other idiotypes were expressed to some extent in all sera. Namely, idiotypes 1305 and 604 were minimally detectible in all sera; 103-1 was found in a low amount in normal sera, in medium amounts in RA sera (between 4000 and 7000 cpm), and in a wider range in SLE sera (between 500 and up to 7000 cpm); and 100-1 was found in low amounts in normal and RA sera but in a wide range in SLE sera (0 to 6000 cpm). Contrary to this, idiotype 134 was expressed in high amounts in all sera (over 5000 to 13000 cpm). Consequently, a serum was found to express several idiotypes, but in different amounts, reflecting the heterogeneous population of serum antibodies. For example, the serum from fusion 13 had high levels of idiotypes 134, 103-1, and 1400, medium levels of 100-1 and very low levels of idiotype 1305, which originated from that fusion. Interes-tingly, all of these idiotypes were affected by the presence of

anti-1305 antiserum in the hybridoma antibody study. These preliminary data show first that, although anti-idiotype antibodies were raised against SLE anti-DNA antibodies, anti-idiotype antibodies detected shared idiotypes in normal, RA and SLE sera, albeit to different extents; and second, that there seems to be a correlation between the presence of SLE shared idiotypes detected in the hybridoma antibodies derived from different patients and in the sera of these same patients.

TABLE 34

CLINICAL CHARACTERISTICS OF SLE PATIENTS FROM WHOM LYMPHOCYTES WERE OBTAINED FOR FUSION EXPERIMENTS

Patient	Age (years)	Sex F/M	Duration of Disease (years)	SLE Manifestations	ANA $(-)_1$	Anti-Coagulant (<34)	VDRL (-)	C1q (<4%)	C4 (>20 mg/ml)	C3 (>100 mg/ml)	DNA Binding (<30)	Others
1	23	F	1	Arthritis(++), Psychiatric (+++), Raynaud's(+), Lymphadenopathy(+)	+ homo	35	-	5	12	105	80	
6	26	F	1	Arthritis(+++)	+	32	+	N.D.*	30	130	38	↓Platelets(+++)
7	21	F	1	Arthritis(+++), Pleuroperi-carditis(+), Lymphadenopa-thy(+)	+ homo	34	-	25	7	60	60	Proteinuria(+++) Bx: diffuse GN
8	38	F	13	Rash(+), Vasculitis(+++), Pleuropericarditis(++), Raynaud's(+), Lymphadenopathy(+)	+	50	-	N.D.	11	95	0	↓Hgb(++),↓ECC(++) ↓HPP(+) Proteinuria(+++) Focal GN
10	39	F	15	Arthritis(++), Rash(+), Pleuropericarditis(+), Raynaud's(+), Lymphadenopathy(+)	+ homo	23	-	42	3.5	45	80	Proteinuria(+++) ↑Serum creatinine(+) Bx: proliferative GN
11	44	F	5	Arthritis(++), Psychiatric (+), Alopecia(+), Rash(+), Pleurocarditis(++), Raynaud's(+)	+ speckled	30	N.D.	70	14	75	85	↓Hgb(+), ↓WBC <3500(+) Proteinuria(+++) Focal GN
12						30	+	7	20	120	48	
13	38	F	2	Arthritis(++), Alopecia(+)	+	N.D.	N.D.	17	5	115	57	HBP(+), Proteinuria (+++), Menbranous GN,
14	36	M	14	Arthritis(++), Rash(+) Vasculitis(+), Pleuroperi-carditis(++), Lymphadenopa-thy(avascular necrosis, hips)	weakly + homo	30	-	8.3	15	120	27	WBC(+), Proteinuria (+++), Serum crea-tinine(+), Glome-rulonephritis, RF(-)

TABLE 34 (Continued)

Patient	Age (years)	Sex F/M	Duration of Disease (years)	SLE Manifestations	ANA (-)	Anti-Coagulant (<34)	VDRL (-)	C1q (<4%)	C4 (>20 mg/ml)	C3 (>100 mg/ml)	DNA Binding (<30)	Others
16	61	F	3	Arthritis(+++), Neurological (+), Alopecia(+)	+	27	-	33	36	145	70	WBC(+), Serum creatinine(+), Focal GN
19	29	M	5	Arthritis(+++), Alopecia(+), Pleuropericarditis(++)	+ Speckled	30	-	1.8	66	180	0	HBP(++), Protenuria (+++), Membranous GN
23	26	F	2	Arthritis(+++), Rash(+)	+ homo	32	-	22	21	95	15	Hgb(+), Protenuria (+), Serum creatine (+), RBC casts
26	23	M	2	Arthritis(+++), Rash(+)	+ nucleolar	40	-	9	24	120	61	

( )[1] gives the normal values.

\+ minimal feature of SLE disease
++ moderate feature of SLE disease
+++ severe feature of SLE disease

FIGURE 39. dDNA BINDING OF SOME NORMAL, SLE AND RA SERA

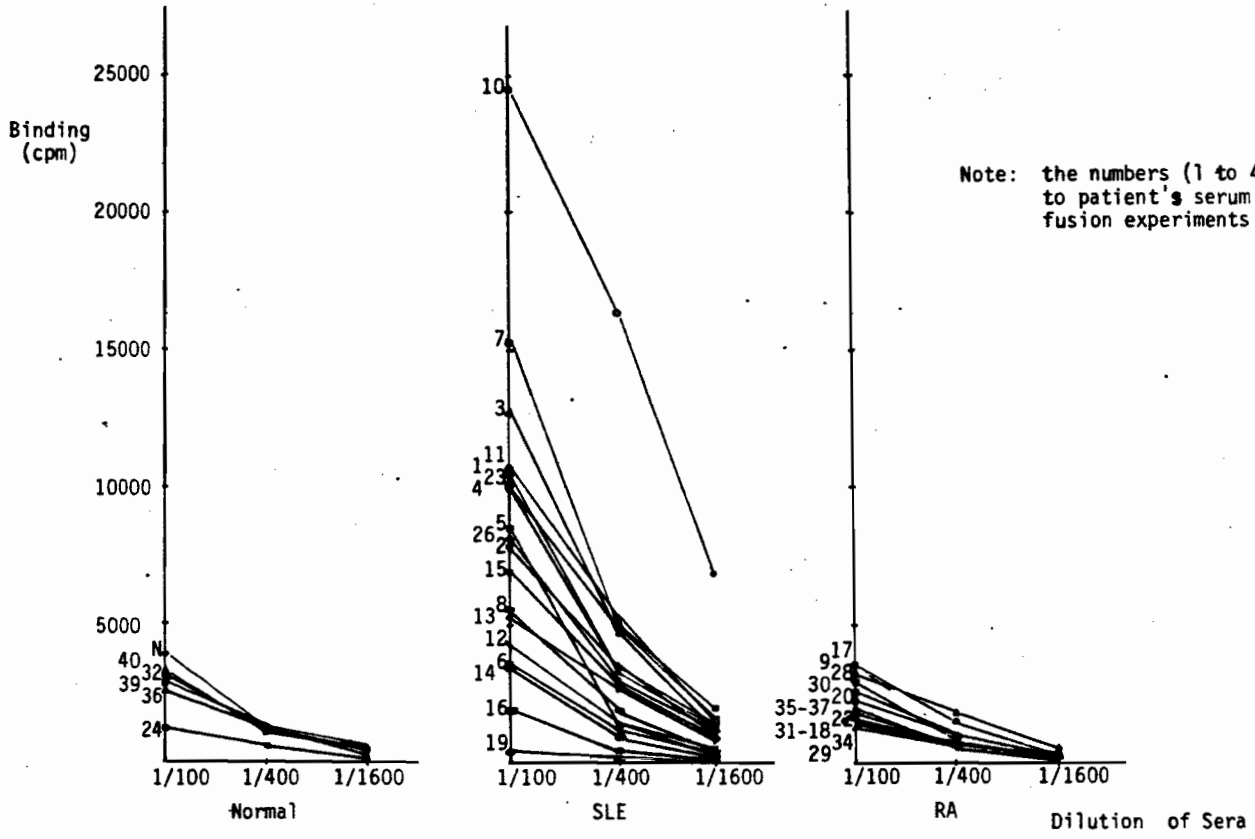


FIGURE 40. nDNA BINDING OF SOME NORMAL, SLE AND RA SERA

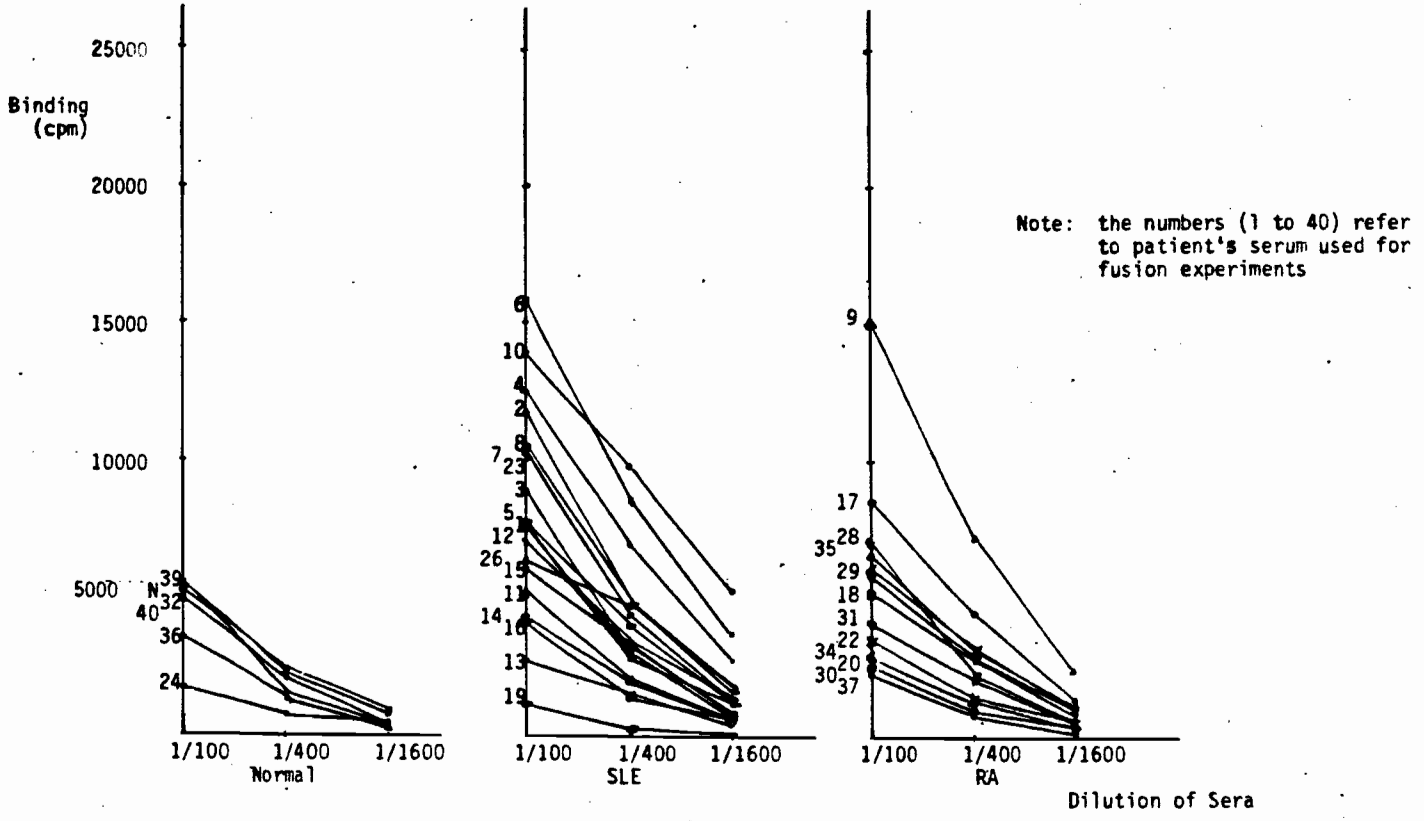
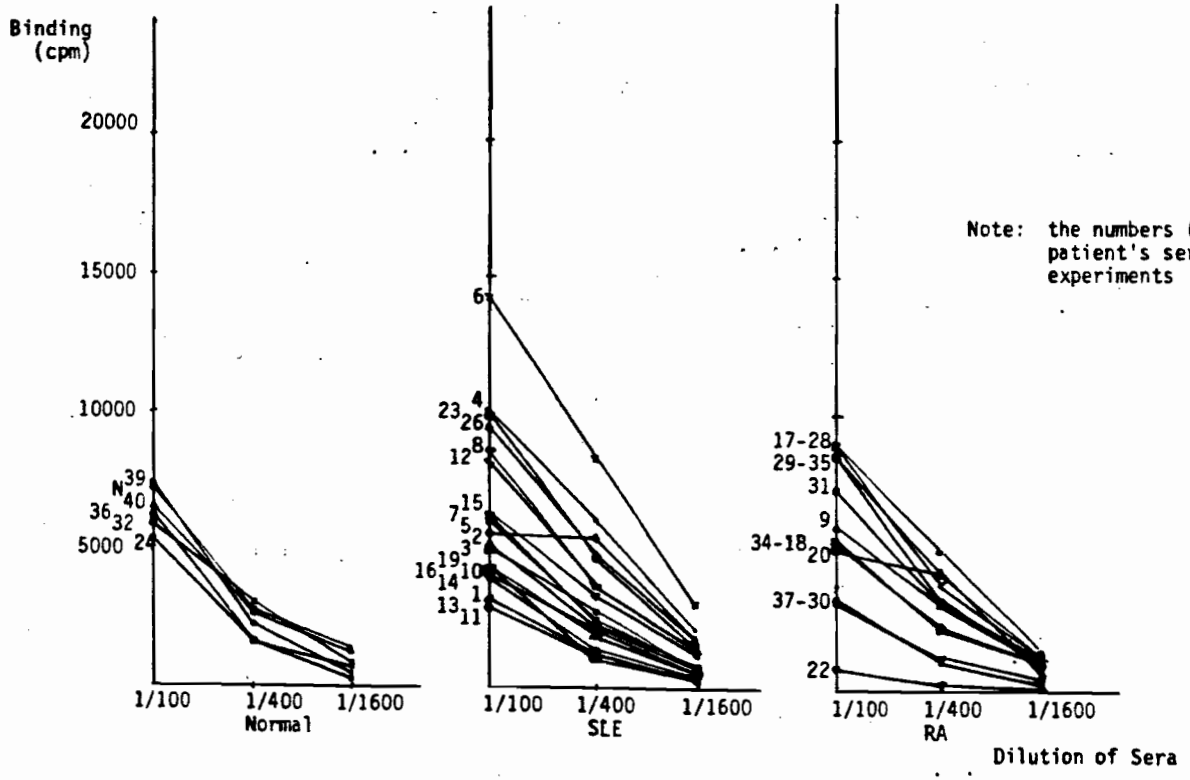
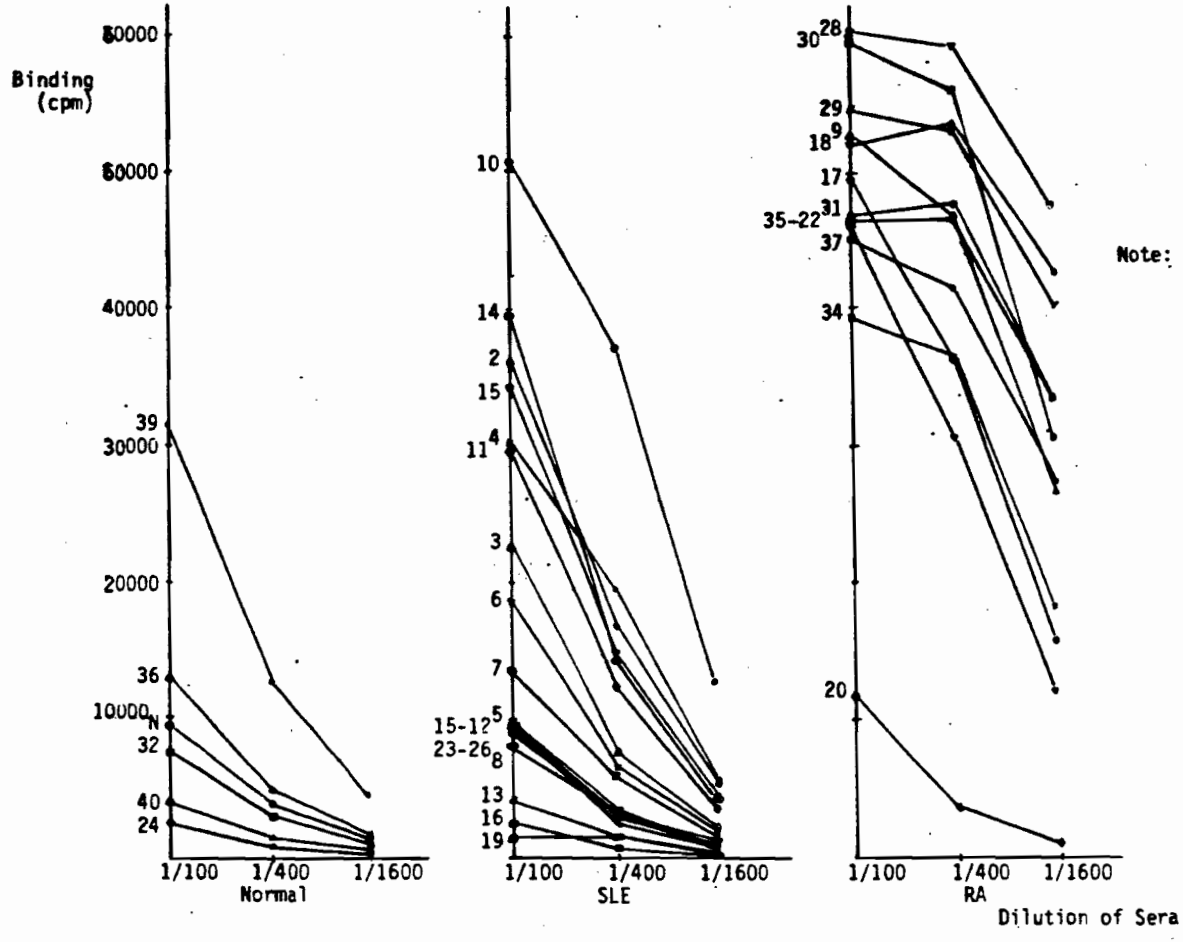


FIGURE 41. CARDIOLIPIN BINDING OF SOME NORMAL, SLE AND RA SERA



Note: the numbers (1 to 40) refer to patient's serum used for fusion experiments

FIGURE 42. RF ACTIVITY OF SOME NORMAL, SLE AND RA SERA



Note: the numbers (1 to 40) refer to patient's serum used for fusion experiments

TABLE 35

RIA DIRECT BINDING ACTIVITIES FOUND IN SLE AND RA SERA

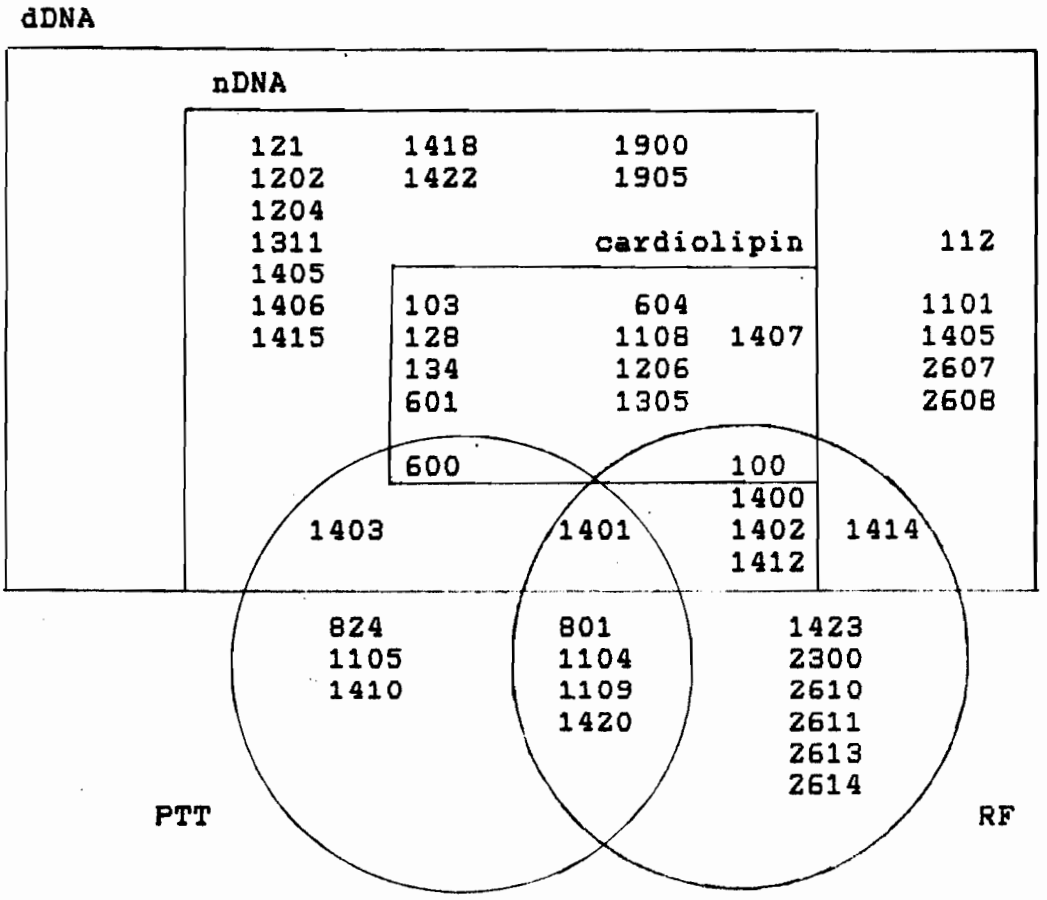
Binding activities found in sera	Fusion number *	
	SLE	RA
dDNA + nDNA + cardio[1] + RF	4	-
dDNA + nDNA + cardio	8,23	-
dDNA + nDNA + RF	2,10,26.	-
dDNA + nDNA	1,3,5,7,15	-
nDNA + cardio + RF	6	17,29,35
nDNA + cardio	12	-
nDNA + RF	-	9,18,28
cardio + RF	-	22
dDNA	11,13	-
RF	16	31,31,34,37
no reactivity	14,19	20

* a serum was recorded to have a binding activity to the antigen when its binding to it at the 1/100 dilution was higher than all the normal sera values at the same dilution

[1] cardiolipin

TABLE 36

DIRECT BINDING ACTIVITIES FOUND IN SLE HYBRIDOMAS

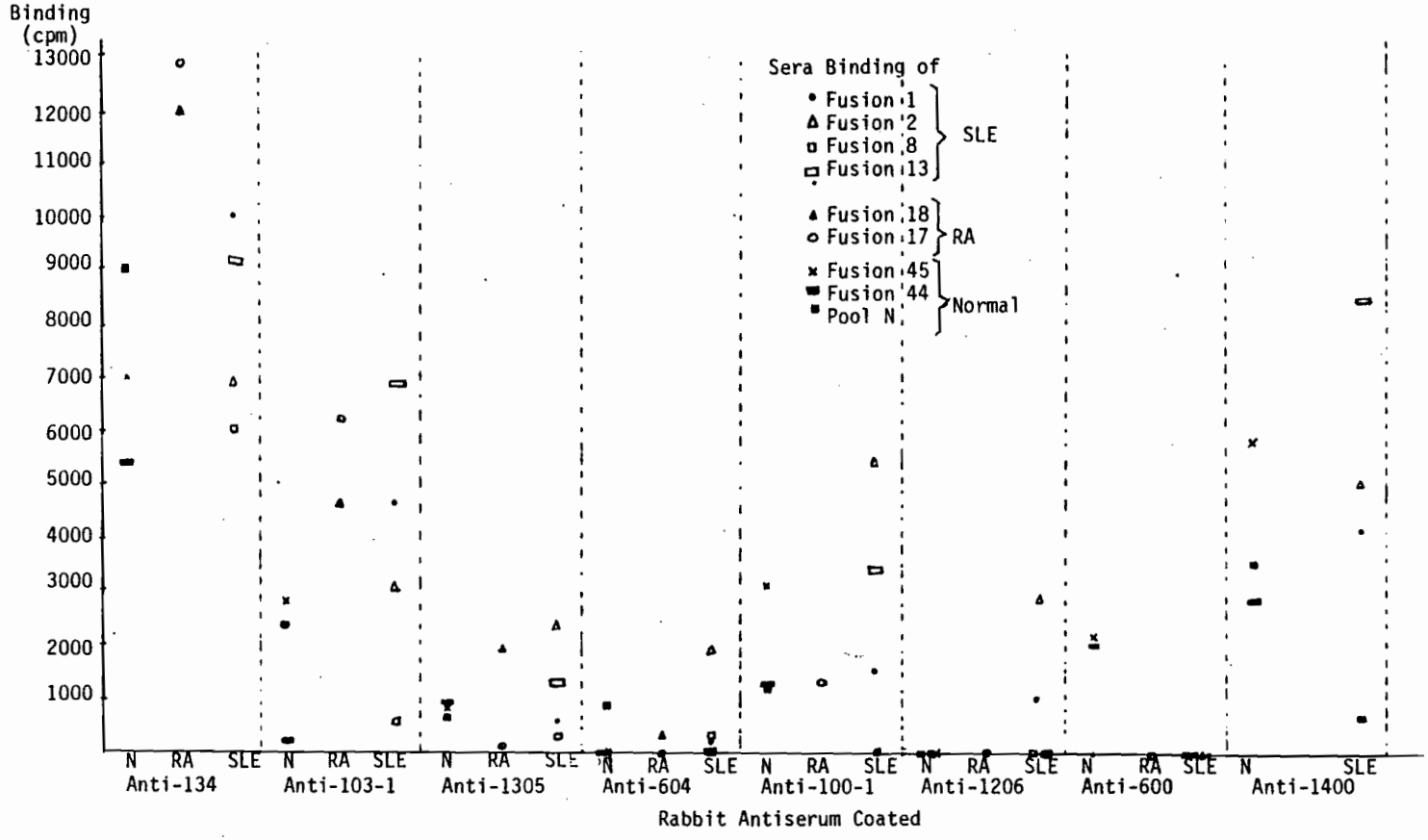


* 215 hybridomas without any of these activities

TABLE 37. REACTIVITIES OF HYBRIDOMA ANTIBODIES PER FUSION

	Number of Hybridomas per Fusion Reactive to														
Binding Activities	1	6	7	8	10	11	12	13	14	15	16	19	23	26	Total
dDNA+nDNA+Cardi+PTT		1													1
dDNA+nDNA+Cardi+RF	1														1
dDNA+nDNA+Cardi	3	2				1	1	1	1						9
dDNA+nDNA+PTT+RF									1						1
dDNA+nDNA+PTT									1						1
dDNA+nDNA+RF									3						3
dDNA+nDNA	1						2	1	5			2			11
dDNA+RF									1						1
dDNA	1					1			1					2	5
RF+PTT				1		2			1						4
RF									1				1	4	6
PTT				1		1			1						3
Total of Reactive Hybridomas	6	3	0	2	0	5	3	2	16	0	0	2	1	6	46
Total of Non-Reactive Hybridomas	30	3	3	46	3	3	5	19	9	36	37	10	3	8	215

FIGURE 43. IDIOTYPE ACTIVITIES IN SERA



CHAPTER 17. MISCELLANEOUS EXPERIMENTS

17.1 Choice of Buffer for Radioimmuniassays

17.11 Effect of Tween 20 Concentration in the RIA Buffer

The effect of Tween 20 in the binding of 2 hybridoma anti-DNA antibodies (103-1 and 134) to uncoated and dDNA coated tubes is shown in Figure 43. The dotted line represents the binding of a non-DNA binding hybridoma antibody (1500) in the RIA. In the absence of Tween 20, the anti-DNA antibodies displayed high binding to uncoated tubes. The addition of 0.01% Tween 20 resulted in a decrease in the binding of 103-1 and 134 to uncoated tubes by 93.7% and 82.8% respectively. Increasing concentrations of Tween 20 in the RIA buffer did not further decrease the binding of the antibodies to the uncoated tubes. However, the addition of 0.01% Tween 20 to the RIA buffer reduced the binding to dDNA coated tubes of these two anti-DNA antibodies by 78.6% and 7.9%, respectively. In the case of 103-1 (Figure 43A, upper panel), the ratio of cpm bound to dDNA tubes over control uncoated tubes was 1.3 (6826/5186) in the absence of Tween 20, compared with 4.4, (1458/329) in the presence of 0.01% Tween 20. An even greater augmentation was noted for 134 (Figure 43B, lower panel), which had a ratio of 2.6 (4154/1626) in the absence of Tween 20 and 13.7 (3727/280) in the presence of 0.01% Tween 20. Thus, Tween 20 was deemed a necessary component of the RIA buffer for the elimination of the non-specific binding of immunoglobulin to uncoated polystyrene tubes and for rendering greater specific of anti-DNA antibodies to the dDNA coated

tubes. For all subsequent experiments, a concentration of 0.01% Tween 20 was chosen as optimal since higher concentration of Tween 20 did not add anything to the specificity of the assay.

17.12 Effect of Different Buffers

The effect of different buffers (all supplemented with 0.1% BSA and 0.01% Tween 20) is shown in Table 38. The highest binding of both 103-1 and 134 anti-DNA antibodies was obtained using the KPO_4-Tween buffer. A comparison of the cpm bound to dDNA coated tubes in the 3 buffers demonstrated that the binding of 103-1 in the KPO_4-Tween buffer was 2.5 times higher than TBS-Tween, and 1.5 times higher than in PBS-Tween. Similarly, the binding for 134 in the KPO_4-Tween buffer was 4.8 and 1.4 times higher than in TBS-Tween and PBS-Tween buffers, respectively.

Thus, the optimal buffer in the present anti-DNA RIA was determined to be 0.1M KPO_4-0.1% BSA-0.01% Tween 20 buffer (0.01% Tween), and was used in all subsequent experiments.

17.13 Effect of PLL Pretreatment Concentration

Table 39 shows the effect of PLL precoating on the binding of the same hybridoma anti-DNA antibodies (103-1 and 134) to tubes coated with PLL alone and PLL followed by dDNA. The values shown represent the mean cpm bound to duplicate tubes, minus the cpm bound by control coated tubes containing buffer only (0.01% Tween). The cpm bound in buffer tubes ranged from 208-322 cpm. The binding of a non-DNA binding hybridoma IgM antibody (1500) did not exceed 441 cpm on all the tubes.

For both antibodies, 134 and 103-1, the maximum binding to dDNA coated tubes with the minimum binding to PLL coated tubes occurred at a PLL concentration of 50 µg/ml. Based on these results, 50 µg/ml of PLL was chosen for use in subsequent experiments.

17.14 Retention of ^{125}I-Radiolabelled DNA on Polystyrene Tubes in the Presence or Absence of PLL

Table 40 shows the amount of denatured and native ^{125}I-DNA retained on polystyrene tubes alone or on tubes precoated with PLL (50 ug/ml). The results given in Table 40 represent the mean standard deviation (S.D.) of three separate experiments. It can be observed that for dDNA, precoating with PLL resulted in the binding of 63.4% of the added ^{125}I-DNA, compared with 17.3% binding occurring in the absence of PLL. Similarly, tubes precoated with PLL bound 30% more nDNA than untreated tubes, which bound only 15% of the added radiolabelled DNA.

17.15 Binding Characteristics of Hybridoma Anti-DNA Antibodies to dDNA and nDNA Coated Tubes With and Without PLL Pretreatment

Table 41 shows that antibodies 103-1 and 134 demonstrated very similar binding to dDNA tubes coated with or without PLL pretreatment. However, the binding to nDNA tubes pretreated with PLL was significantly higher than the binding to tubes coated with nDNA alone for antibody 134, but not for antibody 103. The data in Table 41 represent the mean values of 3 separate experiments.

17.16 Binding of Anti-DNA Antibodies in Serum to dDNA and nDNA Coated Tubes With or Without PLL Pretreatment

A high DNA binding SLE serum determined by the clinical DNA binding test was assessed on dDNA and nDNA coated tubes with and without PLL pretreatment, as well as a pool of normal human sera. Figures 44 and 45 illustrate the results. The dotted line indicates the maximum binding value for normal human sera at a 1/100 dilution. The anti-DNA antibody binding of SLE whole serum showed approximately 20% higher binding to dDNA tubes precoated with PLL (Figure 44). Similar to the hybridoma antibodies, the serum antibodies reactive with nDNA exhibited 2-3 times greater binding when assayed on nDNA tubes precoated with PLL than on tubes coated with nDNA alone (Figure 45).

17.2 ^{125}I-Labelling

The iodination of a protein by the chloramine T method involves in a mixture of cold protein, ^{125}I-radiolablelled protein and free ^{125}I. To separate the components, the mixture was applied to a Sephadex G25 column. This column has the property of separating substances according to their molecular weight. The high molecular weight substances pass rapidly through the column, while the low molecular weight substances are retarded. Two peaks are thus expected if one measures the radioactivity of the eluted fractions from the fractionation of iodinated protein: the first peak corresponds to ^{125}I-radiolabelled protein, while the second corresponds to the free ^{125}I.

Figure 46 shows the iodination-elution profile of

rabbit anti-HIgM (in a dotted line) and rabbit anti-HIgG (in a solid line) from a passage on a Sephadex G25 column. The results are representative of all iodinations done and correspond to what was expected. In this case, the £4 tubes which contain ^{125}I-rabbit anti-HIgG and ^{125}I-rabbit anti-HIgM respectively were kept for RIA. Each month, the iodination of each immunoglobulin was repeated, since the lifespan of an ^{125}I-radiolabelled protein is approximately one month.

17.3 Profile of Purification of Hybridoma Antibodies

To purify the hybridoma IgM antibodies, spent culture fluid was passed over a Sepharose rabbit anti-HIgM column. This affinity column retained all IgM antibodies from the supernatant by non covalent bonding. The IgM antibodies were then eluted from the column by an eluant, 0.1M glycine-HCl, pH 2.3, which break the non covalent bonds. The OD_{280} of each fraction obtained was measured to locate the protein. Figure 47 shows the elution profile of the purification of 103-1 antibody (panel A) and 134 antibody (panel B). Both profiles are representative of all purifications done. All fractions with an OD_{280} greater than 0.01 were kept, pooled, dialysed, concentrated, aliquotted and stored at -4°C.

TABLE 38

EFFECT OF BUFFERS ON THE BINDING OF ANTI-DNA ANTIBODIES TO dDNA COATED TUBES

Sample	Binding (in cpm) to dDNA coated tubes using the following buffers *		
	KPO_4-Tween	TBS-Tween	PBS-Tween
buffer	355	299	273
103-1 antibody	1766	856	1184
134 antibody	3999	1060	2903

* all buffers contained 0.1% BSA and 0.01% Tween 20

TABLE 39

DETECTION OF ANTI-DNA ANTIBODIES ON POLY-L-LYSINE (PLL) PRECOATED TUBES

	Binding (in cpm) of			
	134 antibody to tubes coated with		103-1 antibody to tubes coated with	
Concentration of PLL Coated (µg/ml)	PLL alone	PLL + dDNA	PLL alone	PLL + dDNA
0	458	6602	124	2924
5	700	7089	208	1865
25	920	7337	396	1375
50	633	7580	113	2915
100	737	7324	119	2931

TABLE 40

RETENTION OF ^{125}I-RADIOLABELLED DNA ON POLYSTYRENE TUBES IN THE PRESENCE OR ABSENCE OF PLL

	^{125}I-dDNA			^{125}I-nDNA		
	cpm	S.D.	% bound *	cpm	S.D.	% bound
PLL	1243	381	63.4	947	202	46.2
no PLL	339	7	17.3	322	25	15.7

* the numbers of cpm added to each tubes were 1961 235 cpm for dDNA and 2049 268 for nDNA.

TABLE 41

EFFECT OF PLL ON DNA BINDING ASSAYS

Hybridoma culture fluid *	Binding (in cpm) to tubes coated with: PLL alone	dDNA alone	PLL + dDNA	nDNA alone	PLL + nDNA
GM 4672	482	494	510	579	569
1500	604	503	586	543	681
103-1	1301	15776	15787	1203	1404
134	2410	30356	29242	2083	5392

* the concentration of immunoglobulin in the hybridoma culture fluids were 1.7, 1.4, and 2.0 µg/ml for 1500, and 134, respectively

FIGURE 44. EFFECT OF THE TWEEN 20 CONCENTRATION IN THE RIA BUFFER ON THE BINDING OF ANTI-DNA ANTIBODIES TO UNCOATED AND dDNA COATED TUBES

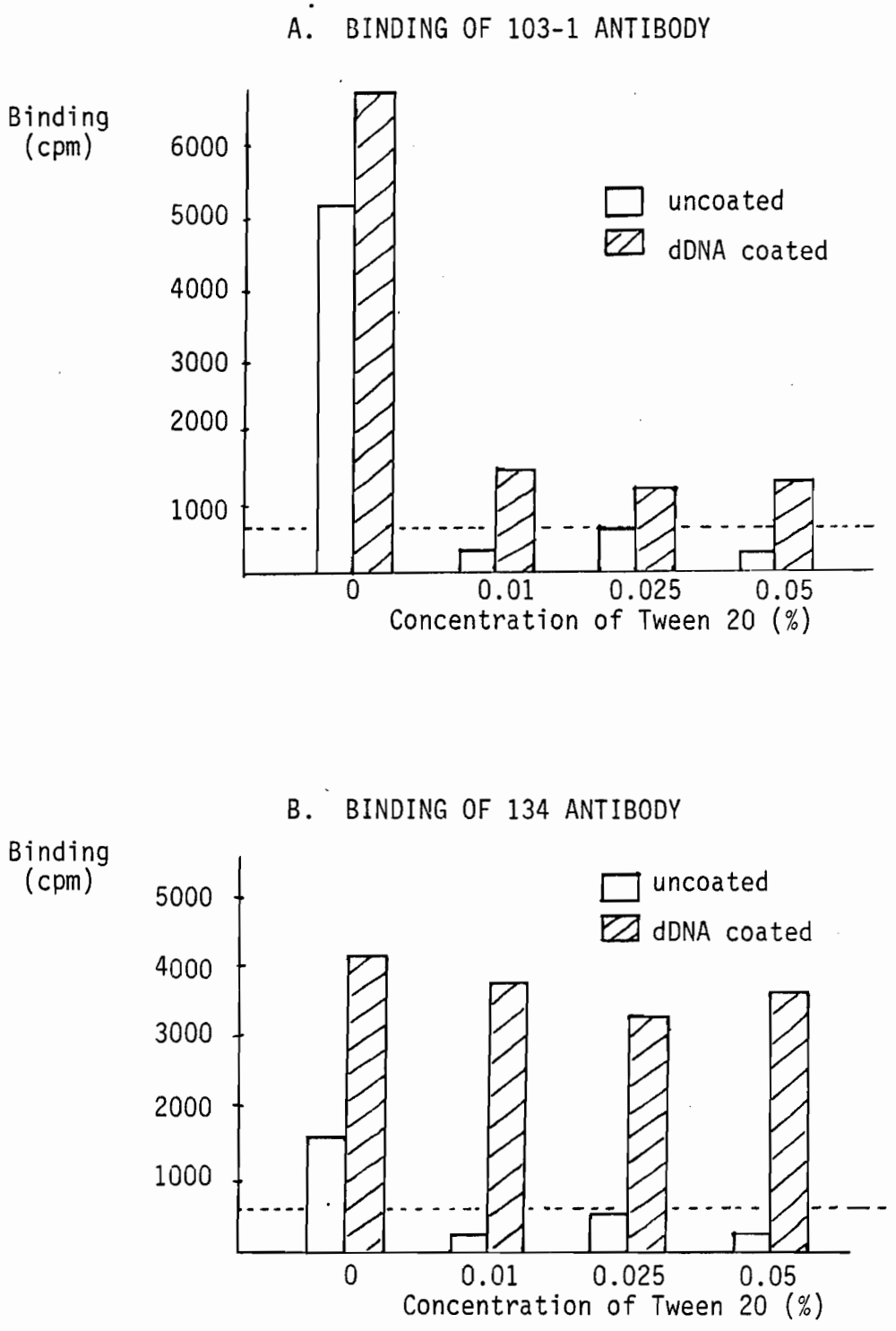


FIGURE 45. BINDING OF A HIGH DNA BINDING SLE SERUM TO PLL, dDNA AND PLL+dDNA COATED TUBES

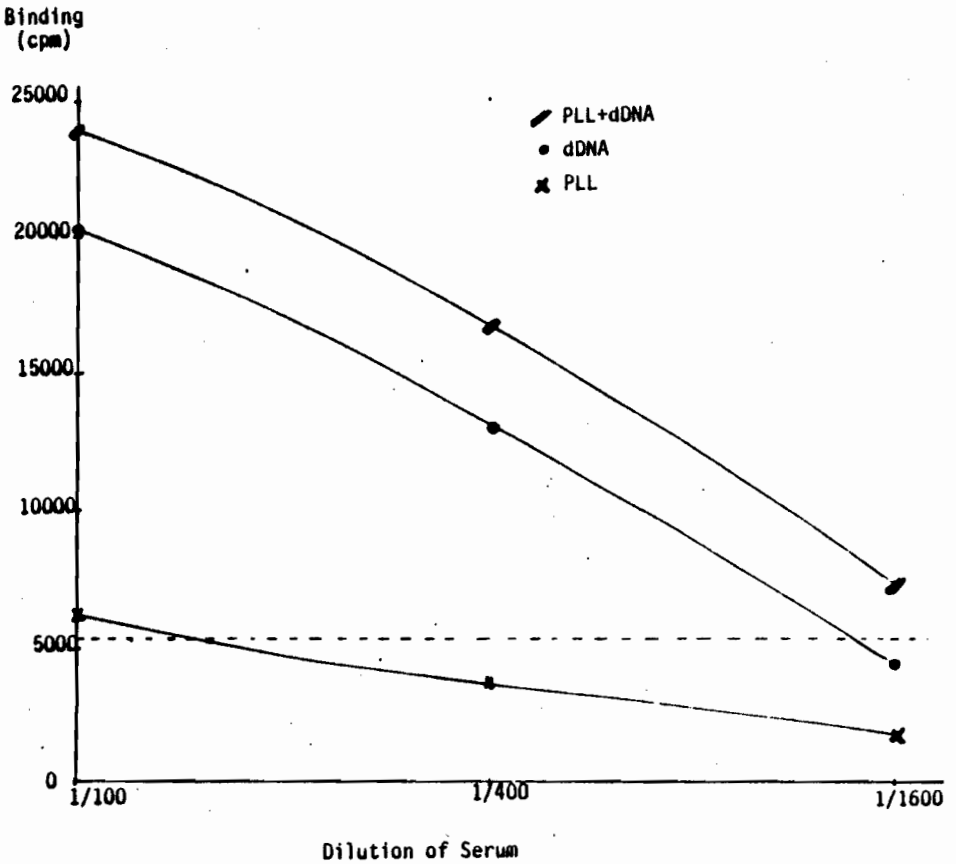


FIGURE 46. BINDING OF A HIGH DNA BINDING SLE SERUM TO PLL, nDNA AND PLL+nDNA COATED TUBES

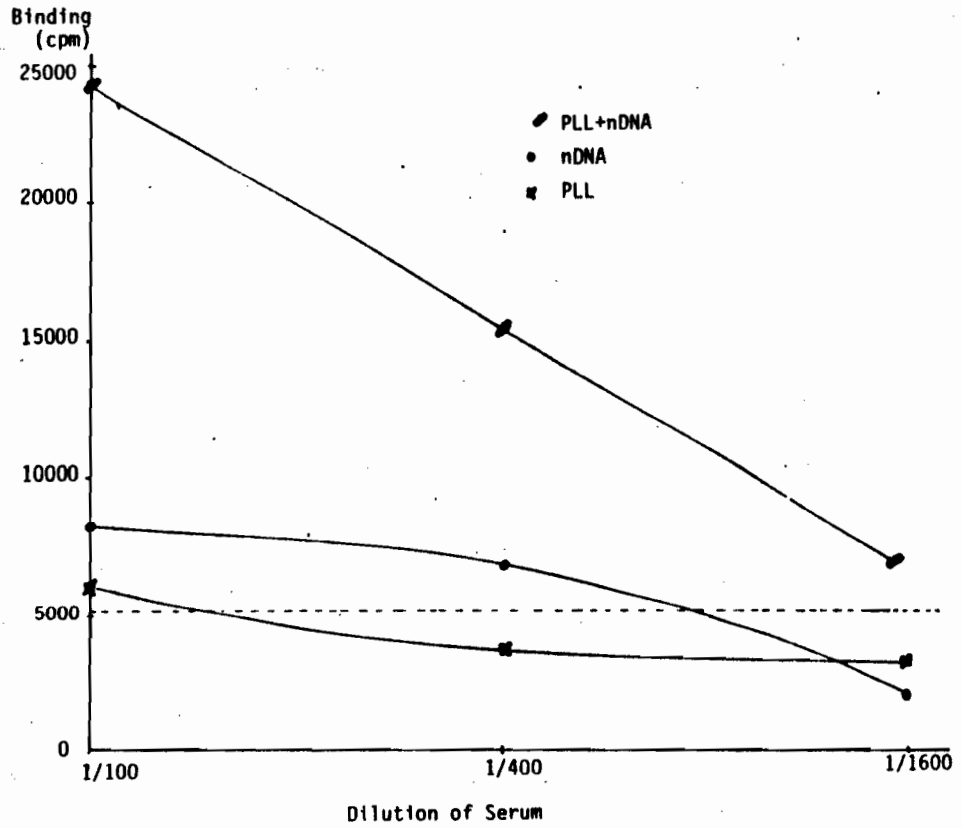


FIGURE 47. ELUTION PROFILES ON A SEPHADEX G25 COLUMN

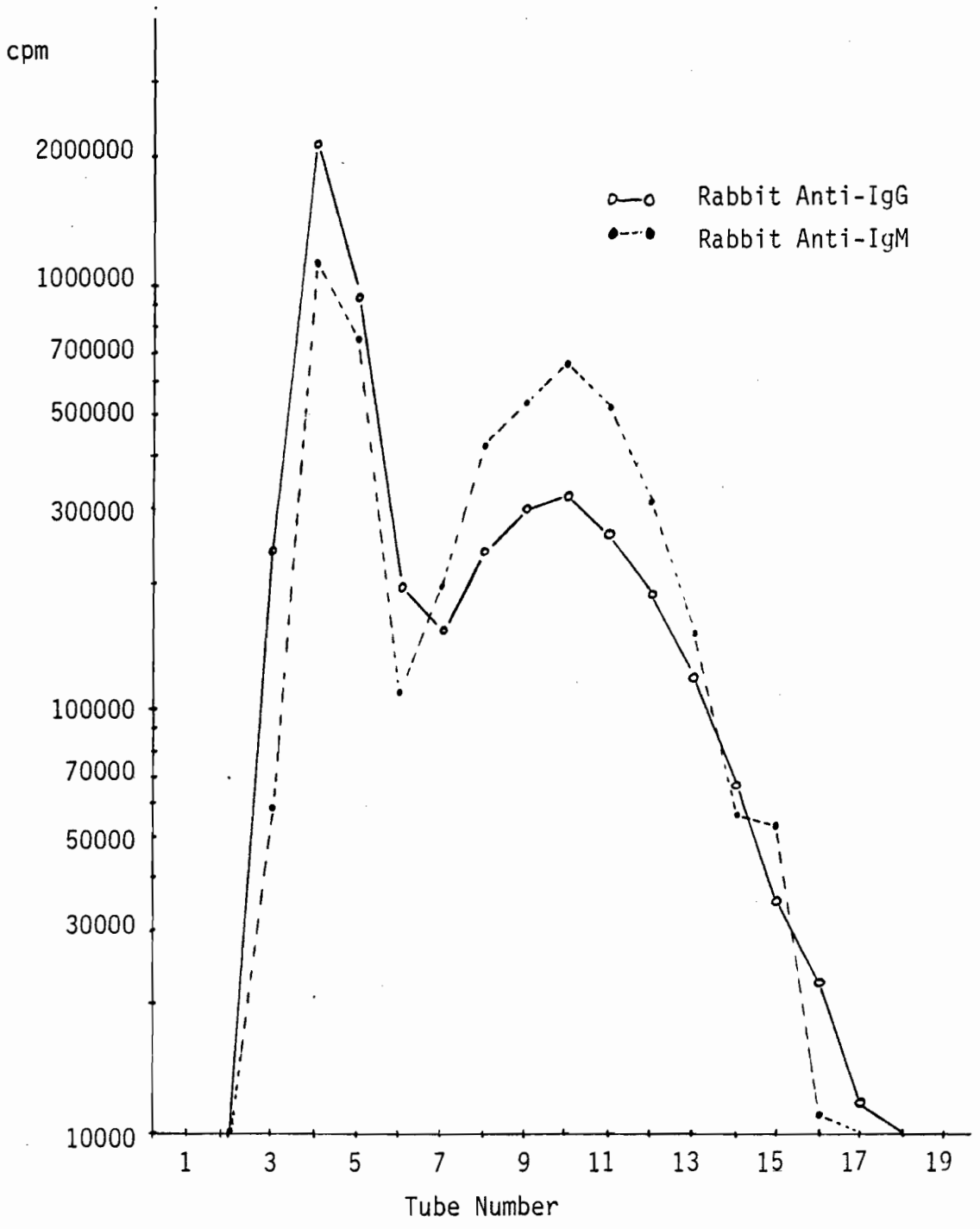
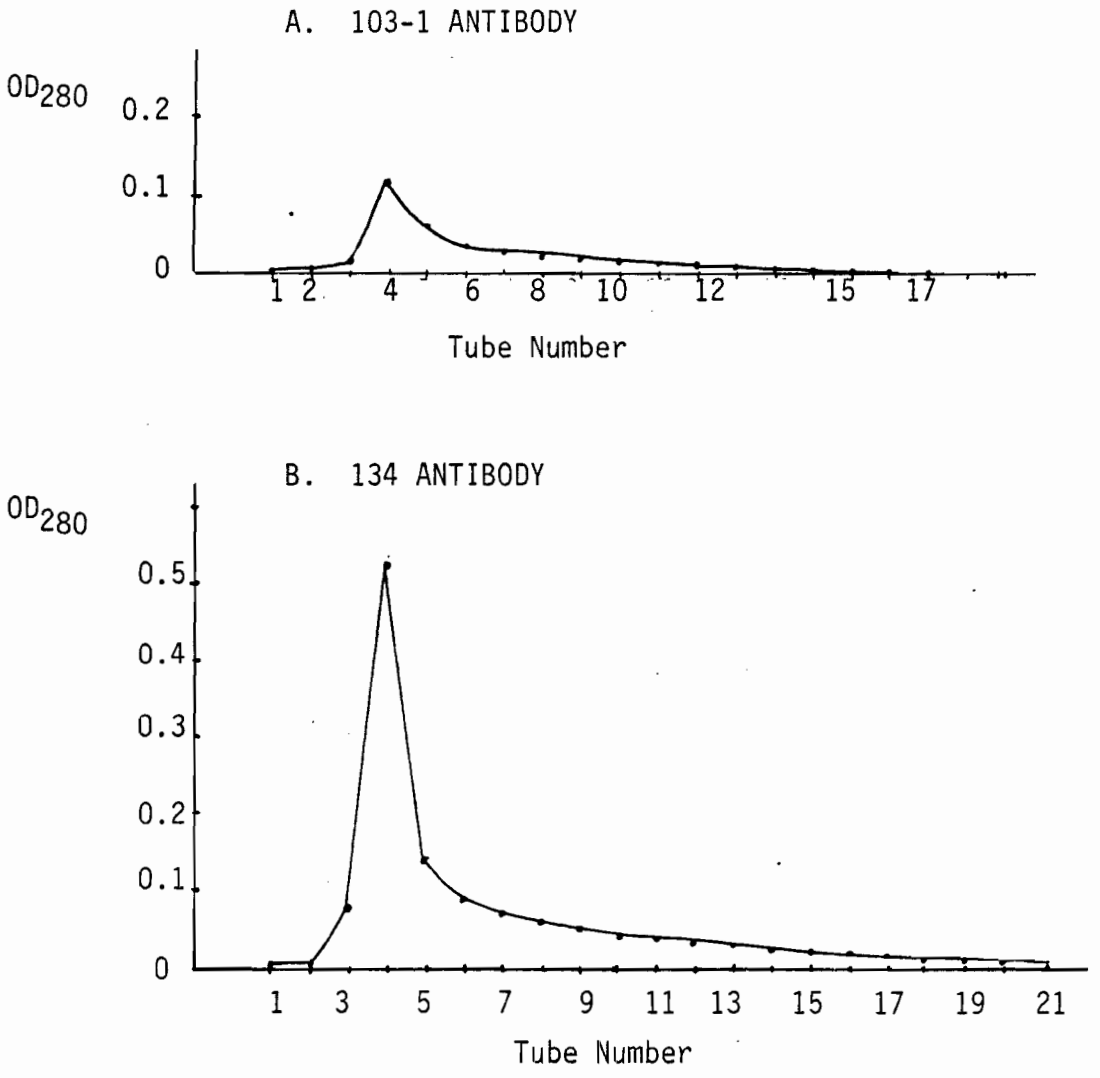


FIGURE 48. PROFILES OF PURIFICATION ON A SEPHAROSE RABBIT ANTI-HUMAN IgM COLUMN



Part IV. Discussion

Chapter 18. PRODUCTION OF HUMAN AUTOANTIBODIES

18.1 Some Conditions of a Successful Fusion

There are several human cell lines of plasmacytoma or lymphoblastoid cell origin which have been successfully fused to produce human-human hybridomas, capable of synthesizing human monoclonal antibodies without the aid of Epstein-Barr virus transformation. These are U-266 AR_1 (or SK0-007), a mutant of the U-266 myeloma cell line (Olsson 1980); GM 1500 6TG-A12 and GM 4672, mutants of the GM 1500 cell line (Croce 1980); LICR-LON-HMy2, a mutant of the ARH-77 cell line (Edwards 1982); 8226 AR/NI P4-1, a mutant of the RPMI 8226 human myeloma line (Pickering 1982); GK-5 human myeloma line (Satoh 1983); UC 729-6 and WIL2/729 HF, mutants of the WIL-2 well line (Glassy 1983); RH-L4 cell line (Olsson 1983); LSM 1.1 and LSM 1.2, mutants of the CRL1484 cell line (Schwaber 1984) and the LTR 228 cell line (Larrick 1983). These cell lines were developed by adding 8-azaguanine or 6-thioguanine to the parent cell line to select strains which are deficient in the hypoxanthine guanosine phosphoribosyl transferase (HGPRT) enzyme. These mutants are thus incapable of surviving in a hypoxanthine-aminopterin-thymidine (HAT) medium, the selective medium employed to segregate hybridomas from the parental unfused cells, the myeloma, and the lymphocytes. The latter cells cannot be maintained in tissue culture and die in a few days regardless of the medium used. Each of these human

cell lines differs in immunoglobulin secretion, phenotypic characteristics, and Epstein-Barr nuclear antigen (EBNA) expression (Kozbor 1983).

The GM 4672 lymphoblastoid subline of the GM 1500 cell line, which produces an IgG2k immunoglobulin and is EBNA-negative (Shoenfeld 1982), was available at the time the present study began. Thus, the GM 4672 cell line was used as a fusion partner to produce human-human hybridoma antibodies from SLE patients. Peripheral blood lymphocytes of 18 SLE patients satisfying the revised ARA criteria for the classification of SLE (Tan 1982) were fused to GM 4672.

Shoenfeld (1982) and Cairns (1984), using the GM 4672 cell line, reported yields of hybridoma growth of 19-57% and 26%, respectively, which is comparable to the yield of hybridomas of 1 to 40% observed in this study. Moreover, these two authors reported successful growth in each of their fusions, which was also the case in the present report if the 4 fusions contaminated before the appearence of hybridomas are excluded. Successful growth without any failure and 83-100% yields of hybridomas were reported with the mutant GM 1500-6TG-A12, from which GM 4672 was derived (Croce 1980, Eisenbarth 1982 and Littman 1983). In contrast, Houghton (1983) and Abrams (1983), using the GM 4672 cell line, reported no clones in the 3 fusions done by Abrams while only one clone per 10^7 cells fused from the 8 fusions done by Houghton.

Houghton and Abrams also evaluated the yield of

hybridomas obtained from another cell line, LICR-LON-HMy2. These two authors reported successful fusion with this cell line, with a 12 to 28% yield of hybridomas by Abrams for the 3 fusions done, while Houghton just mentioned that better results were obtained with this cell line than with the SK0-007 and GM 4672 cell lines. This HMy2 cell line was also used by Sikora (1982), Edwards (1982), and Cote (1983) who observed, respectively, a 0 to 36% yield of hybridomas with success in 5 of 12 fusions; a 0 to 100% yield of hybridomas with success in 27 of 34 fusions; and a 55% yield (no range given) in 38 fusions (no indication of how many were successful). Two attempts were made to produce hybridomas with the HMy2 in the present study. One attempt was made with the PBL of an SLE patient, while the second was made with the PBL of an RA patient. In both cases, no hybridomas arose, while a portion of the same lymphocyte population of these 2 patients fused with the GM 4672 cell line resulted in 48 and 17 hybridomas, respectively. Thus, similar to Sikora and Edwards who did not observe hybridomas with the use of HMy2 in some of their fusions (58% and 21% unsuccessful fusions, respectively), the present study was unsuccessful in the two atttempts at fusions made using HMy2. It appears that successful fusion with HMy2 is more difficult to obtain than with GM 4672, which yields more consistent results among investigators. It is unknown what factors contribute to the discrepancies between these 2 cell lines.

Table 42 summarizes, in chronological order, the fusion

and some plating conditions of published reports on successful human-human hybridoma fusions performed without the use of Epstein-Barr virus transformation. Different human myeloma or lymphoblastoid cell lines were used. Lymphocytes for fusion were obtained from spleens, peripheral blood, tonsils, lymph nodes, and solid tumors. Donors have included normal subjects, as well as patients suffering from SLE, cold agglutinin disease, atopic allergy, diabetes mellitus, subacute sclerosing panencephalitis, cancer, and gliomas, among others. Ratios of lymphocytes to myeloma cells ranged from 1:3 to 10:1; PEG concentrations varied from 30 to 50%; and cell plating densities between $2x10^5$ cells/well and $2x10^6$ cells/well were used. The yield of hybridomas and successful fusions varied among these investigations. The differences in the source and state of lymphocytes, PEG concentration, cell density and cell ratio, the type and state of myeloma cell, the size of the well, the use of feeder layers, and polyclonal mitogen stimulation of lymphocytes prior to fusion may account for the variable results observed. Some of these factors have been evaluated to determine the optimal conditions for human-human fusions.

Shoenfeld (1982), using the GM 4672 cell line and PEG concentrations of 38%, 44%, and 50%, demonstrated that 44% PEG resulted in the highest production of clones. Based on his observation and the fact that the same myeloma line was used, a PEG concentration of 44% was selected as optimal for the present study. Satoh (1983), Littman (1983), Abrams (1983),

Croce (1980), Edwards (1982), and Strike (1984) used a PEG concentration of 50% with different myeloma cell lines. None of these investigators obtained comparable results. The percent of successful fusion as well as the yield of hybridomas varied from one author to the other, indicating that the optimal PEG concentration may vary depending on the fusion cell partner used. In the mouse-mouse fusion system, Lane (1984) and Davidson (1976) showed, using the same myeloma line throughout their experiments, that the molecular weight as well as the time of contact of PEG with the cells to fuse had an effect on the yield of hybridomas. Low molecular weight PEG and longer time of cell contact yielded more hybridomas. These facts further indicate the need to evaluate the optimal concentration of any PEG preparation on each cell fusion partner and also the optimal PEG contact period in the production of human-human hybridomas.

Mitogenic substances were not used in the present study, where the interest was focused on the production of hybridoma autoantibodies which may reflect the state of the lymphocytes <u>in vivo</u>, and not on what the lymphocytes of an SLE patient can be stimulated to produce <u>in vitro</u>.

Shoenfeld (1982) as well as Denis (1983) and Kaplan (1982) showed that mitogenic lymphocytes gave greater yield of hybridomas when fused than non-mitogenic ones. However, the yields of hybridomas from mitogen stimulated lymphocytes found by Shoenfeld (using GM 4672 and SLE lymphocytes) and Kaplan (using U-266 AR_1 and Hodgkin's disease lymphocytes) are not

much higher than the range of yield of hybridomas obtained from unstimulated lymphocytes observed here (using GM 4672 and SLE lymphocytes) and by Olsson (1980); using U-266 AR_1 and Hodgkin's disease lymphocytes). This indicates that the state of these lymphocytes was not really changed by the mitogenic stimulation; they were probably already in an activated state and thus unable to respond further. Fusion of PBL from normal persons, done by a co-worker, gave very few hybrids. Cairns (1984), however, succeeded in producing hybridomas from tonsillar lymphocytes of normal origin with a yield comparable to the fusions done in this study. This indicates that normal tonsillar lymphocytes are not in the same state as peripheral blood lymphocytes. Coté (1983), Glassy (1983), Houghton (1983), and Kaplan (1982) also found, using lymphocytes from normal and patients with different disease states, that PBL gave very poor yields of hybridomas when compared to lymph node or splenic lymphocytes. On the other hand, one should keep in mind that the B-cell fraction of PBL is much lower than the B-cell fraction of tonsil. Thus, the fusion frequency of B-cells is higher with higher B-cell enriched organs.
One should be cautious and carefully document the lymphocyte source used when discussing the yield of hybridomas produced. Edwards (1982), using the HMy2 line, and lymphocytes derived from tonsils, lymph nodes, peripheral blood and tonsillar lymphocytes, sensitized <u>in vitro</u> to TNP-SRBC (2,4,6-trinitrophenylated sheep red blood cells) did not find any difference in the frequency with which hybrids were

obtained from a comparable number of cells.

Denis (1983) fused mitogenic PBL from normal individuals and from patients with common variable immunodeficiency (CVI). He obtained hybridomas from all the fusions done with different mitogenic preparations of normal PBL, whereas CVI PBL produced hybridomas only when stimulated with a mixture of mitogen (PWM and Cowan I). These CVI PBL may be in a suppressed state, compared to SLE PBL, since they required stimulation prior fusion to produce hubridomas.

No feeder layer was used in the present study. Other studies, however (Brodin 1983 and Edouards 1982), demonstrated that the use of a feeder layer at the beginning of fusion might increase the yield of hybridomas.

18.2 Influence of Fusion Cell Ratio and Cell Plating Density

The effects of the lymphocyte-to-fusion partner cell ratio and the cell plating density on the outcome of hybridoma growth have been evaluated by only 4 investigators among the 23 listed in Table 42. Cell ratios ranging from 1:1 to 10:1 have been used by these 23 authors.

Shoenfeld (1982), with the GM 4672 cell line, studied cell ratios of 5:1 and 10:1 and determined that maximal hybridoma growth occurred using a 5:1 cell ratio. Further analysis on the influence of fusion cell ratios using the same GM 4672 line was done in the present study. Lymphocyte-to-GM 4672 cell ratios of 1:1 and 5:1 were used and compared (Section 10.3). The results demonstrated that the optimal production of clones (7.5%) occurred at a lymphocyte-to-GM 4672

cell ratio of 1:1. In fact, the use of a 1:1 cell ratio, compared with a 5:1 ratio of lymphocyte-to-GM 4672 cells, was found to double the yield of clones. Cairns (1984), using the GM 4672 cell line and cell ratios of lymphocyte-to-GM 4672 of 1:1 and 5:1, also found that the 1:1 ratio gave a better yield of hybridoma. Coté (1983) evaluated cell ratios of lymphocyte-to-HMy2 cell line of 1:1, 2:1, 5:1, and 10:1 and determined that the 1:1 and 2:1 ratios resulted in 2 to 8 times more hybrids than 5:1 and 10:1 ratios. Strike (1984) used cell ratios of lymphocytes-to-WIL2-729 HF cell line of 10:1, 5:1 and 1:1 and also found that the 1:1 ratio was the optimal condition to produce the best yield of hybridomas. With the results of these 4 investigators, it can be concluded that regardless of the myeloma cell line employed, the best yields of hybridoma in human-human fusion are obtained with the use of a ratio of lymphocytes-to-fusion partner of 1:1.

The influence of cell density (number of cells seeded per well and well size) on the successful generation of hybridoma clones was evaluated in the present study. Three cell densities were used; $1x10^5$, $2x10^5$ and $4x10^5$ cells/well with two well sizes, the two first cell densities plated in a 0.3 ml well and the other in a 2 ml well. Maximal clonal growth of 16.3% occurred when cells were plated at $4x10^5$ cells/well in 2.0 ml tissue culture wells. Lower cell numbers plated in smaller wells resulted in yields of 5.5 and 6.6% respectively, which are not statistically different (Appendix I). These results indicate that surface area as well as the concentration

of cells are important in determining the viability and growth of clones. The plating of approximately equal concentrations of cells ($3.3x10^5$/ml and $4x10^5$/ml) in 0.3 ml and 2.0 ml wells, respectively, resulted in a 2.5-fold greater yield (16.3% compared to 6.6%) in the larger wells. As the total cell numbers plated in these 2 types of wells differed by a factor of 4, these results cannot distinguish between the effects of the surface area and those of increased cell numbers. Systematic evaluation of equal cell numbers and equivalent cell concentrations plated in 0.3 ml and 2.0 ml wells is necessary to establish whether a larger surface area is, in fact, critical in determining increased hybridoma production.

Shoenfeld (1982) had previously noted hybridoma yields of 32%, compared with 1.8% when equal numbers of cells ($2x10^5$) were plated in 2.0 ml wells and 0.3 ml wells, and suggested that the bigger wells may be advantageous to hybridoma formation by providing more medium and nutrients per cell and a large surface area for hybridoma growth. Olsson (1980) also found cell concentration to have an important effect on hybridoma growth. He used cell densities ranging from $5x10^4$ to $1x10^6$ cells/well in 0.3 ml and reported that the optimal plating concentration for 0.3 wells was $2x10^5$ cells/well. The same cell density used in this study did not differ statistically from $1x10^5$ cells/well. Olsson found that higher cell numbers in these small wells resulted in poor hybridoma yields.

Cairns (1984), using the same cell densities of the

present study, also found that the best yield of hybridoma was obtained when $4x10^5$ cells per well in a 2.0 ml well were used. Strike (1984) compared 2 cell densities of $5x10^5$ and 10^6 cells/well in 2.0 ml wells and found that the best yield of hybridomas was with the use of $5x10^5$ cells/well in a 2.0 ml well. The above evidence suggests that the density of cells and available surface area are important in determining the successful growth of hybridoma clones. The best conditions were found with the plating of 10^5 cells/well in a 2.0 ml well

In the same way that both cell ratio and cell plating density had notable effects on the yield of hybridoma clones, there was a significant difference in the frequency of anti-DNA clones produced (Tables 6 and 7). A higher percentage of clones producing anti-DNA antibodies was also observed with the 1:1 cell ratio (17%), compared to the 5:1 cell ratio (11%). The cell density of $2x10^5$ cells/well with the use of the optimal cell ratio of 1:1, however, gave the best yield of anti-DNA hybridoma antibodies, 24% compared to 13% and 15% at cell densities of $4x10^5$ and $1x10^5$ cells/well respectively. Nonetheless, Table 8 shows that both cell ratio and cell density produced approximately equal proportions of weak and strong anti-DNA binding antibodies, which, moreover, secreted similar quantities of IgM (ranging between 0.26 and 19.9 µg/ml). If the percent of all seeded wells positive for both hybridoma growth and anti-DNA antibody production is calculated and compared for the optimal 1:1 cell ratio, a yield of 2.0% is obtained at the cell density of $4x10^5$ cells/well,

compared to 1.95% and 0.9% at cell densities of $2x10^5$ and $1x10^5$ cells/well, respectively. The same calculation, done for the 5:1 cell ratio, gave a yield of 1.3, 0.5, and 0.5% at cell densities of $4x10^5$, $2x10^5$, and $1x10^5$ cells/well respectively. Consequently, the best conditions to produce anti-DNA hybridoma antibodies are achieved with the use of a cell ratio of 1:1 with cell plating density of $4x10^5$ cells/well. Cairns (1984) also observed similar results in the yield of anti-DNA hybridoma antibodies.

18.3 Production of Hybridoma Antibodies with Specific Activity

Initially, hybridoma antibodies were screened for anti-dDNA, anti-nDNA, and anti-cardiolipin activities. Later, the new fusions were also screened for anticoagulant and rheumatoid factor activities. These 2 last activities were also determined in some stored culture fluids from earlier fusions. Of the 14 successful fusions done, 8 produced hybridoma antibodies with anti-DNA, anti-cardiolipin, anticoagulant and/or rheumatoid factor activities. Two additional fusions gave rise to anticoagulant and/or rheumatoid factor antibody activities. The remaining 4 fusions did not produce hybridoma antibodies with SLE antibody-related activity. Production of SLE-related hybridoma antibody properties was thus successful in 79% of the fusions done and represented 18% of all hybridoma produced. Cairns (1984) found, in one fusion, that 11.8% of the hybridomas produced SLE-related antibodies. Shoenfeld (1982) also observed that 15% of

his SLE hybrids produced autoantibodies. Although these two authors used different conditions (Cairns used normal tonsillar lymphocytes while Shoenfeld used pokeweed mitogen stimulated SLE PBL), their results were similar to those obtained in the present study. All hybridoma autoantibodies produced in this report and by Shoenfeld and Cairns are of the same class: IgM. No IgG autoantibodies were found. The amount of IgM produced by the hybridomas in the present study varied from 0.29 μg/ml to 19.9 μg/ml, with one culture fluid producing 124 μg/ml. The clones of Shoenfeld produced 1 to 15 μg of IgM/ml while the clones from Cairns produced 0.5 to 8 μg/ml. No differences were seen in these 3 studies regarding production of hybridoma SLE-related autoantibodies, their classes, and their immunoglobulin production.

The fact that normal PBL produced some hybridoma autoantibodies and that normal tonsillar lymphocytes gave a similar yield of autoantibodies as SLE PBL suggests that: (1) all normal individuals have the genetic information to produce autoantibodies, and (2) that a suppressive mechanism exists in normal individuals which is absent in SLE patients, allowing autoantibodies to be expressed by SLE PBL.

The immunoglobulin produced by the hybridoma antibodies which did not have any SLE-related autoantibody activities in this study was, in most cases, of the IgM class. The IgM level produced by the non-SLE-related antibody was similar to the SLE-related autoantibody (Tables 8, 9). A few hybridoma non-SLE-related antibodies produced only IgG (on a level similar

to GM 4672 line), while a few hybridomas did not produce either IgG or IgM antibody. Occasionally hybridoma SLE-related autoantibodies expressed both IgM and IgG antibodies, but the IgG had no autoantibody activity. Cairns, however, found a difference between the production of IgM by the hybridoma SLE-related autoantibodies and the non-SLE-related antibodies. All hybridomas without any SLE-related activity produced an IgM level lower than 0.5 μg/ml. Cairns did not indicate whether any hybridomas had no production of immunoglobulin or only IgG immunoglobulin, but did record an IgG and IgM producer of non-SLE-related autoantibodies.

Most of the investigators (Table 42) found similar levels of IgM immunoglobulin production among their hybridomas. Cavagnaro (1983) fused the GM 4672 line with B-cells immunized in vitro with different antigens. All hybridoma antibodies produced were of the IgM class, except for the hybridoma antibodies directed against $Rh_0(D)$, an antigen found on human erythrocytes. They were of the IgG class and of very low titer (10-100 ng/ml). Coté (1983) detected secretion of immunoglobulin in 80 to 90% of the hybridoma obtained; only one class of Ig (either IgM, IgG or IgA) was expressed by a clone. The level of immunoglobulin produced by 70 to 75% of these hybridomas was 1 to 10 μg/ml, while the 25 to 30% left produced 11 to 100 μg/ml. Strike (1984) also detected immunoglobulin in 86 to 94% of the hybridomas obtained; when only one class was expressed, half of the clones produced IgG and the other half, IgM. Olsson (1984) detected secreted immunoglobulin in

only 59% of the hybridomas produced; 72% produced IgG and 28% IgM. 70% of the hybridomas of Pickering (1982) showed IgG production in the range of 3 to 10 µg/ml, with one exception of 30 µg/ml. Littman (1983) found that all the 30 clones arising from the use of the GM 1500-6TG-A12 line were positive for IgG, but also that 10 of them were positive for IgA and 23 for IgM. It was observed in this study and those of other investigators that no detectable IgG was coexpressed in the IgM producing clones. That fact is curious since the the myeloma line fusion partner is an IgG secretor cell. An explanation could be that the level of IgG produced by these hybridomas was below the sensitivity of the assay, or, more likely, that there was a repression of the expression of the IgG from myeloma cells in the hybridomas. This would also explain the existence of non-immunoglobulin secretor hybridomas.

Most of the investigators have produced hybridomas of unknown specificities and failed to obtain any of the desired hybridoma antibodies. Edwards (1982) did not obtain anti-SRBC antibodies; Denis (1983) and Glassy (1983) produced hybrids of unknown and unstudied specificity, and Sikora (1983) had no success in finding an antibody that would differentiate each type of glioma.

18.4 Subcloning

Subcloning by the limiting dilution technique was performed on 15 hybridoma clones. A total of 79 clones arose (0 to 15 clones by parental cell subcloned), representing

a yield of 0 to 13% (mean 5.3%). Olsson (1983) obtained with his hybridoma antibodies a subcloning efficacy of 3 to 42%, Larrick (1983) obtained 10%, and Strike (1984) obtained 3 to 20%, while Abrams (1983) and Shoenfeld (1982) were unable to subclone any of their hybridomas. Brodin (1983) improved his subcloning efficiency with the use of human monocyte as feeder layer, achieving 6.5% compared to less than 1% without the use of a feeder layer, and 2.37% with the use of a Balb/c thymocytes as feeder layer. The use of a feeder layer improved Brodin's yield; but overall, his yield was similar to the mean yield of the present study, where limited dilution was done without the use of a feeder layer. The subcloning procedure by the limited dilution technique is therefore hazardous for a human hybridoma. It seems that human cells need the proximity of other cells to survive.

The aim of subcloning is to ensure monoclonality and viability of an interested clone. According to Bastin (1982) and Zola (1982), this procedure is essential since the recently fused cells were seeded at a cell density of 10^5 cells/well which might lead to overgrowth or co-culturation of irrelevant colonies. Edwards (1982) and Shoenfeld (1982) observed that all the clones derived from the same parental cell behave like the parental cell. Similarly, the subclones of the present study and their original uncloned cells demonstrated similar binding characteristics (direct and indirect) and expressed the same idiotype, suggesting that the original cell was the only positive cell among the other negative

cells plated in the same well. Only one difference was observed between subclones and uncloned parental cells. This difference was seen in competition assays where subclones required and reacted with a lesser amount of inhibitors than the parental cell, but the order of inhibitors remained the same for subclones and parental cells. These results may be explained by the fact that one gets rid of hindrance from negative clones growing with the positive parental clone.

Interestingly, Coller (1983) did a statistical analysis of subcloning by the limiting dilution technique. The table derived by him from the Poisson equation and based on the percent of wells showing growth allows the rapid evaluation of the likelihood of monoclonality. From the observed data of 13% of wells showing growth and this table, it can be concluded that 92% of these wells contained only a single cell per well, and thus, were monoclonal. Since all the subclones behaved like the parental cell, again it can be concluded that the parental cells were probably monoclonal.

18.5 Proof of the Hybrid Nature

It had been shown (Section 14.1) that the IgG2K produced by the GM 4672 cell line used as fusion partner had no anti-DNA activity. Consequently, the fact that IgM antibodies were produced by the majority of the clones obtained and that some of these IgM antibodies had anti-DNA activity suggests that true hybridomas were produced. The electrophoresis on the urea formate gel further demonstrated the production of new synthesized immunoglobulins: the

tissue-culture fluids contained biosynthetically labeled IgM and IgG. The presence of both parental immunoglobulins indicated that the clones were indeed products of cell fusions. Further proof of the existence of true hybrid cells can be achieved by HLA typing and karyotyping. Both these procedures were applied to the hybridoma cells. The HLA typing did not work as the cells died during the assay procedure. These procedures are presently in the process of being adapted to hybridoma cells. However, because hybridoma cells are unstable and undergo the loss of chromosomes in tissue culture (Kohler 1978), it has been observed by Cairns (1984), Shoenfeld (1983) and Edwards (1982) that karyotyping showed great chromosome variation between clones as well as within them. Thus the HLA typing of the hybridoma cells will be a better criterion than karyotyping to fully prove the hybrid nature of hybridoma cells.

TABLE 42

SYNOPSIS OF HUMAN HYBRIDOMA FUSION EXPERIMENTS

Investigator	Human Cells	Disease State	Fusion Partner	Cell Ratio Lymphocyte: Myeloma	Cell Density Cells/Well	Well Size	PEG Concentration	Yield*	Successful Fusion/ Total Fused
Olsson (1980)	Normal Splenic Lymphocytes (devoid of disease)	Patients with Hodgkin's Disease Sensitized to DNCB[1]	U-266AR1 (SKO-007)	1:1	$2x10^5$***	0.3 ml	38% (w/v)	38-64%	3/3
Croce (1980)	Peripheral Lymphocytes	Subacute Sclerosing Panencephalitis	GM 1500-6TG-A12	N.I.**	N.I.	2.0 ml	50% (w/v)	83%	1/1
Sikora (1982)	Intraglioma Lymphocytes	Glioma	LICR-LON-HMy2	1:3	N.I.	2.0 ml	30% (v/v)	0-36%	5/12
Shoenfeld (1982)	Peripheral or Splenic Cells	SLE, cold Agglutinin, Atopic allergy	GM 4672	5:1	$2x10^5$	2.0 ml	44% (v/v)	19-57%	5/5
Pickering (1982)	Lymph node, Spleen cells	Renal transplant Recipients	8266 AR/ NIP4-1	2:3	$2.5x10^5$	0.3 ml	40% (w/v)	22%	N.I./2
Eisenbarth (1982)	Peripheral Lymphocytes	Diabetes mellitus	GM 1500 6TG-AL2	2:1	N.I.	2.0 ml	N.I.	92%	1/1
Edwards (1982)	Lymphocytes from Tonsils, Lymph nodes and Blood	Breast carcinoma (lymph nodes) or N.I.	LICR-LON-HMy2	10:1	$2x10^6$	2.0 ml	50% (w/v)	0-100%	27/34
Handley (1982)	Peripheral Lymphocytes	Patients with CLL[2]	UC729-6	2.5:1	N.I.	2.0 ml	40%	N.I.	N.I.

It has been argued that 8266AR/NIP4-1 could be a mouse myeloma and not a human myeloma (Kozbor 1983).

TABLE 42 (continued)

Investigator	Human Cells	Disease State	Fusion Partner	Cell Ratio Lymphocyte: Myeloma	Cell Density Cells/Well	Well Size	PEG Concentration	Yield	Successful Fusion/ Total Fused
Kaplan (1982)	Peripheral or Splenic cells	Patients with Hodgkin's disease Sensitized to DNCB	U-266 AR, (SKO-007)	N.I.	N.I.	N.I.	N.I.	18-36%	2/3
Glassy (1983)	Lymphocytes from Tonsils, Lymph nodes, Spleen & blood	Cancer patients Lymphoma, malignant and Normal	UC729-6	2:1	$1x10^5$	0.3 ml	35%	N.I.	15/16
Satoh (1983)	Peripheral Lymphocytes	Diabetes mellitus	GK-5	1:1	$2x10^5$	0.3 ml	50% (w/v)	5.8 %	N.I./4
Littman (1983)	Peripheral Lymphocytes	SLE	GM 1500-6TG-AL2	1:1	$1x10^6$	2.0 ml	50% (w/v)	100 %	1/1
Cote (1983)	Lymphocytes from Lymph Nodes, spleens, Blood & tumor Specimens	Normal and patients with different Types of cancer	LICR-LON-HMy2	2:1	$1-2x10^5$	0.3 ml	42% (w/v)	55 %	38/38
Denis (1983)	Peripheral Lymphocytes	Patients with CVI	WIL2/729HF	1:1	$3x10^5$	0.3 ml	30%	0-80%	N.I./9
Abrams (1983)	Peripheral Lymphocytes	Patients with CLL	UC729-6	10:1	$2.5x10^4$	0.3 ml	50% (w/v)	38-58%	3/3
Brodin (1983)	Peripheral Lymphocytes	Normal	SKO-007	1:1	$2x10^5$	0.3 ml	37%	N.I.	1/1
Larrick (1983)	Peripheral Lymphocytes	Normal persons Sensitized to Tetanus toxoid	LRT228	1:1	10^5	0.3 ml	40%	100%	1/1

TABLE 42 (continued)

Investigator	Human Cells	Disease State	Fusion Partner	Cell Ratio Lymphocyte: Myeloma	Cell Density Cells/Well	Well Size	PEG Concentration	Yield	Successful Fusion/ Total Fused
Olsson (1983)	Peripheral Lymphocytes	N.I.	RH-L4	1:1	$2x10^5$	0.3 ml	37% (w/v)	4-56%	21/100
Houghton (1983)	Lymphocytes from Lymph nodes, Blood and tumor Specimens	Patients with Malignant Melanoma	LICR-LON-HMy2	2:1	10^5	0.3 ml	41.5% (w/v)	N.I.	N.I./158
Cairns (1984)	Lymphocytes from Tonsils	Normal	GM 4672	1:1	$4x10^5$	2.0 ml	44% (v/v)	26%	1/1
Schwaber (1984)	Lymphocytes from Spleen and blood	Patients with Hodgkin's disease Sensitized to Pneumovax	CRL1484	N.I.	N.I.	0.3 ml	40% (v/v)	N.I.	24/24
Strike (1984)	Lymphocytes from Tonsils and Blood	Normal	WIL-2-729HF$_2$	1:1	10^6	2.0 ml	50%	86-94%	2/2
Massicotte (1984)	Peripheral Lymphocytes	SLE	GM 4672	1:1	$4x10^5$	2.0 ml	44% (v/v)	1-40%	14/14

* Yield= $\frac{\text{number of wells producing hybridomas}}{\text{number of wells seeded}}$

** N.I.- not indicated in publication

*** Only the optimal value for cell ratio, plating density, and PEG concentration was noted in Table 42 in instances where more than one value was reported in the publication

1. DNCB: 2,4-dinitrochlorobenzene
2. CLL: Chronic lymphocytic leukemia
3. CVI: Common variable immunodeficiency

Chapter 19. CHARACTERIZATION OF HUMAN HYBRIDOMA ANTIBODIES

19.1 RIA

At the beginning of this work, the buffers tested and used in the screening assays were 0.1M KPO_4 buffer, supplemented with 1% normal horse serum for the determination of anti-DNA activity, and Tris buffer, supplemented with 1% BSA for the determination of anti-cardiolipin activity. At that time both BSA and normal horse serum were buffer components currently employed to block non-specific binding in assays. However, with time, it was observed that some anti-DNA hybridoma antibodies had high unspecific binding to uncoated tubes as well as tubes coated with PLL, PLG or PLL+PLG alone (which were used as pretreatments) and that it was sometimes impossible to obtain inhibition of dDNA binding of certain anti-DNA hybridoma antibodies by dDNA and nDNA. These data prompted the re-evaluation of the optimal buffer and precoating conditions of the RIA required to detect antibodies to nDNA and dDNA.

Nonspecific binding of Ig to plastic is a major problem of both solid phase RIA and ELISA (Lange 1976, Pesce 1978). In reviewing the different solid phase immunoassays for the detection of anti-DNA antibodies, it becomes evident that there is a large number of published protocols used to measure specific anti-DNA antibody binding. Pretreatment of polystyrene or polyvinyl chloride plastic surfaces with PLL, protamine sulfate, or other plastic-DNA "linkers" has been reported to be both necessary and unnecessary for the binding of DNA to plastic (Aotsuka 1979, Eaton 1983, Fish 1981, Kanai

1982, Klotz 1979, Pesce 1974, and Tan 1973). It was demonstrated that Tween 20, a nonionic detergent, was able to effectively block unoccupied protein binding sites on nitrocellulose membranes, and was more effective than BSA or serum in reducing the non-specific binding of Ig to these membranes (Batteiger 1982). It was also found that the addition of 2% bovine gamma globulin reduced the non-specific binding of Ig to PLL coated plate, (Fish 1981).

In order to minimize the non-specific binding to uncoated tubes observed in this study, the effects of the addition of Tween 20 in the buffer, different buffers and poly-L-lysine on the solid phase RIA for the detection of anti-DNA antibodies were investigated. The use of buffer containing 1, 2, or 5% normal horse serum (data not shown) was found to be ineffective in reducing the non-specific binding to the polystyrene tubes. However, it was demonstrated that the addition of Tween 20 at a concentration of 0.01% was sufficient to reduce unspecific binding of hybridoma anti-DNA antibodies to polystyrene tubes by 80-90%. Higher concentration of Tween 20 did little to improve the RIA (Figure 43). It was also demcnstrated that KPO_4-Tween buffer was more effective in improving the anti-DNA antibody binding than TBS-Tween or PBS-Tween buffer (Table 38). No attempt was made to identify the factor important in the different buffers used. The experiments that led to these conclusions were performed at least twice and were found reproducible with a maximum variation of 10-20% in all cpm

values. This variation was primary a function of the age of the radiolabelled second antibody.

It was demonstrated, using radiolabelled nDNA and dDNA, that pretreatment of the polystyrene tubes with 50 µg/ml of PLL increased the binding of nDNA and dDNA by 3 fold and 4 fold respectively (Table 40). This improved binding of nucleic acid was associated with a three-fold increase in the sensitivity of the RIA for the detection of antibodies to nDNA, but had little or no effect on the binding of antibodies to dDNA. The absolute quantity of hybridoma anti-DNA antibodies detected in the anti-DNA RIA ranged from 0.01-10 µg/ml. In sera, the RIA was capable of detecting anti-DNA antibodies at dilutions ranging from 1/100 to 1/1600. However, since the demonstration that the retention of radiolabelled nDNA and dDNA to polystyrene tubes was maximal when the tubes were precoated with PLL was done very recently, the PLL pretreatment step was not used in the present study (the pretreatment was stopped when the observations mentioned above in the first paragraph occurred).

The fact that nDNA binds poorly to polystyrene tubes, may explain why no strict nDNA binding antibodies as well as no high nDNA-binding antibodies were obtained. On the other hand, Pisetsky (1984) showed that the binding properties of anti-DNA antibodies of human or murine origin can be markedly influenced by the conditions of the assay. Depending on the presence of certain salts, activities may be enhanced, inhibited or unchanged. The present study also showed

a difference in the anti-DNA binding activity of antibodies, depending of the buffer used.

Fish (1981) demonstrated the necessity of including a specificity control for the binding of each Ig sample to a surface not coated with DNA but treated in an identical manner to the DNA-coated surface. In the present study, however, because of the limited number of tubes which could be done simultaneously without loss of accuracy, control non-DNA coated tubes were not included in all experiments. The binding of each different culture fluid was, however, checked separately. In spite of the presence of Tween 20, several hybridoma antibodies continued to adhere to plastic and may be analogous to the lymphoma protein described by Merlini (1979), which reacted with acryl particles. These antibodies were regarded as "non-specific binders" in the RIA system and were discarded from further analysis.

PLL pretreatment was not used in the cardiolipin RIA; instead 0.01% Tween buffer was used. Both were tested and demonstrated to be unnecessary and necessary respectively (data not shown). The amount of cardiolipin retained on the tubes was not tested.

19.2 Types of Anti-DNA Antibodies

The screening of all hybridoma antibodies on 3 types of antigen coated tubes (dDNA, nDNA and cardiolipin) revealed that the positive hybridomas antibodies could be divided into 3 groups. The first group is composed of anti-DNA antibodies that recognize a determinant strictly found on dDNA molecule (18% of

the clones). The second group is constituted by anti-DNA antibodies, which recognize a determinant shared by dDNA and nDNA (49% of the clones). The last group of anti-DNA antibodies recognizes a determinant common to dDNA, nDNA, as well as to cardiolipin (33% of the clones). No antibodies with strict anti-nDNA and strict anti-cardiolipin or strict anti-nDNA and anti-cardiolipin activity have been produced by any hybridomas obtained.

These results are in agreement with the classification of anti-DNA antibodies detected in the serum of patients suffering of SLE disease. Cohen in 1971 divided the SLE sera he tested into 2 categories. The first category of SLE sera expresses anti-DNA antibodies directed against both native and denatured DNA, while the second category of SLE sera encompasses anti-DNA antibodies reacting only with dDNA. Cohen found that none of those SLE sera tested reacted exclusively with nDNA. Koffler (1971) also demonstrated that the anti-DNA antibodies contained in the SLE sera tested, reacted more avidly with dDNA than nDNA, except for one serum. Arana (1967) showed that some SLE sera reacted strictly with dDNA while others reacted either similarly to dDNA and nDNA, or more effectively with nDNA than dDNA. But again, no strict anti-nDNA antibodies were found. Cohen and Arana attributed the anti-DNA activity in their sera to both IgM and IgG classes.

Andrzejewski (1982) suggested that the anti-DNA antibodies which reacted with both kinds of DNA, native and

denatured, must be directed against the sugar-phosphate backbone of the DNA molecule. Steinmann (1978) also proposed the same kind of determinant for antibodies directed against both dDNA and nDNA, and further suggested that those directed against dDNA only are directed against determinant involving specific bases.

The idea of reactivity of antibodies against nDNA and dDNA with phosphate backbone structure could also be applied to the third group of anti-DNA antibodies produced here, the one which reacted with all 3 antigens. The similitude between DNA and cardiolipin is based on the same phosphate backbone: two phosphodiester bonds separated by 3 carbon atoms. It seems, thus, that some anti-DNA antibodies detect a difference between DNA (native and denatured) and cardiolipin. In fact, it has been shown that most patients with active SLE have serum antibodies that react with cardiolipin (Harris 1983); moreover, cardiolipin-binding activity in lupus serum is present in the DNA-binding fraction (Koike 1982).

This direct binding analysis in the present study demonstrated that the anti-DNA antibodies produced by the human-human hybridomas mimic the anti-DNA antibodies found in SLE sera. Thus the study of human hybridoma autoantibodies should be relevant to the analysis and understanding of SLE antibodies.

19.3 Specificity of Anti-DNA Antibodies

To characterize the specificity of these anti-DNA hybridoma antibodies, competitive assays were performed.

The results obtained from these assays do not always correlate with the direct binding assays, but may yield different information concerning the strength of reactivity of a particular antibody to other antigens, relative to a given ligand (i.e. dDNA). The competitors used were polynucleotides and phospholipids, which have some similarity with dDNA. There were three forms of DNA: the native form which is a double-stranded B form helix (Watson 1953), the denatured form which is a single-stranded structure (Bagchi 1969), and the left double-stranded structure called zDNA (Lafer 1981, Wang 1979). Also poly (dT), a synthetic single-stranded structure (Gilliam 1980), RNA, a double stranded structure, cardiolipin, a phospholipid, and poly (I) were tested. This latter compound can assume a variety of conformations, from a single-stranded structure to a four stranded helix (Arnott 1974) and different poly (I) preparations show heterogeneity in serological reactions with either anti-poly (I) or anti-dsRNA antibodies (Stollar 1962).

The study of the inhibition of dDNA binding of these anti-DNA hybridoma autoantibodies showed that: 1) all hybridoma antibodies had their own pattern of reactivity; 2) all hybridoma antibodies had multiple antigen-binding specificities, implying a common determinant on the different polynucleotide and cardiolipin antigens (4 of 7 antigens used were inhibiting the reaction); 3) even if the hybridoma antibodies were selected for their anti-DNA activity, the preferred antigen in the inhibition test in 50%

of the cases was not dDNA or nDNA but poly (dT), while for another 38%, it was zDNA; and 4) there were a maximum of 30% inhibition of dDNA binding by nDNA or RNA.

Denatured DNA was prepared by heat denaturation of calf thymus DNA. This preparation proceeds, within the melting range, from: dissociation of hydrogen bonds between the complementary strands of the double helix, through loss of helical stability and, finally, to separation of the individual strands. Reversal of the above process leads to rejoining of the complementary bases over short or long regions (Bagchi 1969). The antigenic activity of denatured DNA is similar to but not identical with that of native DNA (Arana 1967). To keep DNA in its denatured form, the heated preparation is chilled rapidly in an ice bath, which minimizes double-stranded regions (Bagchi 1969). However, as demonstrated by Shishido (1972) and Stollar (1980), some double-stranded regions are found in dDNA preparations. Similarly, some single-stranded regions are found in nDNA preparations, as demonstrated by Steinman (1978) and Hasselbacher (1975), with the storage of nDNA with time. This fact leads these authors to observe a decrease of the specificity of antigen preparation.

One could argue, with these data in mind, that the nDNA direct binding observed in this report was not a true binding, but due to a denatured region in the preparation of nDNA. In this study, double-stranded DNA (nDNA) was prepared by removing all single-stranded regions of calf thymus DNA with S1 nuclease enzyme treatment (Stollar 1980, Papalian 1980).

The preparation, aliquoted in volumes of 2 ml and stored at -4°C, was renewed every 2-3 months. A maximum of 2 thawings were performed on each frozen vial. In this way, denaturation by storage and thawing was thought to be minimized. Actually, no non-specific binding to nDNA occurred. Thus, the binding to nDNA observed here is considered to be true because: 1) fresh native DNA was used; 2) not all anti-dDNA antibodies bound to nDNA; 3) the binding to nDNA could be diluted; 4) mouse monoclonal anti-ssDNA (H43; Andrzejewski 1980) did not react with tubes coated with S1 nuclease treated DNA, while another mouse monoclonal anti-ssDNA and anti-nDNA (HZ41) did; and 5) inhibition of nDNA binding by nDNA was observed in one system (Figure 26). The possibility that the anti-DNA antibodies have more affinity for dDNA than nDNA may explain the observed non inhibition of dDNA binding by nDNA.

The dDNA or nDNA binding was not inhibited by RNA, a finding which is in agreement with the results of Cohen (1971) and Arana (1967). The anti-DNA antibodies must recognize a determinant found on DNA that is not expressed by RNA.

The fact that half of the antibodies preferred poly (dT), a single-stranded compound without any secondary structure (Gilliam 1980), to dDNA suggests that the single-stranded structure is more important than the presence of DNA; consequently, DNA is possibly not the immunogen. This latter idea is reinforced by the finding that the immunization

of normal mice with cardiolipin led to the production of anti-DNA and anti-cardiolipin antibodies (Rauch 1984). Anti-DNA antibodies were also produced in response to a polyclonal B-cell activator (Fish 1982). Thus, the anti-DNA response may be a secondary event. Moreover, nDNA is a poor immunogen while helical nucleic acid polymers other than B-DNA (as well as dDNA) are immunogenic (review in Stollar 1975). But these immunogens induced antibodies specific for conformational features that differ from those of nDNA (with which they do not react).

Lafer, in 1981a, demonstrated that zDNA was immunogenic and resulted in the production of anti-zDNA antibodies or anti-zDNA and anti-BDNA antibodies, tested in direct binding. The zDNA binding of these antibodies could be inhibited by only the zDNA form, while the BDNA binding of the same antisera could be inhibited by BDNA only or BDNA and zDNA, depending on the animal. In 1983, Lafer showed that zDNA binding antibodies found in the serum of SLE patients were of two types: antibodies reactive solely with zDNA, and antibodies reactive with both dDNA and zDNA. In that study, Lafer suggested that high-affinity antibodies to zDNA were limited to SLE sera; they did not appear frequently in other rheumatic diseases such as rheumatoid arthritis (RA), Sjogren's syndrome, juvenile rheumatoid arthritis, scleroderma, osteoarthritis or in normal individuals. The competition assays here showed that all the dDNA binding of hybridoma antibodies selected for their reactivity to dDNA could be inhibited by the presence of both

dDNA and zDNA. This was also true for 80% of the anti-dDNA hybridoma antibodies obtained from the fusion of RA PBL (Rauch 1985). One notable difference between the present study and the one of Lafer (1983) is that the present hybridoma antibodies were asseyed for zDNA reactivity by competition assay only, while Lafer measured the anti-zDNA serum antibodies by direct binding assays. It would have been interesting to know if anti-zDNA hybridoma antibodies were produced. Sibley (1984) noted high levels of antibodies to zDNA in the sera of RA patients and, with the results of the SLE and RA hybridoma anti-DNA antibodies produced in this laboratory, these findings suggest that zDNA reactive antibodies may not be specific for SLE, similar to anti-dDNA hybridoma antibodies, which could be derived from SLE and RA patients, as well as from normal individuals.

In direct binding assays, only 18% of the anti-DNA antibodies also bound cardiolipin, a ubiquitous antigen in living organisms (MacFarlane 1964). In contrast, 79% showed inhibition of dDNA binding by cardiolipin. In the clinical laboratory, false positive Wasserman tests and circulating lupus anticoagulants are observed in SLE sera. This phenomena may be explained here by the observation of the binding of antibodies to cardiolipin, which is a phospholipid involved in both tests (Harris 1983).

Thus, the anti-DNA antibody polyspecificities imply that these antibodies recognize a determinant common to polynucleotides and phospholipids. This suggests that some of

of the diversity of autoantibody reactions in SLE may be a result, in part, of the presence of a relatively simple antigenic structure, in a variety of biological molecules (Lafer 1981b).

The lupus autoantibodies produced by Shoenfeld (1983) behaved similarly to the hybridoma autoantibodies in this report. Also, Cairns (1984) showed that most of her clones derived from a normal individual behaved similarly, having polyspecific antigen-binding reactivity, by direct binding assays. In contrast to the findings in the present study, Cairns was able to isolate a clone that had only an anti-cardiolipin reactivity. In addition, some of her clones also showed binding to RNA.

19.4 Stability

Three levels of long term stability in hybridomas can be discussed: 1) the quantity and class of an antibody produced, 2) the reactivity of the antibody produced, and 3) the idiotype expression.

In regard to the quantity and class of antibody produced, four groups of hybridomas were seen in this study. There were: (1) hybridomas whose supernatant did not contain any detectible IgM or IgG; (2) hybridomas whose supernatant contained only IgG; (3) hybridomas whose supernatant contained only IgM; and (4) hybridomas whose supernatant contained both IgM and IgG.

The group of non-Ig producing hybridomas is of interest. It was seen in low frequency in each fusion. It is difficult

to explain why these hybridomas did not secrete at least the IgG of the GM 4672 myeloma partner in the fusion, and also why this myeloma IgG was not expressed in the IgM producing hybridomas. Experiments performed on these hybridomas will provide insight into the ability of hybridoma to produce Ig. These types of non-Ig producer hybridoma were used as new myeloma-like partners in fusions by three investigators. Tucker (1984) fused NS0 (mouse Ig-non-producer subline of NS1/Ag4.1) mouse myeloma cells with lymph node cells from a calf immunized with red blood cells. He selected hybridoma of unspecified characteristics, rendered it HAT-sensitive and then re-fused it with lymph node cells from a second calf immunized with red blood cells. The percentage of hybridomas secreted, and the amount of antibodies produced by them over a period of time was greater in the second fusion, with the myeloma-like cells, than in the first fusion. Foung (1984) fused a Sp2/0-8Ag mouse myeloma line with human peripheral B lymphocytes isolated from a normal donor. He isolated an hybridoma which stopped producing immunoglobulins (unidentified), and rendered it HAT-sensitive. This new cell line was then fused with an EBV-transformed lymphocyte cell line that had an anti-blood group A activity. Here again, the number of hybridomas along with the amount of anti-blood group A antibodies produced by each of them was much higher than that observed in the fusion of the same EBV-transformed lymphocytes with Sp2/0-8Ag. Schwaber (1984) fused CRL1984 (Harris 1974), a non-Ig producer

human myeloma line, with peripheral blood lymphocytes from a normal individual. All hybridomas obtained were non-human Ig producers. Schwaber selected an hybridoma (LSM 2.7) and rendered it HAT-sensitive. This myeloma analog was then fused with splenocytes of 10 Hodgkin's patients who had been immunized with pneumovax 1 to 11 days prior to splenectomy. All ten fusions resulted in hybrids producing human Ig. Three fusions yielded hybridomas whose antibodies reacted specifically with pneumococcal capsular polysaccharide. It appeared that the 3 successful fusions with specific antibodies used splenocytes of patients who had been immunized only 3 to 4 days prior to surgery, suggesting again a particular, so far unidentified, stage of differentiation or activation of B lymphocytes.

Several questions arose from these findings: What did the myeloma-like partner possess that, when fused with sensitized lymphocytes, it would render almost 100% successful positive hybridomas with higher amounts of Ig produced? Did the anti-SRBC antibody produced originate from the myeloma-like partner or from the second sensitized lymphocytes fused? Was the antibody produced in both of the second fusions monoclonal?

Schwaber had demonstrated that refusion of a HAT-sensitive clone, derived from a hybrid which had secreted antibodies, with peripheral blood mononuclear cells resulted in reactivation of specific antibody secretion.

No attempts were made in the present study to

identify the IgG produced by any hybridomas. Thus it is impossible to say whether there was production of IgG other than myeloma IgG. However, certainly no IgG-SLE-related antibody reactivity was produced by any hybridomas in the present report. The quantity of IgG produced by the hybridomas was equal or slightly higher than the amount produced by the parental cell GM 4672 (0.26 μg/ml).

The IgM producing hybridomas were of two kinds. Some produced constant amounts of IgM, as seen with 1311, which produced a constant amount of IgM non-anti-DNA binding antibody of 2.94 ± 0.32 μg/ml, while others produced fluctuating amounts of IgM, as seen with 134; both were followed over a period of 8 months. It would be of great value to determine what renders a clone consistent in the level of antibody produced.

The IgM and IgG producing hybridomas were a transient group of the hybridomas observed. These hybridomas, at first, were producing only IgM antibodies. They then started to produce lower and lower amounts of IgM. At one point, detectable IgG appeared in the supernatant along with the decrease in IgM production. Finally, the hybridomas produced only IgG; IgM was undetectable. Thus, IgM and IgG appeared in the supernatant only when the hybridoma had its IgM production gradually turned off, an event accompanied by the turning on of IgG production. No attempts were made to identify this IgG as the myeloma product.

However, each time that these hybridomas were noticed, they were IgM anti-DNA hybridomas losing their activity. This anti-DNA activity was linked to the presence of IgM antibodies in the supernatant. No further anti-DNA activity was detected when only IgG was produced. Moreover, a study of the presence of the idiotype found on the anti-DNA antibody revealed the link of the idiotype with the IgM antibody originally produced by the hybridoma. Thus, this class "switch" is not a real class switch from an IgM response to an IgG response but cessation of production of one class of antibodies in favor of the production of another class. It seems that the expression of IgG in the IgM producer hybridomas could only be detected when the IgM production was gradually ceasing. The fact that IgG and IgM were not coexpressed is surprising.

All positive SLE-related antibody activities produced in the present study were due to IgM-class antibodies. The majority of the hybridomas demonstrated binding activities on the first screening that remained constant for at least 10 months. However, it was observed, on occasion, that some hybridomas, on initial screening, bound only to nDNA or cardiolipin coated tubes. But at subsequent screenings, they bound to all three antigens. On two occasions, an IgM-non-DNA binding hybridoma, grown as negative control to produce non-binding hybridoma Ig for tests, became positive, showing anti-DNA activity after 1-2 months of culture or after purification. There were no changes

in the specificity of antibodies obtained from hybridomas producing fluctuating amounts of antibodies. However, the affinity of the antibodies for the antigens changed slightly, which was demonstrated with the follow-up of 134 antibody antibody production. A change of affinity was suggested by the decrease in binding seen after purification of the antibody. On the other hand, subcloning always gave antibodies of higher affinity than the parental hybridoma, as seen with 103 subclones. However, fluctuating amounts of IgM produced, purification of antibodies, as well as subcloning did not affect the expression of idiotypes. The idiotype was always present in any productive culture fluids, in the purified form of the antibody and in the subclones of a clone, as seen with 134 and 103-1.

Several reasons may be advanced to explain the non-Ig producing hybridomas : 1) the non-IgG producing hybridoma (producing IgM or not) resulting from the fusion of a myeloma producer, may have lost the structural genes to produce the myeloma IgG; 2) these hybridomas may be able to synthesize the myeloma IgG but not to secrete it (incomplete IgG molecule); 3) the hybridomas have lost a positive regulatory site distant from the structural genes; or, 4) the results can be due to intrinsic properties of the B lymphocytes, such as the production of an inhibitory substance acting on the synthesis and secretion of any Ig.

The loss of the structural genes for the myeloma IgG

production is most likely inapplicable here, since an IgM producing hybridoma may become an IgG producer (presumably myeloma IgG) in time. Thus, the genes must be present but they are not expressed. The loss of structural genes as an explanation of the cessation of antibody secretion by an hybridoma is likely also inapplicable in some cases, as demonstrated by Schwaber who reactivated a specific secretion by fusion of the hybridoma with peripheral blood mononuclear cells from a normal individual. The specific antibody secretion seen in all the wells plated after fusion can not come from the new lymphocyte partner, which had not been presensitized with pneumovax. It must come from the reactivation of the myeloma-like partner, suggesting that an intrinsic factor was lost from the myeloma-like partner which was supplied by the normal lymphocyte. Intrinsic properties of B lymphocytes, such as regulatory sites, inhibitory or activating substances, may only explain fluctuating or constant amounts of antibody produced, cessation of antibody production, inhibition of secretion of myeloma Ig and production of higher affinity subclones, since all the hybridomas were made with the same myeloma and they were treated similarly.

The change of specificity, observed on two occasions, of an IgM non-DNA binding hybridoma producer into an IgM anti-DNA hybridoma producer may be explained by the occurrence of a mutation. It was observed by Diamond (1984) that a point mutation of one amino acid change may transform an anti-

phosphocholine myeloma Ig into an anti-DNA antibody. A study of the amino acid sequences of the Ig produced before and after the new specificity will help to achieve some insights.

The fact that only IgM-SLE related antibodies were produced may result from intrinsic properties of the myeloma partner used, or may be due to the lack of an *in vitro* substance that switches the immune response from an IgM to an IgG.

19.5 Correlation with Sera and Serologic Activities

Eighteen SLE sera, 12 RA sera and 6 normal individual sera were screened in this study for the presence of antibodies to dDNA, nDNA, cardiolipin and HIgG (rheumatoid factor). Reactivity with both native and denaturated DNA was found in 11/18 (61%) of the SLE sera, while 2 (11%) sera each reacted with either nDNA or dDNA alone. Thus, an equal proportion of SLE sera 13/18 (72%) reacted with either dsDNA or ssDNA but a total of 15/18 (83%) reacted with either form of DNA. In contrast, 6/12 (50%) of the RA sera reacted with nDNA while none of them showed reactivity with dDNA. Reactivity with cardiolipin was found in 5/18 (28%) of the SLE and 4/12 (33%) of the RA sera. Rheumatoid factor activity was found in 6/18 (33%) of the SLE, 11/12 (92%) of the RA and, 1/6 (17%) of the normal sera.

The level of antibodies in a serum depends on many factors. For patients, these include the kind and status of disease and for normal individuals, their overall health and

their contact with sick patients. In a randomly selected population of SLE and RA patients, Epstein (1975) found that 60.8% of the SLE and 18.7% of the RA sera had antibodies to dsDNA while 39.4% of the SLE and 20.6% of the RA sera reacted with ssDNA; Koffler (1973) found that 52% of the SLE and 24% of the RA sera had antibodies to ssDNA; Bell (1975) found that 42% and 56% of the SLE sera had reactivity to dsDNA and ssDNA respectively, while reactivity to dsDNA only was found in 9% of the RA sera; Talal (1975) found that 76% of the SLE and 20% of the RA sera had antibodies to nDNA and Sharp (1971) found that 24% of the SLE and 18% of the RA sera had antibodies to cardiolipin. In a randomly selected population of SLE patients, Alarcon-Segovia (1975) found that 75% and 83% of the SLE sera reacted to dsDNA and ssDNA respectively; Arana (1967) found that 41% and 29% of the SLE sera reacted to dsDNA and ssDNA respectively and Tan (1982) found the same production of 50-60% of the SLE sera reacting with either or ssDNA. However, in a group of SLE sera divided into active and inactive, Sharp (1971) found that, of the active SLE sera, 96% and 68% reacted with nDNA and dDNA respectively, while only 4% of inactive SLE sera reacted with ssDNA, with no sera having activity to nDNA. In a group of SLE patients with either anticoagulant, thrombosis, cerebral infarction or thrombocytopenia, 80-91% of them had anti-cardiolipin antibodies (Harris 1983).

In a group of randomly selected normal sera, Talal

(1975) found that 29% were reacting with nDNA while Sharp (1971) found an equal proportion (1.4%) reacting with either form of DNA. In a study by Koffler (1971) who studied a population of normal sera, divided into groups according to their place of employment, the sera of normal individuals who were not working in hospitals, had 0.3% and 3.7% reactivity with nDNA and dDNA, respectively, while sera of normal individuals who did work in hospitals had 0% and 16.8% reactivity with nDNA and dDNA, respectively. Similar results were also observed by Carr (1975). Thus, there are elevated levels of antibodies to DNA in individuals working in hospital areas compared to individuals working elsewhere.

It is apparent from these studies that antibodies to both forms of DNA can be found in any population. However, the quantity of antibodies found in each population may be determined by their proximity to disease. Thus, antibodies to DNA will be found in lower quantity in normal than in RA patients. These will be, in turn, lower than for SLE patients, whose antibody levels are lower in patients whose disease is in an inactive state while they are high in those patients with active disease. Also, the variation of proportions of anti-DNA antibodies in a population from one author to the other may be related to the type and the sensitivity of assay employed by them, as demonstrated by Locker (1977).

Thus, the findings in this study of high levels of anti-DNA antibodies in both SLE and RA sera group is logical, since these patients were not randomly chosen. They

were selected because of a relapse of their disease activity. Also, the 6 normal persons selected were individuals working in an hospital area, a fact which may explain the high RF activity found with one normal serum.

Also the reactivity of RA and SLE sera to cardiolipin observed in the present study was not surprising because of the similarity between cardiolipin and DNA. It was demonstrated by Koike (1984) that the cardiolipin-binding activity of lupus serum was present in the DNA-binding fraction.

Finally, it was demonstrated in this study that the reactivities found in a patient's serum were not always reflected in the hybridoma antibodies derived from the fusion of the patient's lymphocytes to GM 4672. The cells of a patient, for example, with no serum anti-DNA activity (14) gave rise to hybridoma anti-DNA antibodies, while that from a patient with serum RF reactivity (16) did not give rise to any RF hybridoma antibody reactivity. However, the reactivity of the monoclonal anti-DNA antibodies was similar to the reactivity of serum anti-DNA antobodies. They possess wide polyreactivity; binding to dDNA, nDNA, polynucleotides, nucleosides, and cardiolipin; and are competed by these same substances. Monoclonal anti-DNA hybridoma antibodies as well as non-DNA binding hybridoma antibodies were capable of demonstrating other types of antibody activities, such as anticoagulant and rheumatoid factor reactivities. The surprising fact about the monoclonal hybridoma antibodies were that only 30% of them were monospecific. Thus, 70% of the hybridoma antibodies were able to demonstrate

polyspecificities towards autoantigen; 55% had in addition other activities (RF and/or PTT).

Chapter 20. ANTI-IDIOTYPE ANTIBODIES

20.1 Rabbit Anti-idiotype Antisera

Eight anti-DNA antibodies were selected at random to raise anti-idiotype antibodies in rabbit. Seven (100-1, 103-1, 134, 1206, 600, 604, and 1305) of them had similar binding characteristics to the 3 antigens, dDNA, nDNA and cardiolipin, but, with different affinities. The last one (1400) bound to dDNA and nDNA only. In addition to these binding characteristics, three had other activities: 100 and 1400 had RF activity and 600 had a PTT activity. Three (100, 103-1, and 134) hybridoma antibodies were derived from the same patient, two (600 and 604) from another, and the last 3 from 3 different patients. Thus, 8 clones coming from 5 different patients were purified over a Sepharose rabbit anti-HIgM column and were injected into rabbits biweekly as described (see Section 5.1).

The antisera collected from each rabbit were negatively absorbed several times over a Sepharose pooled normal HIgM column and over a Sepharose GM 4672 IgG column. These absorptions were intended to remove all reactivities to normal IgM and to myeloma IgG, such as anti-isotype and anti-allotype activity. The process of absorption varied for each antiserum, in that some required more passages than others to remove reactivity to normal and myeloma immunoglobulins. After complete absorption, the sera no longer bound to these Ig , although they showed binding to their immunogens. They also did not bind

significantly to polynucleotides (dDNA and others) and the slight binding observed was not sufficient to interfere with their binding to the immunogen.

To localize the idiotype recognized by each antiserum, 2 types of competition assays were used. The first assay involved the inhibition of dDNA binding of the idiotype by antisera. An inhibition of binding suggested that the anti-idiotype antiserum was directed against an idiotype located within the dDNA binding site of Ig, or interfering with it. In this case, the binding of the anti-idiotype antiserum to the idiotype prevented the binding of Ig to dDNA. Six antisera were able to inhibit the binding of their homologous idiotypes to dDNA, indicating that they were antigen-binding site related. The last 2 antisera (anti-600 and anti-1305) were not able to compete the binding of their homologous idiotype to dDNA, even at a concentration of 2 mg/ml, meaning that the idiotype recognized by them was located outside the dDNA binding site of the Ig, probably in the framework region.

The second assay involved the inhibition of idiotype binding to anti-idiotype antiserum by polynucleotides and cardiolipin. The binding of 5 idiotypes (103-1, 134, 1400, 1206, and 604) to their homologous anti-idiotype antiserum was inhibited by the presence of polynucleotides and cardiolipin, again suggesting the antigen-binding site location of these idiotypes. There was no inhibition of the binding antibody 1305 to its anti-idiotype

antiserum by the presence of polynucleotides or cardiolipin. This result again implied no relation of the idiotype recognized by anti-1305 antiserum to the antigen-binding site of 1305 antibody. Unfortunately, this type of assay could not be performed on the last 2 idiotypes, 100-1 and 600, since the binding of these antibodies were too weak when diluted.

In fact, the binding of 1305 antibody to dDNA in the presence of antisera or to anti-idiotype antisera in the presence of polynucleotide was, in both cases, increased instead of inhibited. In other words, the binding of the antigen or the anti-idiotype antiserum to 1305 antibody increased the accessibility of the second site. Thus, it can be deduced that the antigen-binding site and the 1305 idiotype are two different entities on 1305 antibody, but that the affinity of both sites for their respective ligand is affected and increased if one or the other sites is occupied.

Thus, from 2 types of inhibition assays, it was possible to demonstrate the antigen-binding-site location of the idiotype for 5 anti-idiotype antisera and the non-antigen-binding-site location of the 1305 idiotype, for homologous idiotype/anti-idiotype system. For 2 idiotype/anti-idiotype systems, only one type of inhibition assay could be used, which demonstrated that the 100-1 idiotype system was antigen-binding site related while the 600 idiotype system was not.

20.2 Shared Idiotypes on Hybridoma Antibodies

Using 3 types of assays, it was shown that the

idiotype recognized by one anti-idiotype antiserum was also present on other hybridoma antibodies. These 3 assay, involving 2 inhibition assays and one direct binding assay, are summarized in Table 43 for easier reference as well as presented in Figures 24 A to E and Tables 23 and 24. Similar and different information were expected from these 3 assays because: i) the sensitivity of the 2 kinds (competittion and direct binding) of assays are different; ii) one assay looks at the dDNA site while the other 2 assays look at the idiotype site; iii) the site exposed in solution may differ from the one exposed when the molecule is coated to a solid phase; iv) each antiserum is composed of heterogeneous population of anti-idiotype antibodies; v) each antibody may express different idiotypes; vi) the degree of identity of the idiotype site on different antibodies affects the binding; and vii) the degree of affinity of the anti-idiotype antibody for its idiotype also affects the binding.

In the first inhibition assay, it was seen that anti-idiotype antisera were able to inhibit the dDNA binding of the non-homologous immunogens used. In fact, the dDNA binding of each immunogen (Table 43A) was inhibited by at least one non-homologous anti-idiotype antiserum. Furthermore, the anti-134 antiserum recognized a cross-reactive idiotype in the dDNA-binding site of 4 out of 7 (57%) non-homologous antibodies.

Moreover, an idiotype located in the dDNA-binding site of the 100-1 antibody was recognized by all anti-idiotype

antisera. Conversely, the anti-100-1 antiserum did not recognize its idiotype in all the antigen-binding sites of non-homologous-immunogenic antibodies, but only in 3 (42%) of them. In addition, the 2 antigen non-inhibitable antisera (anti-600 and anti-1305) were able to inhibit the dDNA binding of other non-homologous antibodies, suggesting that the idiotypes recognized by them in these cases were located in the dDNA binding site of the antibodies. These 2 antisera thus recognized an idiotype which could be located at a different site on a different antibody.

These assays showed that there are shared idiotypes among the antibodies used as immunogens, that those shared idiotypes were situated in their dDNA-binding site, and that non-antigen-inhibitable anti-idiotype antiserum was able to inhibit the dDNA-binding of non-homologous antibodies, detecting an idiotype translocated (Kabat 1978, Wu 1982, Takashiba 1984). This translocation rendered a non-antigen-inhibitable anti-idiotype antiserum as able to react similarly to an antigen-inhibitable antiserum.

Cross-reacting idiotypes were tested in a direct binding assay for 3 idiotype/anti-idiotype systems (100-1, 103-1, and 134, Figures 20 to 22). By comparing the two assays, the inhibition of dDNA binding and direct idiotype binding by anti-idiotype antiserum (Figures 49 A and B), it was seen that although anti-134 antiserum inhibited the dDNA binding of 100-1, 103-1, and 134

antibodies, it did not bind directly to all 3 antibodies but only to 134 and 100-1. Similarly, although anti-103-1 antiserum inhibited the dDNA binding of the same 3 antibodies, it did not bind directly to all 3, but only to 103-1 and 134. In contrast, anti-100-1 antiserum inhibited the dDNA binding of all 3 antibodies cited and bound directly to all 3.

These differences in data may be explained by the fact that in the direct binding assay, the purified form of an antibody was used for coating while its culture fluid form was used in the inhibition assay. It was seen here that an antibody in its purified or unpurified form was able to interact successfully with its homologous anti-idiotype antiserum. However, both forms were not reactive with the non-homologous antiserum. It is generally admitted and shown that an antibody may bear different idiotypes (Shilling 1980, Suzan 1982). For example, one can say that an antibody expesses one major and one minor idiotype. The homologous reaction of an idiotype and anti-idiotype antiserum may involve the major idiotype, while the non-homologous reaction may involve the minor idiotype. This minor idiotype may be, somehow, altered by the purification procedure or may be hidde or inaccessible on the coated tube, resulting in no reaction with the non-homologous antiserum. Thus, the major idiotype is easily recognized by its homologous anti-idiotype antiserum, while the minor idiotype is not, because of steric hindrance and/or of affinity.

Using a second inhibition assay, it was seen that the presence of non-homologous idiotypes could inhibit the homologous idiotype binding of an anti-idiotype antiserum. A maximum of 15 purified anti-DNA hybridoma antibodies and 3 purified non-DNA binding hybridoma antibodies were tested, in 6 idiotype/anti-idiotype systems, as seen in Table 23. Non-homologous anti-DNA idiotype antibodies succeeded in inhibiting the binding of anti-idiotype antiserum to homologous idiotype-coated tubes, while none of the non-DNA-binding antibodies (except in one case) succeeded. These results again suggest that shared idiotypes are found among hybridoma antibodies. Moreover, they suggests that the shared idiotypes are specifically found only on anti-DNA antibodies (except for the only inhibition of an antiserum, anti-1305 by 1207, a non-DNA binding antibody), but not necessary in the DNA-binding site (not tested).

That assay also revealed that some idiotypes are more wide spread and shared than others. For example, anti-100-1, anti-604, anti-134, and anti-1305 idiotype antisera reacted with 58%, 47%, 33%, and 27%, respectively, of the anti-DNA antibodies derived from up to 5 patients (Figure 43 B). It could also be seen that one anti-DNA antibody (such as 100-1, 1305, etc.) could inhibit the homologous idiotype-binding of several antisera. This does not imply, however, that the same structure on that anti-DNA antibody was recognized by the different antisera. Otherwise, all these antisera

would have recognized the same group of anti-DNA antibodies. Moreover, not all anti-DNA antibodies from one fusion were recognized by the same anti-idiotype antiserum, and even not all 33 anti-DNA antibodies were recognized. This implies that the 7 autoantibodies not recognized by any of the 8 anti-idiotype antisera expressed a different idiotype or failed to express the idiotype.

If one looks more closely at the 3 idiotype/anti-idiotype systems which cross-reacted, 100-1, 103-1, and 134 antibodies and their antisera, again some differences in the results are noted between the direct anti-idiotype binding and the inhibition of that binding by antibodies (Figure 49 B and C). The binding of anti-100-1 antiserum to its homologous idiotype was inhibited by the 3 antibodies to which it bound directly, and of which it inhibited the dDNA binding. In contrast, the binding of anti-103-1 antiserum was inhibited by 100-1 and 103-1, but not by 134, although it bound directly to 134 and 103-1, but not to 100-1, and was able to inhibit the dDNA binding of all 3 antibodies. Similarly, the binding of anti-134 antiserum to its homologous idiotype was inhibited only by 134, although it bound directly to 134 and 100-1 and was able to inhibit the dDNA binding of all 3 antibodies.

The following assignments of private and unique idiotypes, the size of the idiotype site expressed and recognized (important in the inhibition assay), and the affinity of the antiserum for its homologous idiotypes

(important in the direct and inhibition binding assay) can explain the data observed: 1) Anti-134 antiserum recognizes a public or shared idiotype called IdX-134. This idiotype is expressed entirely on 134 antibody, but partially on 100-1 and 103-1 antibodies (represented there as IdX-134a). The high affinity of anti-134 antiserum for its idiotype enables the antiserum to recognize every expressed form of IdX-134. 2) Anti-100-1 antiserum recognized strictly, with high affinity, the form of the public idiotype expressed on 100-1 antibody, IdX-134a. 3) Anti-103-1 antiserum recognized a private or unique idiotype called IdI-103-1. This idiotype, entirely expressed on the 103-1 antibody, was only partially represented on 100-1 and 134 antibodies (IdI-103-1a). 4) Finally, the expression of IdI-103-1a on 100-1 antibody and IdX-134a on 103-1 antibody, were not preponderant and easily accessible when these antibodies were coated to tubes.

To get around the necessity of purifying every clone for demonstration of shared idiotypes, a third assay was used where anti-idiotype antiserum was coated to tubes. Using this method, 23 anti-DNA antibodies and 26 non-DNA binding antibodies were tested for direct binding to each anti-idiotype antiserum. Eight to 42% of all anti-DNA antibodies seemed to express and share the same idiotype (Table 43 C). Moreover, this shared idiotype was found mostly on hybridoma anti-DNA antibodies derived from up to 5 different patients. There was binding of 3 (14%) of the

non-DNA-binding antibodies to the anti-134 antiserum, the only case where non-DNA binding antibodies were reacting with an anti-idiotype antiserum.

The anti-600 idiotype antiserum must recognize an idiotype to which it has a weak affinity since it did bind to 600 (idiotype coated) tubes, but only at low dilution, while 600 antibody did not bind to anti-600 antiserum (anti-idiotype coated). Also, none of the hybridoma antibodies tested succeeded in binding to the anti-600 antiserum, suggesting that none were expressing the 600 idiotype, and questioning the existence of an immune response by the rabbit immunized with it. Similarly, the affinity of 1206 idiotype for its homologous anti-idiotype antiserum was also low, since the binding of 1206 antibody to its antiserum did not show in the direct binding assay. However, non-homologous antibodies succeeded in binding to anti-1206 antiserum.

Figure 49 D completes the study for cross-reacting idiotype among 103-1, 100-1 and 134 antibodies. Again, the data observed reflected the degree of affinity and sharing (size of the idiotype site) of an idiotype expressed on non-homologous antibodies for an anti-idiotype antiserum.

From these 3 assays, one can see that: 1) IdX-134, the public idiotype, was expressed on 53% and 12% of all anti-DNA and non-DNA binding hybridoma antibodies tested respectively; 2) that anti-1400 and anti-103-1 antisera each recognized a private

idiotype which was expressed on their homologous antibodies in addition to the shared idiotype IdX-134; 3) although anti-1305 antiserum seemed to recognize a framework determinant of 1305 antibodies, that idiotype was not found in all of the antibodies; and 4) that anti-100-1, anti-1305, anti-1206 and anti-604 antisera recognized a subgroup of the family of antibodies sharing the IdX-134 with only few additions.

In conclusion, the binding characteristics of anti-DNA antibodies did not enable one to predict the behaviour of an anti-idiotype antiserum regarding the location of the idiotype recognized by the latter on the former. Moreover, there were also no associations between a serologic activity or binding characteristics among hybridoma antibodies and reactivity with the anti-idiotype antisera, since the latter failed to recognize specific group of antibodies. However, no doubt exists that the recognition of an idiotype was almost (except 134 idiotype) always associated with the presence of an anti-DNA activity.

20.3 Shared Idiotypes on Hybridoma Antibodies from Different Diseases

Anti-DNA and non-DNA binding hybridoma antibodies derived from 2 RA patients were available to the author to test whether antibodies from a disease other than SLE may share idiotypes found on a SLE anti-DNA hybridoma antibody. In the inhibition of idiotype binding of anti-103-1 and anti-134 antisera, 8 purified RA anti-DNA and 2 purified RA non-DNA binding hybridoma antibodies were tested. Two

anti-DNA and 1 non-DNA binding antibodies from the same RA patient succeeded in inhibiting the binding of anti-134 antiserum to its homologous idiotype, while none succeeded in interfering with the binding of anti-103-1 antiserum. Thus, a structure resembling idiotype 134 was also seen in RA hybridoma antibodies.

In a direct binding assay, 10 RA anti-DNA and 11 RA non-DNA binding antibody culture fluids were tested for reactivity with the anti-idiotype antisera, except anti-600 antiserum, which was dropped from this study. Forty % (4/10) of the anti-DNA and 20% (2/11) of the non-DNA binding RA hybridoma antibodies, derived from both RA patients, bound to the anti-134 antiserum. No other RA hybridoma antibodies, whatever their activities, succeeded in interacting with any other anti-idiotype antisera. Thus, these antisera seem to recognize idiotypes expressed only on SLE anti-DNA hybridoma antibodies.

These results suggest that although an SLE antibody was used to raised an anti-idiotype antiserum, the latter may recognize an idiotype that may not be restricted to one disease. On the other hand, if one accepts the fact that any individual has the potential to make any kind of antibody, one could expect to find that an idiotype found in a disease may be also expressed in a normal person. In fact, 2 anti-DNA and 6 non-DNA binding hybridoma antibodies derived from the blood peripheral lymphocytes of 2 normal individuals were available and tested for direct binding to anti-idiotype antisera. Reactivity of one anti-DNA hybridoma antibody derived from a normal

individual was observed with anti-134 and anti-1400 antisera. None of the other normal hybridoma antibodies succeeded in binding to any other anti-idiotype antisera.

20.4 Idiotype Activity in Sera

It was seen in a preliminary experiment that 6 of the 8 idiotypes recognized by the anti-idiotype antisera were present in normal, RA, and SLE sera while the last two idiotypes (1206 and 600) were only detected in SLE and normal sera respectively. However, the magnitude of expression of an idiotype among these sera varied. For example, there was a tendency of idiotypes 1305 and 604 to be equally and minimally expressed in all sera, while 134 was highly expressed in all sera. In general, there was a tendency for a wider range of expression of an idiotype in SLE sera than in the other sera.

Further studies are required to assess the importance of one idiotype, its association with a clinical feature and/or a group of antibodies and its strict expession in SLE.

20.5 Mouse Anti-idiotype Antibodies

Unsuccessful results were obtained from attempts to raise mouse monoclonal anti-idiotype antibodies. Although a response to the immunogen was obtained in all injected Balb/c mice and mouse hybridoma antibodies arose, not one of the latter reacted specifically with only the immunogen. It appeared that when a hybridoma antibody reacted with the immunogen, it also reacted with normal IgM and myeloma IgG. Subcloning procedures did not succeed in isolating the anti-idiotype reactivity from the other

reactivities. Thus, most likely, the mouse response to the immunogen was directed towards a structure shared by different classes of immunoglobulins, which might be either in the light chain, a D or J region or Fc fragments. None of these possibilities were tested to further characterize the mouse monoclonal antibodies.

20.6 Literature Review of Shared Idiotypes

Private and shared idiotypes among human SLE anti-DNA antibodies were also observed by Solomon (1983), Shoenfeld (1983b), Zouali (1984) and Isenberg (1984). Solomon (1983) reported on a mouse monoclonal anti-idiotype antibody (3I) produced by injection into a Balb/c mouse of purified IgG anti-dsDNA antibodies, fraction isolated from a SLE patient serum. This monoclonal 3I antibody was shown to be specific for the immunogen and non-antigen-binding site-related. With the use of 3I antibody, Solomon detected shared idiotypes in 8 of 9 sera of patients with active SLE and measurable anti-dsDNA antibodies, in 4 of 9 sera of patients with active SLE and undetectable anti-dsDNA antibodies, and in none of the 10 healthy subject sera tested. This recognition of shared idiotypes by 3I antibody was shown to be irrespective of the IgG class of the anti-DNA antibodies present in the sera (not isotope related).

Similarly, Zouali (1984) produced an anti-idiotype antibody by injecting rabbits with the affinity purified serum IgG anti-dsDNA, isolated from a patient (TOF) with severe SLE

submitted to therapeutic plasmapheresis. The affinity purified rabbit IgG anti-idiotype was shown to be specific to TOF anti-DNA antibodies. It failed to react with a pool of normal human IgG after having been absorbed on it, with anti-tetanus toxoid antibodies, and with TOF Ig depleted of anti-DNA activity. This rabbit anti-idiotype antiserum was directed against the DNA-binding site of TOF anti-dsDNA antibodies. Using that anti-idiotype antibody, Zouali showed that 1/25 (4%) of normal human sera and 31/34 (91%) of SLE sera shared the TOF idiotype. Zouali and Solomon have thus shown that anti-DNA antibodies from non-related SLE patients share common idiotypes that may or not be associated with the binding site.

Shoenfeld (1983b) reported on the production of 3 anti-idiotype antibodies, one in mice (anti-32/15) and 2 in rabbits (anti-32/15 and anti-16/6), by injection of 2 monoclonal SLE-anti-DNA antibodies. These anti-idiotype antibodies were shown to be specific for the immunogen (they did not react with normal IgM and IgG) and antigen-binding site-related (polynucleotides other than DNA were used to obtain inhibition). Shoenfeld showed that 39 of 59 (66%) of anti-DNA hybridoma atibodies, derived from 7 patients, shared idiotypes. The rabbit anti-16/6 antiserum reacted with 30 of the hybridoma antibodies which were derived from 4 patients; the rabbit anti-32/15 antiserum reacted with 22 of them, derived from 3 patients; and finally, the mouse monoclonal anti-32/15 antibody reacted with 25 of them derived from 6 patients. The latter result included 5 anti-DNA

hybridoma antibodies, derived from 2 patients of the present study. Mouse monoclonal anti-32/15 antibodies also reacted with 1 non-DNA binding hybridoma antibody (113-3) of the present study. It was deduced that the mouse and rabbit anti-idiotype antibody, raised against the same immunogen, reacted with a different idiotype on the same molecule. Similar to the results of the present study, Shoenfeld found shared idiotypes among the population of anti-DNA hybridoma antibodies, but also failed to find a relationship between the antigen-binding properties and idiotype cross-reactions.

Isenberg (1984) further characterized the anti-idiotype antibodies studied by Shoenfeld by studying the idiotype expression in sera. In that report, the rabbit anti-16/6 antiserum reacted with 54% of the active SLE sera, 25% of the inactive SLE sera, 24% of the RA sera, and 3% of the normal sera; the rabbit anti-32/15 antiserum reacted with none of the RA, normal and inactive SLE sera but with 1% of the SLE active sera, while the mouse monoclonal antibody reacted with 28% of the active SLE sera, 4% of the inactive SLE sera, 10% of the RA sera, and none of the normal sera. Thus, similar to this study, shared idiotypes were not only observed in SLE patients, active or inactive, but also in RA patients and normal subjects. However, Isenberg further showed that high levels of 16/6 idiotype occurred concurrently with the elevation of anti-nDNA and anti-ssDNA antibodies during clinical antivity, but failed to show a correlation between serum levels of 16/6 idiotype and those of either total IgG and

IgM.

Cross-reactive idiotypes were also shown among monoclonal lupus autoantibodies derived from either $(NZB \times NZW)F_1$ (Marion 1982, Tron 1982 & 1983) or MRL-lpr/lpr mice (Rauch 1982), two lupus-prone mice. These shared idiotypes were again not confined to SLE anti-DNA antibodies, but were also found on other antibodies as well, as in normal mice (Datta 1983). Cross-reacting idiotypes have also been reported with other human autoantibodies, including IgM cold agglutinins (Williams 1968), rheumatoid factors (Kunkel 1973, Agnello 1980, Chen 1985), mixed cryoglobulins (Geltner 1980), anti-acetylcholine receptor antibodies from patients with myasthenia gravis (Lefvert 1981), and cerebrospinal fluid immunoglobulins from patients with multiple sclerosis (Tachovsky 1982).

Cross-reactive idiotypes are not restricted to autoantibodies only but are also found on all other antibodies produced in response to any antigens or haptens. For example, they were found on anti-Val (Karol 1977), anti-GAT (Ju 1978), anti-dextran (Schilling 1980), anti-galactan (Mushinky 1977), and anti-MOPC315 myeloma protein (Jorgensen 1977) antibodies. The latter study, as well as that of Kraus (1978), demonstrated that there were no general rules according to which the antibody-antigen complex induces synthesis of anti-idiotype antibodies against idiotypes associated with framework residues and soluble or polymerized antibodies are able to induce synthesis

of anti-idiotype antibodies specific for idiotypes associated with the combining site. Again, these reports showed that the expression of an idiotype was not restricted to the population of antibodies of the antigen-specificity studied.

Studies of the amino acid composition and sequence, as well as the association of V_L and V_H gene segments of antibodies (directed against self, exogeneous or unknown antigens) with shared idiotype, have revealed that: 1) the V_H only or the combination of V_H and V_L may be responsible for the expression of a particular shared idiotype. Thus, the combination of one V_H to a different V_L gene (and vice versa) segments can lead to the expression of different idiotypes (Suzan 1982, Capra 1983, Kranz 1981); 2) the same set of V germ lines may be used to make antibodies with different antigenic specificities (Schelling 1980, Rocca-Serra 1983, Margolies 1983, Juszczak 1984, Atkinson 1985, Kofler 1985, Legrain 1985); 3) the heterogeneity of a group of antibodies with shared idiotypes resides in the amino acid sequences of the D segment (Schilling 1980, Auffrey 1981, Berck 1984, Kofler 1985); 4) there is, in some cases, translocation of the D gene segments into the HV2 region of an antibody (Kabat 1978, Wu 1982, Takahashi 1984); 5) in some systems, one can lose expression of the idiotype as a result of one or 2 amino acid variations while in others, the variation can be extensive yet the idiotype is retained. These findings indicate that the three-dimensional structure is crucial and

that the correlation established between amino acid sequence and presence of the idiotype will likely only hold for a particular system (Suzan 1982, Capra 1983); 6) the V region is probably composed of minigenes defined as a DNA segment coding for a portion of a complete V region, and shows some evidence of segregation as a functional unit independent of the rest of the DNA coding for the V region (Kabat 1979). These minigenes are involved in rearrangement (Takahaski 1984). 7) Both somatic recombination of scattered gene segments of an Ig and mutation contribute greatly to an increase in the diversity of antibodies synthesized by a single organism (review in Tonegawa 1983).

There is considerable evidence that suggests the true existence of an idiotype/anti-idiotype immune network (Geha 1981 & 1982, Rodkey 1974, Zanetti 1983). Paul (1982) postulated the existence of a regulatory idiotype for the network hypothesis. This regulatory idiotype is an idiotype found on a relatively high proportion of Ig molecules and/or lymphocytes and is capable of acting on a site for a receptor-specific regulatory system. Manser (1985) explained the production of shared or private idiotypes of the immune response in all individuals by the existence of clonotypes with high levels of inherent fitness for participation in the immune response to the antigen.

20.7 Speculations

Obviously, the tendency of the presence of high levels of IdX-134 in normal sera was not associated with the presence

of anti-DNA antibody levels, which was low. Thus, IdX-134 in normal sera is expressed on antibodies of unknown specificities. One can postulate that because of its high level of expression in normal sera, that IdX-134 should be a regulatory idiotype well expressed on a relatively high proportion of Ig molecules. It is also possible to postulate that, because of the high levels of IdX-134 in SLE and RA sera as well as among the majority (60%) of anti-DNA and 10% of non-DNA hybridoma antibodies and its association with the DNA-binding site, that in autoimmune disease a mechanism occurred (mutation and/or rearrangement) that induced the switch of a regulatory idiotype to an autoantibody. This mechanism has induced the expression of silent idiotypes (Miller 1982, Hahn 1984, Teitelbaum 1984, Urbain 1982, Briles 1984, Norton 1985). This switch could also explain the partial expression of IdX-134 on some hybridoma anti-DNA antibodies.

This phenomenon may be the result of the production of a substance by a virus or other pathogenic agent which could induce a malfunctional reading of DNA gene codes (in splicing and/or joining mechanisms) and which could, in turn, lead to the formation of autoantibodies. Rearrangement has been shown to produce differences in antigen-binding specificities and idiotype expression (Legrain 1985, Kofler 1985, Capra 1983, Rudikoff 1982 and 1983).

There is a transitory effect of anti-idiotypic immunizations (Miller 1982, Hahn 1984, Teitelbaum 1984, Thorbeeke 1984, Page 1985). This immunotherapy succeeded to

stop the production of antibodies bearing the injected idiotype. However, a recrudescence of activity of autoantibodies occurred which were negative for the idiotype which had been injected. This treatment may have been ineffective because the wrong idiotype was selected. One should choose the idiotype that is found responsible for the disease and not simply a major cross-reactive idiotype, since the latter may be an idiotype of a normal immune response. The injection of that major cross-reactive idiotype may suppress a beneficial immune response of the animal, leading to the recrudescence of activity of an autoimmune response.

Thus, the study of the expression of idiotypes on normal as well as "abnormal" antibodies will help to better understand autoimmunity.

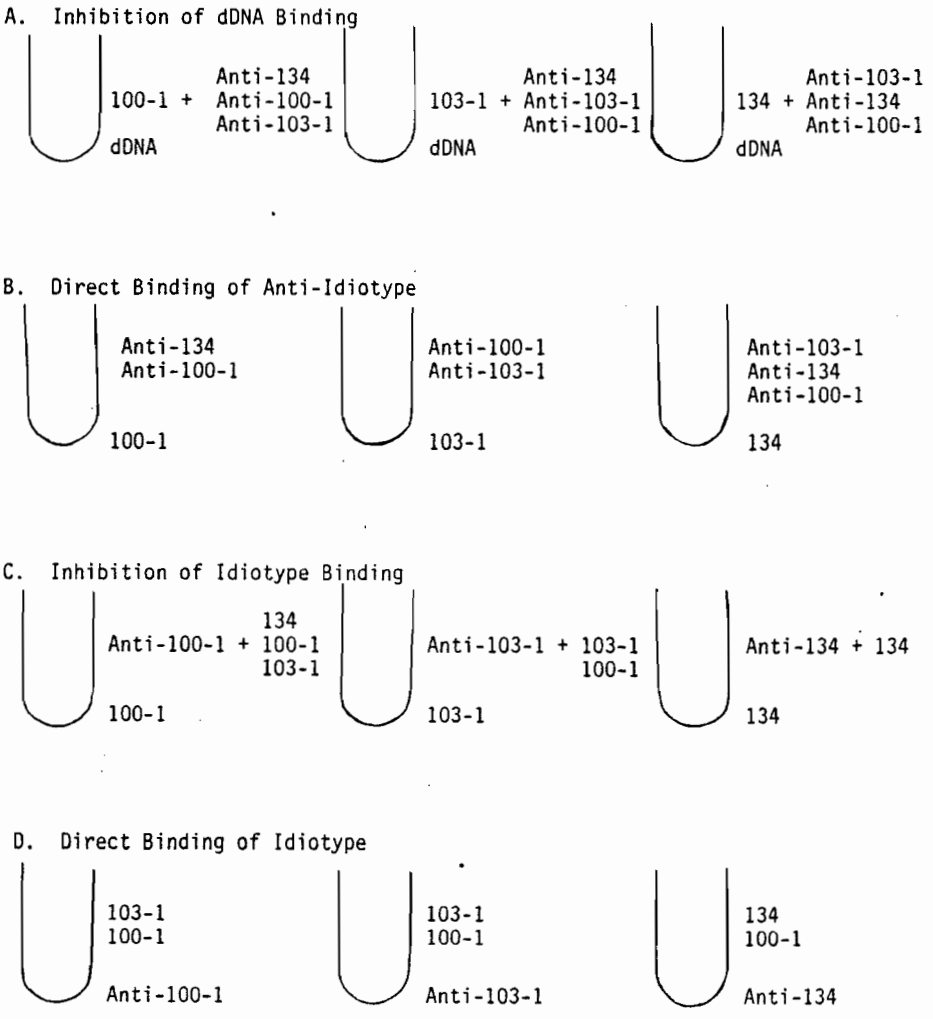
TABLE 43

HYBRIDOMAS WITH SHARED IDIOTYPE IN THREE TYPES OF ASSAY

	Assays	Anti-100-1	Anti-103-1	Anti-134	Anti-600	Anti-604	Anti-1206	Anti-1305	Anti-1400
A	Inhibit the dDNA Binding of	100-1 103-1 134 604	100-1 103-1 134	100-1 103-1 134 604 1400	100-1	100-1 604	100-1 134	100-1 103-1 1400	100-1 1400
B	The Idiotype-Binding is Inhibited by	100-1 103-1 134 1305 1202 112-2 1108	100-1 103-1	134 1206 1305 121 128	N.P.	100-1 600 604 1206 601 1400 1311	1206	604 1305 1311 1108 1207	N.D.
	% Reactive Clones	58	14	33	-	47	7	27	-
C	Direct Binding to Anti-Idiotype by	100-1 103-1 1108 1206 1305 1407	103-1 1406	100-1 134 600 604 1108 1400 1404 1406 1407 2607 2608 1201 2600 2613	None	134 600 604 1305 2607	100-1 103-1 600 604 1305	103-1 134 1305 1403	1400 1401
	% Reactive Clones	23	8	42	0	19	19	15	8

N.P. Not possible to do
N.D. Not done

FIGURE 49. COMPARISON OF DIFFERENT ASSAYS DEMONSTRATING SHARED IDIOTYPE FOR THE SAME 3 IDIOTYPE/ANTI-IDIOTYPE SYSTEMS



CONCLUSION

Autoantibodies to DNA are a common feature of SLE and it is generally accepted that they play an important role in the pathogenesis of glomerulonephritis associated with this disease. Antibodies to DNA were produced by fusion of SLE PBL with the GM 4672 lymphoblastoid cell line. An optimal yield of hybridomas (7.5%) and anti-DNA antibodies (17%) was obtained when an equal number of lymphocytes and myeloma cells and a cell density of $4X10^5$ cells/2ml well were used. The monoclonal lupus autoantibodies obtained bound to a variety of antigens; a single antibody reacted with more than one nucleic acids and/or cardiolipin and/or demonstrated other activities like PTT and RF. Each autoantibody had its own pattern of polyspecific reactivities. This fact indicates that the heterogeneity of serologic reactions in serum from SLE may be a manifestation of a diversity not only in the immune response, but also in the reactivity of individual antibodies with similar epitopes in different molecules. Study of the idiotype expression on these monoclonal lupus autoantibodies revealed that they shared idiotypes. Some shared idiotypes were only expressed in patients with SLE disease, while others were equally and highly expressed in normal patients as well as in those with RA disease. The latter idiotype may be a regulatory idiotype normally expressed on antibody of normal origin that is now expressed on autoantibodies by an unknown mechanism.

Appendix I. Statistical Analysis of the Effect of the Cell Ratio and Cell Density on the Yield of Hybridomas

The effects of cell density and cell ratio on the yield of hybridomas were evaluated in the present study. Ten fusions or experiments were done, using each time 3 cell densities ($4X10^5$, $2X10^5$ and $1X10^5$ cells/well) and 2 cell ratios (lymphocyte:myeloma of 5:1 and 1:1). From Table A I which gives the number of wells plated and hybridomas obtained at each cell ratio and cell density used for each fusion made, the yield of hybridomas (number of hybridomas arisen divided by number of wells plated times 100 = % hybridoma/well seeded) was calculated and put in Table A II. A statistical analysis of the variance (ANOVA) was then made on the results to determined whether there is an effect of the cell ratio and/or the cell density on the yield of hybridomas. Table A III gives the sum (T) of yield of hybridomas for each cell ratio and cell density for the 10 fusions together. Tables A II and A III served to establish the table of ANOVA (Table A IV). The calculations made to build Table A IV were:

- The total sum squares (S^2_T) which measures the variability of each results in a fusion from the general mean was given by the formula:

$$S^2_T = \sum_{i=1}^{3} \sum_{j=1}^{2} \sum_{k=1}^{10} (\%)^2_{ijk} - \frac{T...^2}{pqr}$$

where: i: is the row or the number of cell density used; i=1, 2, 3.

j: is the column or the number of cell ratio used; j=1, 2.

k: is the number of yield of hybridomas obtained for each cell density or cell ratio; k=1, 2, ..., 9, 10.

p: represents the number of cell density used; p=3.

s: represents the number of cell ratio used; q=2.

r: represents the number of experiments done at each cell ratio and cell density; r=10.

%: is the yield of hybridomas expressed in Table A II.

T...: is the total sum of yield of hybridomas obtained for all the columns, rows, and experiments together; t...=593 (Table A III).

$$\text{Thus, } S^2_T = ((30)^2+(7)^2+\ldots+(2)^2+(1)^2) - \frac{(593)^2}{60}$$

$$= 14875 - 5860.85 = 9014.8$$

The degree of freedom of S^2_T was $(pqr - 1) = 59$

- The sum of squares of the row (S^2_p) which measures the variability from one row to another or from one cell density to another was given by:

$$S^2_p = \sum_{i=1}^{3} \frac{Ti...^2}{qr} - \frac{T...^2}{pqr}$$

$$= \frac{(303)^2 + (172)^2 + (118)^2}{20} - \frac{(293)^2}{60}$$

$$= 6765.85 - 5860.82 = 905.$$

The degree of freedom of S^2_p was $(p-1) = 2$.

- The sum of squares of the column (S^2_g) which measures the variability from one column to another or from one cell ratio to another was given by:

$$S^2_g = \sum_{j=1}^{2} \frac{T.j.^2}{pr} - \frac{T...^2}{pqr}$$

$$= \frac{(185)^2 + (408)^2}{30} - \frac{(593)^2}{60}$$

$$= 6689.63 - 5860.85 = 828.81$$

The degree of freedom of S^2_g was $(q-1) - 1$.

- The sum of squares for interaction (S^2_{pg}) which measues the lack of additivity of row and column effects was given by:

$$S^2_{pg} = \sum_{i=1}^{3} \sum_{J=1}^{2} \frac{T.j.^2}{r} - \frac{T...^2}{pqr} - S^2_p - S^2_g$$

$$= \frac{(93)^2 + (58)^2 + \ldots + (84)^2}{10} - \frac{(593)^2}{60} - 905 - 828.81$$

$$= 7732.1 - 5860.82 - 905 - 828.81 = 137.47$$

The degree of freedom of S^2_{pg} was $(p-1)(q-1) = 2$.

- The within group sum of squares (S^2_e) which measures the variance of the six individuals populations (1 cell ratio + 1 cell density) was given by:

$$S^2_e = S^2_T - S^2_p - S^2_g - S^2_{pg}$$

$$= 9014.18 + 905 - 828.81 - 137.47 = 7142.9$$

The degree of freedom of S^2_e was $pq(r-1) = 54$.

- The mean of squares for each case was obtained by dividing the sum of squares found in each case by their degree of freedom.

The observed F ratio to test the lack of additivity F(2, 54) = 0.52 is smaller than the critical value at 5% found in Table A V, F(0.05, 2.54) = 3.17, suggesting that there was no lack of additivity between the population.

The observed F ratio to test the effect of the cell density on the yield of hybridomas F(2, 54) = 3.42 was greater than the critical value at 5% found in Table A V, F(0.05, 2.54)= 3.17, suggesting that there was an effect of the cell density on the yield of hybridomas.

The observed F ratio to test the effect of the cell ratio on the yield of hybridomas F(1, 54) = 6.27 is greater than the critical value at 5% found in Table A V, F(0.05, 1.54)= 4.02, suggesting that there is an effect of the cell ratio on the yield of hybridomas.

The Duncan test was used to determine which cell ratio and cell density was better.

The mean yield of hybridoma calculated for the cell density was:

$4X10^5$ cells/well	15.15 (303/20)
$2X10^5$ cells/well	8.6 (172/20)
$1X10^5$ cells/well	5.9 (118/20)

The difference between $4X10^5$ and $2X10^5$ cells/well was 6.55, the one between $4X10^5$ and $1X10^5$ cells/well was 9.25 and the one between $2X10^5$ and $1X10^5$ cells/well was 2.7. The variance of the cell density was:

$$S = \sqrt{\frac{S^2_e}{20}} = \sqrt{\frac{132.28}{20}} = 2.63$$

This latter value was used to multiply the Duncan value found in Table A VI with the degree of freedom of 54 and at 5%:

$$P(2,54) = 2.84 \times 2.63 = 7.47$$

$$P(3.54) = 2.99 \times 2.63 = 7.86$$

Thus, the difference between $2X10^5$ and $1X10^5$ cells/well of 2.7 is smaller than P(2, 54) = 7.47, suggesting that these 2 cell densities were equal regarding the yield of hybridomas. In contrast, the difference between $4X10^5$ and $1X10^5$ cells/well is greater than P(3, 54) = 7.86 but the difference between $4X10^5$ and $2X10^5$ cells/well is smaller than P(3, 54) = 7.86. These results clearly indicate that the cell density of $4X10^5$ cells/well gave the better yield of hybridomas.

The mean yield of hybridomas calculated for the cell ratio was:

5:1	6:17 (185/30)
1:1	13.6 (408/30)

The difference between these cell ratios was 7.43. The variance of cell ratios was:

$$S = \sqrt{\frac{S}{30}} = \sqrt{\frac{132.28}{30}} = 2.10$$

and was utilized to multiply the Duncan value found in Table AIV with the degree of freedom of 54:

$$P(2, 54) = 2.84 \times 2.10 = 5.95$$

The difference between the two cell ratios of 7.43 is greater than the Duncan value P(2, 54) = 5.95, suggesting that the 1:1 cell ratio is better than the 5:1 cell ratio.

In conclusion, at 5%, the 1:1 cell ratio and $4X10^5$ cells/well statistically gave the best yield of hybridomas.

TABLE AI.

NUMBER OF WELLS PLATED AND HYBRIDOMAS OBTAINED AT EACH CELL RATIO AND CELL DENSITY USED FOR EACH FUSION DONE

Cell Density & Cell Ratio		Fusion 1		10		11		12		13		14		15		16		19		23	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
5:1	A	20	6	30	2	15	1	20	0	10	2	18	1	20	2	40	2	40	3	20	0
	B	40	5	30	0	30	0	40	0	18	3	33	0	40	8	80	4	80	0	40	1
	C	80	6	60	0	60	0	80	0	39	6	66	4	80	1	160	4	160	0	80	1
1:1	A	25	2	27	1	9	2	12	3	10	4	10	5	27	5	38	13	63	5	25	0
	B	50	3	27	0	18	0	30	3	20	4	21	11	54	9	74	6	169	0	100	1
	C	100	14	55	0	37	1	60	2	36	2	39	4	108	11	147	8	169	4	100	1

I. is the number of wells plated for each cell ratio and cell density
II. is the number of hybridomas arose at each cell ratio and cell density
5:1&1:1. lymphocytes/myeloma cell ratio

A. $4x10^5$ cells/well
B. $2x10^5$ cells/well cell density
C. $1x10^5$ cells/well

TABLE A II

YIELD (%) OF HYBRIDOMAS FOR EACH CELL RATIO AND CELL DENSITY USED IN EACH FUSION

Cell ratio & cell density		Fusion 1	10	11	12	13	14	15	16	19	23
5:1	A	30	7	7	0	20	6	10	5	8	0
	B	13	0	0	0	17	0	20	5	0	3
	C	8	0	0	0	15	6	1	3	0	1
1:1	A	8	4	22	25	40	50	19	34	8	0
	B	6	0	0	10	20	52	17	8	0	1
	C	14	0	3	33	6	10	10	5	2	1

Cell Ratio : Lymphocyte:Myeloma; 5:1, 1:1.
Cell Density : A: $4X10^5$ cells/well;
B: $2X10^5$ cells/well;
C: $1X10^5$ cells/well.

TABLE A III

SUM OF YIELD (%) OF HYBRIDOMA FOR EACH CELL RATIO AND CELL DENSITY USED

Cell density \ Cell ratio	5:1	1:1	
A	93	210	= 303
B	58	114	= 172
C	34	84	= 118
	185 +	408	= 593

Cell Ratio : Lymphocyte : Myeloma; 5:1; 1:1
Cell Density : A: $4X10^5$ cells/well;
B: $2X10^5$ cells/well;
C: $1X10^5$ cells/well.

TABLE A IV

ANALYSIS OF VARIANCE: ANOVA

Origin of variation	Sum of squares	degree of freedom	Means of squares	F ratio
Row means	905	2	452.5	$F_{2,54} = 3.42$
Column means	828.81	1	828.81	$F_{1,54} = 6.27$
Interaction	137.47	2	68.74	$F_{2,54} = 0.52$
Error	7142.9	54	132.28	
Total	9014.8	59		

TABLE A V

$F(\nu_1, \nu_2)$ Distributions

ν_1, DEGREES OF FREEDOM FOR NUMERATOR

ν_2, DEGREES OF FREEDOM FOR DENOMINATOR	Cum. prop.	1	2	3	4	5	6	7	8	9	10	11	12	Cum. prop.
29	.0005	.0*40	.0*50	.0*50	.015	.030	.047	.065	.082	.098	.114	.129	.143	
	.001	.0*16	.0*10	.0*80	.022	.040	.060	.080	.099	.117	.134	.150	.164	
	.005	.0*40	.0*50	.024	.050	.079	.107	.133	.156	.178	.197	.215	.231	
	.01	.0*16	.010	.038	.072	.107	.138	.167	.192	.215	.235	.254	.270	
	.025	.0*10	.025	.071	.118	.161	.197	.229	.257	.281	.302	.321	.337	
	.05	.0*40	.051	.116	.174	.222	.263	.296	.325	.349	.370	.389	.406	
	.10	.016	.106	.193	.262	.315	.357	.391	.420	.443	.464	.481	.497	
	.25	.103	.290	.406	.480	.532	.571	.601	.625	.645	.661	.676	.688	
	.50	.466	.709	.807	.858	.890	.912	.927	.939	.948	.955	.961	.966	
	.75	1.38	1.45	1.44	1.42	1.41	1.39	1.38	1.37	1.36	1.35	1.35	1.34	
	.90	2.88	2.49	2.28	2.14	2.05	1.98	1.93	1.88	1.85	1.82	1.79	1.77	
	.95	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.13	2.09	
	.975	5.57	4.18	3.59	3.25	3.03	2.87	2.75	2.65	2.57	2.51	2.46	2.41	
	.99	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	3.07	2.98	2.91	2.84	
	.995	9.18	6.35	5.24	4.62	4.23	3.95	3.74	3.58	3.45	3.34	3.25	3.18	
	.999	13.3	8.77	7.05	6.12	5.53	5.12	4.82	4.58	4.39	4.24	4.11	4.00	
	.9995	15.2	9.90	7.90	6.82	6.14	5.66	5.31	5.04	4.82	4.65	4.51	4.38	
30	.0005	.0*40	.0*50	.0*50	.016	.030	.048	.066	.084	.100	.117	.132	.147	
	.001	.0*16	.0*10	.0*80	.022	.042	.061	.081	.101	.119	.137	.153	.169	
	.005	.0*40	.0*50	.024	.051	.080	.108	.135	.159	.181	.201	.220	.237	
	.01	.0*16	.010	.038	.073	.108	.140	.169	.195	.219	.240	.259	.276	
	.025	.0*99	.025	.071	.119	.162	.199	.232	.260	.285	.307	.327	.344	
	.05	.0*40	.051	.116	.175	.224	.265	.299	.329	.354	.376	.396	.412	
	.10	.016	.106	.194	.263	.317	.360	.394	.424	.448	.469	.488	.504	
	.25	.103	.290	.405	.480	.533	.572	.603	.627	.647	.664	.680	.691	
	.50	.463	.705	.802	.854	.885	.907	.922	.934	.943	.950	.956	.961	
	.75	1.36	1.44	1.42	1.40	1.39	1.37	1.36	1.35	1.34	1.33	1.32	1.31	
	.90	2.84	2.44	2.23	2.09	2.00	1.93	1.87	1.83	1.79	1.76	1.73	1.71	
	.95	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	2.04	2.00	
	.975	5.42	4.05	3.46	3.13	2.90	2.74	2.62	2.53	2.45	2.39	2.33	2.28	
	.99	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.89	2.80	2.73	2.66	
	.995	8.83	6.07	4.98	4.37	3.99	3.71	3.51	3.35	3.22	3.12	3.03	2.95	
	.999	12.6	8.25	6.60	5.70	5.13	4.73	4.44	4.21	4.02	3.87	3.75	3.64	
	.9995	14.4	9.25	7.33	6.30	5.64	5.19	4.85	4.59	4.38	4.21	4.07	3.94	
40	.0005	.0*40	.0*50	.0*51	.016	.031	.048	.067	.085	.103	.120	.136	.152	
	.001	.0*16	.0*10	.0*80	.022	.041	.062	.083	.103	.122	.140	.157	.174	
	.005	.0*40	.0*50	.024	.051	.081	.110	.137	.162	.185	.206	.225	.243	
	.01	.0*16	.010	.038	.073	.109	.142	.172	.199	.223	.245	.265	.283	
	.025	.0*99	.025	.071	.120	.163	.202	.235	.264	.290	.313	.333	.351	
	.05	.0*40	.051	.116	.176	.226	.267	.303	.333	.360	.382	.402	.419	
	.10	.016	.106	.194	.264	.318	.362	.398	.428	.453	.475	.493	.510	
	.25	.102	.289	.405	.480	.534	.573	.604	.629	.650	.667	.680	.692	
	.50	.461	.701	.798	.849	.880	.901	.917	.928	.937	.945	.951	.956	
	.75	1.35	1.42	1.41	1.38	1.37	1.35	1.33	1.32	1.31	1.30	1.29	1.28	
	.90	2.79	2.39	2.18	2.04	1.95	1.87	1.82	1.77	1.74	1.71	1.68	1.66	
	.95	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.95	1.92	
	.975	5.29	3.93	3.34	3.01	2.79	2.63	2.51	2.41	2.33	2.27	2.22	2.17	
	.99	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.72	2.63	2.56	2.50	
	.995	8.49	5.80	4.73	4.14	3.76	3.49	3.29	3.13	3.01	2.90	2.82	2.74	
	.999	12.0	7.76	6.17	5.31	4.76	4.37	4.09	3.87	3.69	3.54	3.43	3.31	
	.9995	13.8	8.65	6.81	5.82	5.20	4.76	4.44	4.18	3.98	3.82	3.69	3.57	

(Dixon 1969)

TABLE A VI

DUNCAN VALUES

TABLE E—Significant Studentized Ranges for a 5-percent Level New* Multiple Range Test

nombre de modalités

n_2 \ n_1	2	3	4	5	6	7	8	9	10	12	14	16	18	20	50	100
1	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18
2	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6.09	6
3	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4
4	3.93	4.01	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4.02	4
5	3.64	3.74	3.79	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3.83	3
6	3.46	3.58	3.64	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3.68	3
7	3.35	3.47	3.54	3.58	3.60	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3.61	3
8	3.26	3.39	3.47	3.52	3.55	3.56	3.56	3.56	3.56	3.56	3.56	3.56	3.56	3.56	3.56	3
9	3.20	3.34	3.41	3.47	3.50	3.52	3.52	3.52	3.52	3.52	3.52	3.52	3.52	3.52	3.53	3
10	3.15	3.30	3.37	3.43	3.46	3.47	3.47	3.47	3.47	3.47	3.47	3.47	3.47	3.48	3.48	3
11	3.11	3.27	3.35	3.39	3.43	3.44	3.45	3.46	3.46	3.46	3.46	3.46	3.47	3.48	3.48	3
12	3.08	3.23	3.33	3.36	3.40	3.42	3.44	3.44	3.46	3.46	3.46	3.46	3.47	3.48	3.48	3
13	3.06	3.21	3.30	3.35	3.38	3.41	3.42	3.44	3.45	3.45	3.45	3.46	3.47	3.47	3.47	3
14	3.03	3.18	3.27	3.33	3.37	3.39	3.41	3.42	3.44	3.45	3.45	3.46	3.47	3.47	3.47	3
15	3.01	3.16	3.25	3.31	3.36	3.38	3.40	3.42	3.43	3.44	3.45	3.46	3.47	3.47	3.47	3
16	3.00	3.15	3.23	3.30	3.34	3.37	3.39	3.41	3.43	3.44	3.45	3.46	3.47	3.47	3.47	3
17	2.98	3.13	3.22	3.28	3.33	3.36	3.38	3.40	3.42	3.44	3.45	3.46	3.47	3.47	3.47	3
18	2.97	3.12	3.21	3.27	3.32	3.35	3.37	3.39	3.41	3.43	3.45	3.46	3.47	3.47	3.47	3
19	2.96	3.11	3.19	3.26	3.31	3.35	3.37	3.39	3.41	3.43	3.44	3.46	3.47	3.47	3.47	3
20	2.95	3.10	3.18	3.25	3.30	3.34	3.36	3.38	3.40	3.43	3.44	3.46	3.47	3.47	3.47	3
22	2.93	3.08	3.17	3.24	3.29	3.32	3.35	3.37	3.39	3.42	3.44	3.45	3.46	3.47	3.47	3
24	2.92	3.07	3.15	3.22	3.28	3.31	3.34	3.37	3.38	3.41	3.44	3.45	3.46	3.47	3.47	3
26	2.91	3.06	3.14	3.21	3.27	3.30	3.34	3.36	3.38	3.41	3.43	3.45	3.46	3.47	3.47	3
28	2.90	3.04	3.13	3.20	3.26	3.30	3.33	3.35	3.37	3.40	3.43	3.45	3.46	3.47	3.47	3
30	2.89	3.04	3.12	3.20	3.25	3.29	3.32	3.35	3.37	3.40	3.43	3.44	3.46	3.47	3.47	3
40	2.86	3.01	3.10	3.17	3.22	3.27	3.30	3.33	3.35	3.39	3.42	3.44	3.46	3.47	3.47	3
60	2.83	2.98	3.08	3.14	3.20	3.24	3.28	3.31	3.33	3.37	3.40	3.43	3.45	3.47	3.48	3
100	2.80	2.95	3.05	3.12	3.18	3.22	3.26	3.29	3.32	3.36	3.40	3.42	3.45	3.47	3.53	3
∞	2.77	2.92	3.02	3.09	3.15	3.19	3.23	3.26	3.29	3.34	3.38	3.41	3.44	3.47	3.61	3

nombre de l.s.d.

From Duncan, D. B., "Multiple Range and Multiple F Tests," *Biometrics*, 11, 19.

*Using special protection levels based on degrees of freedom.

Appendix II. Stastistical Analysis of the Mean IgM Production by DNA and non-DNA Binding Hybridomas

In Tables 8 and 9, the amount of IgM produced by 27 DNA-binding and 14 non-DNA-binding hybridoma antibodies respectively were given. The mean production of IgM and the standard deviation for the DNA-binding antibodies, calculated from Table 8, was 5.11 22.57 μg/ml. The one for the non-DNA-binding antibodies, calculated from Table 9, was 2.99 4.77 μg/ml. The purpose here was to determine if these two means differed. The t student test for the comparison of two means was here used. The observed t value was given by the following equation:

$$t_{(\text{observed},\ N_1+N_2-2)} = \frac{X_1 - X_2}{\sqrt{\frac{s^2_{X_1}(N_1-1)+s^2_{X_2}(N_2-1)}{N_1+N_2-2}}\sqrt{\frac{1}{N_1}+\frac{1}{N_2}}}$$

where: X represents the mean prodution of IgM

N represents the number of antibodies in a group

s^2 is the standard deviation

1 refers to the anti-DNA antibody group

2 refers to the non-DNA binding antibody group

Thus,

$$t_{(\text{observed, } 39)} = \frac{5.11 - 2.99}{\sqrt{\frac{22.57 \times 26 + 4.77 \times 13}{39}} \sqrt{\frac{1}{27} + \frac{1}{14}}}$$

$$= 1.579$$

The mean production of IgM of the two groups of antibodies will differ if the observed t value of 1.579 is greater than the Table t value at 5% of 2.05, given in Table VII. Since it was not the case, the mean production of IgM by DNA antibodies and non-DNA antibodies did not statistically differ ($p > 0.05$).

Table AVII. t distributions

df	$t_{.60}$	$t_{.70}$	$t_{.80}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$
1	.325	.727	1.376	3.078	6.314	12.706	31.821	63.657
2	.289	.617	1.061	1.886	2.920	4.303	6.965	9.925
3	.277	.584	.978	1.638	2.353	3.182	4.541	5.841
4	.271	.569	.941	1.533	2.132	2.776	3.747	4.604
5	.267	.559	.920	1.476	2.015	2.571	3.365	4.032
6	.265	.553	.906	1.440	1.943	2.447	3.143	3.707
7	.263	.549	.896	1.415	1.895	2.365	2.998	3.499
8	.262	.546	.889	1.397	1.860	2.306	2.896	3.355
9	.261	.543	.883	1.383	1.833	2.262	2.821	3.250
10	.260	.542	.879	1.372	1.812	2.228	2.764	3.169
11	.260	.540	.876	1.363	1.796	2.201	2.718	3.106
12	.259	.539	.873	1.356	1.782	2.179	2.681	3.055
13	.259	.538	.870	1.350	1.771	2.160	2.650	3.012
14	.258	.537	.868	1.345	1.761	2.145	2.624	2.977
15	.258	.536	.866	1.341	1.753	2.131	2.602	2.947
16	.258	.535	.865	1.337	1.746	2.120	2.583	2.921
17	.257	.534	.863	1.333	1.740	2.110	2.567	2.898
18	.257	.534	.862	1.330	1.734	2.101	2.552	2.878
19	.257	.533	.861	1.328	1.729	2.093	2.539	2.861
20	.257	.533	.860	1.325	1.725	2.086	2.528	2.845
21	.257	.532	.859	1.323	1.721	2.080	2.518	2.831
22	.256	.532	.858	1.321	1.717	2.074	2.508	2.819
23	.256	.532	.858	1.319	1.714	2.069	2.500	2.807
24	.256	.531	.857	1.318	1.711	2.064	2.492	2.797
25	.256	.531	.856	1.316	1.708	2.060	2.485	2.787
26	.256	.531	.856	1.315	1.706	2.056	2.479	2.779
27	.256	.531	.855	1.314	1.703	2.052	2.473	2.771
28	.256	.530	.855	1.313	1.701	2.048	2.467	2.763
29	.256	.530	.854	1.311	1.699	2.045	2.462	2.756
30	.256	.530	.854	1.310	1.697	2.042	2.457	2.750
40	.255	.529	.851	1.303	1.684	2.021	2.423	2.704
60	.254	.527	.848	1.296	1.671	2.000	2.390	2.660
120	.254	.526	.845	1.289	1.658	1.980	2.358	2.617
∞	.253	.524	.842	1.282	1.645	1.960	2.326	2.576
df	$-t_{.40}$	$-t_{.30}$	$-t_{.20}$	$-t_{.10}$	$-t_{.05}$	$-t_{.025}$	$-t_{.01}$	$-t_{.005}$

When the table is read from the foot, the tabled values are to be prefixed with a negative sign. Interpolation should be performed using the reciprocals of the degrees of freedom.

(Dixon 1969)

APPENDIX III. COMPETITION ASSAY CURVES OF 16 ANTI-DNA HYBRIDOMA ANTIBODIES

FIGURE A1. INHIBITION OF dDNA BINDING OF 1400 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

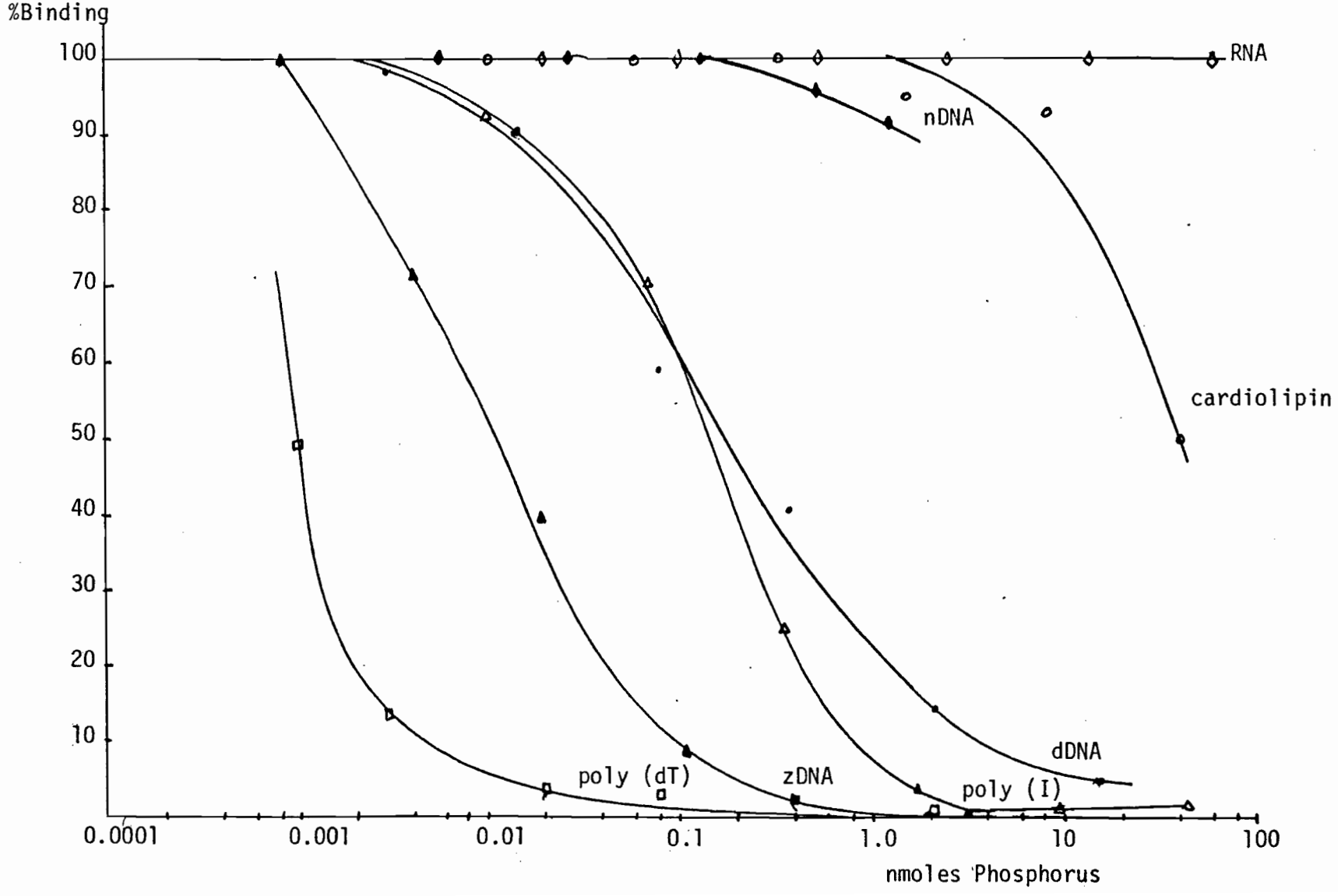


FIGURE A2. INHIBITION OF dDNA BINDING OF 1900 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

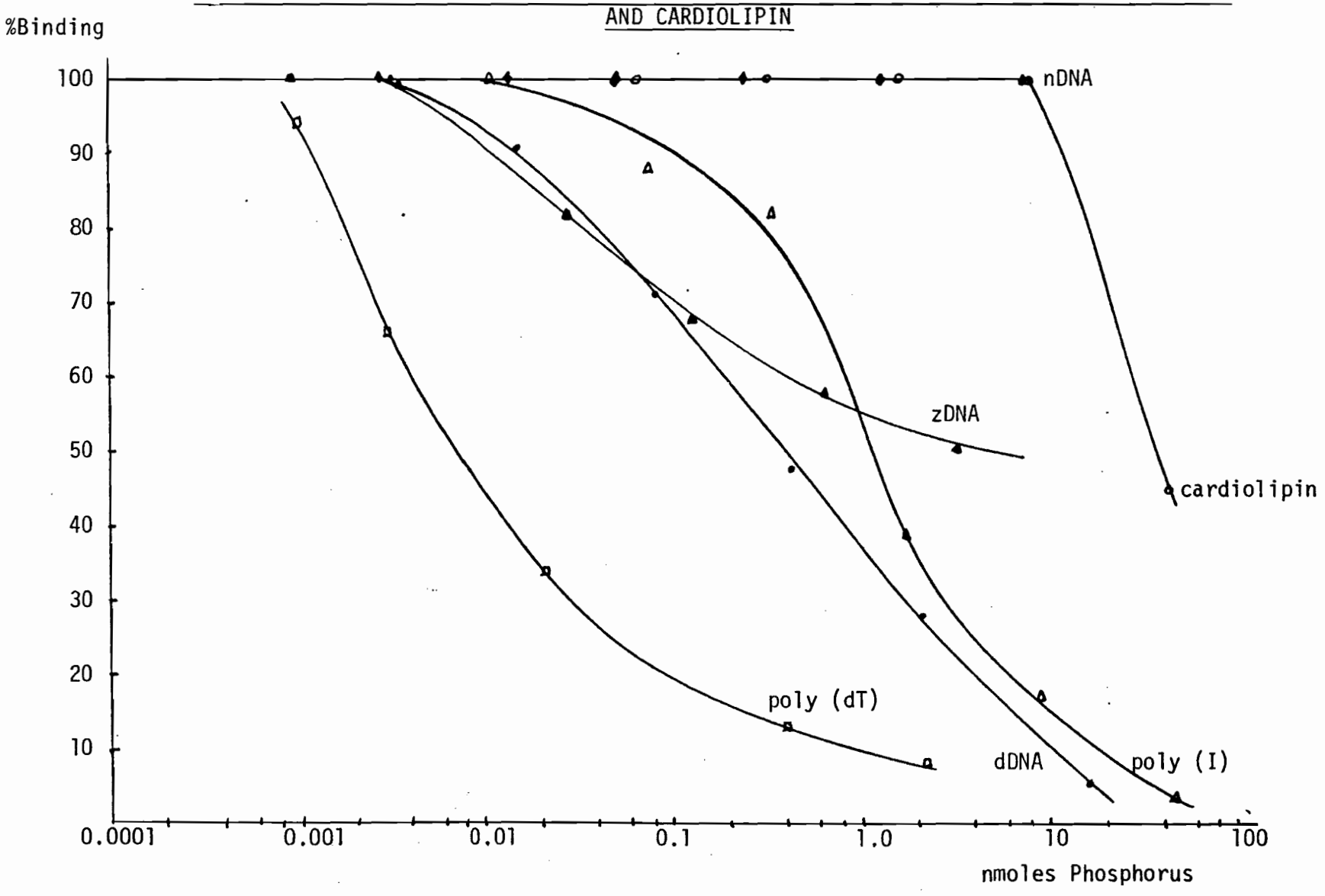


FIGURE A3. INHIBITION OF dDNA BINDING OF 1418 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

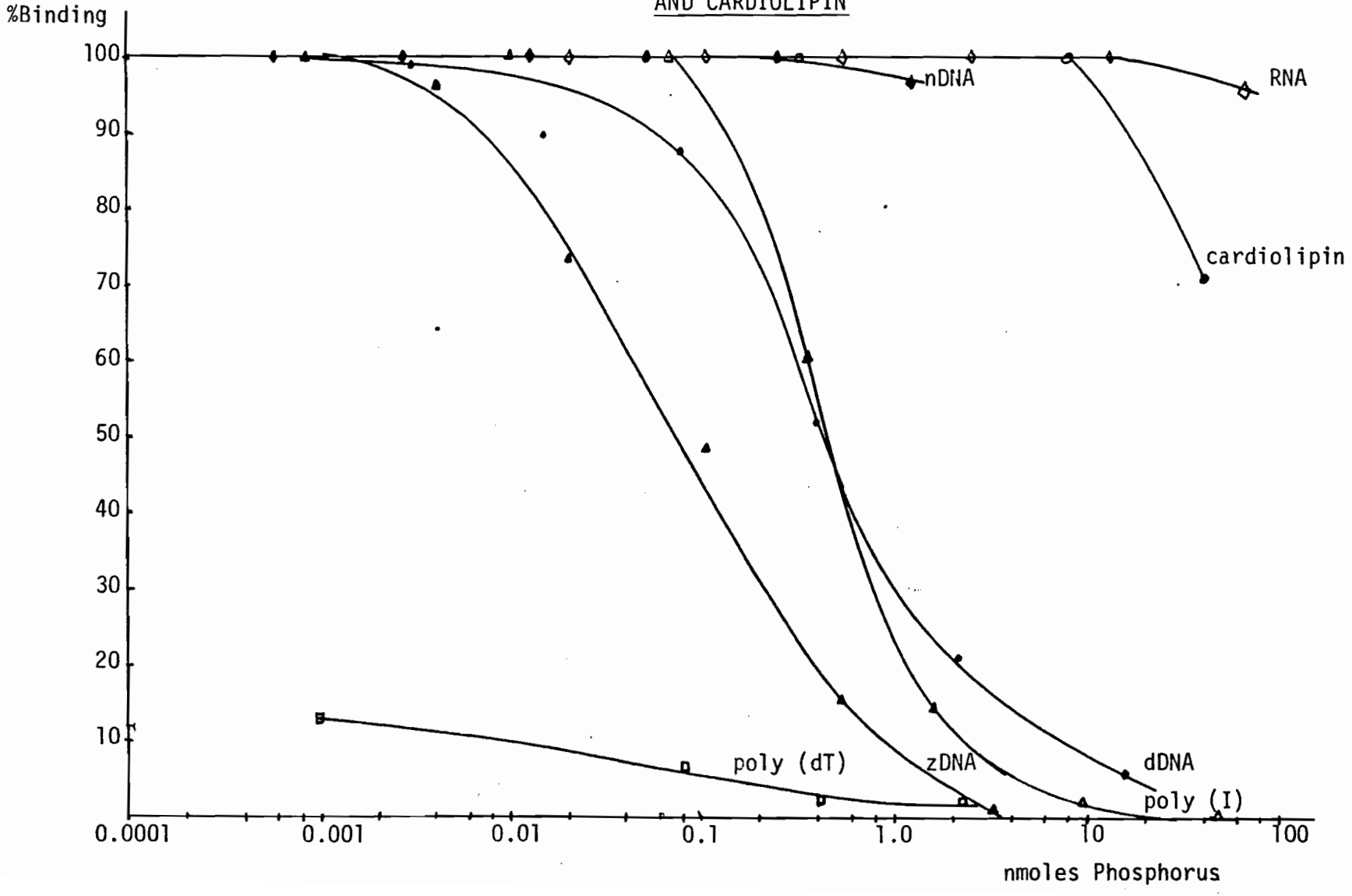


FIGURE A4. INHIBITION OF dDNA BINDING OF 1407 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

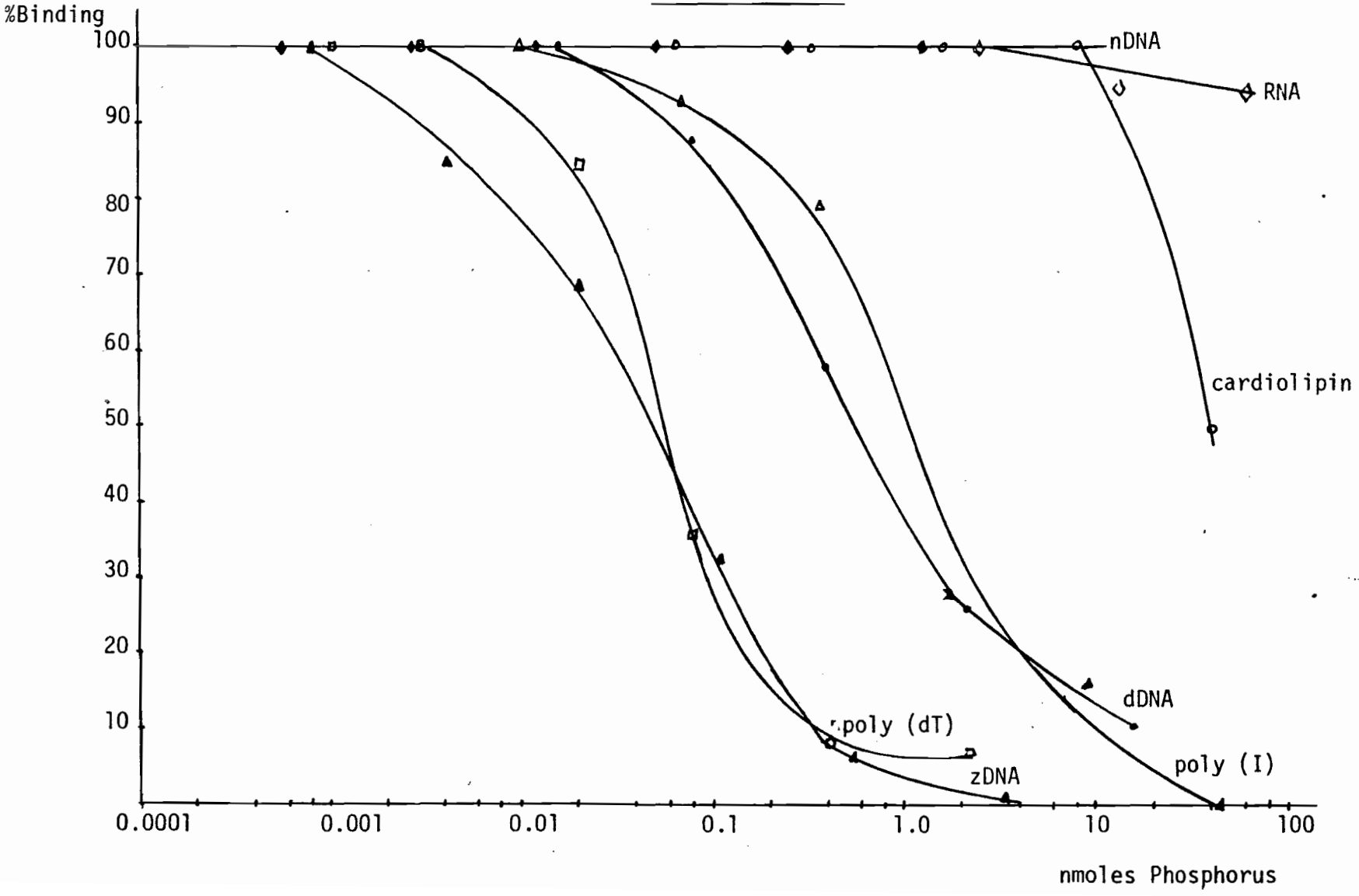


FIGURE A5. INHIBITION OF dDNA BINDING OF 1422 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

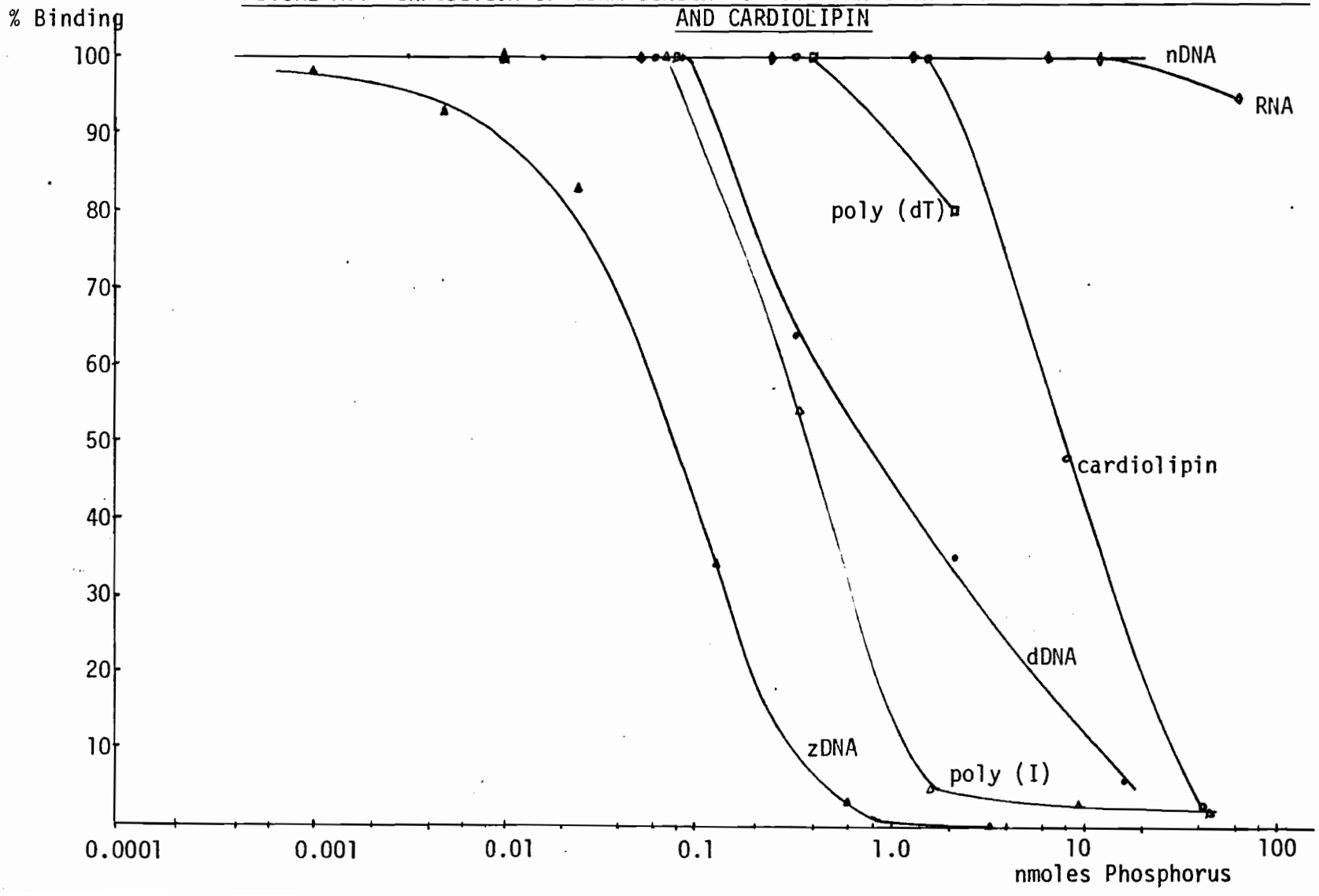


FIGURE A6. INHIBITION OF dDNA BINDING OF 121 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

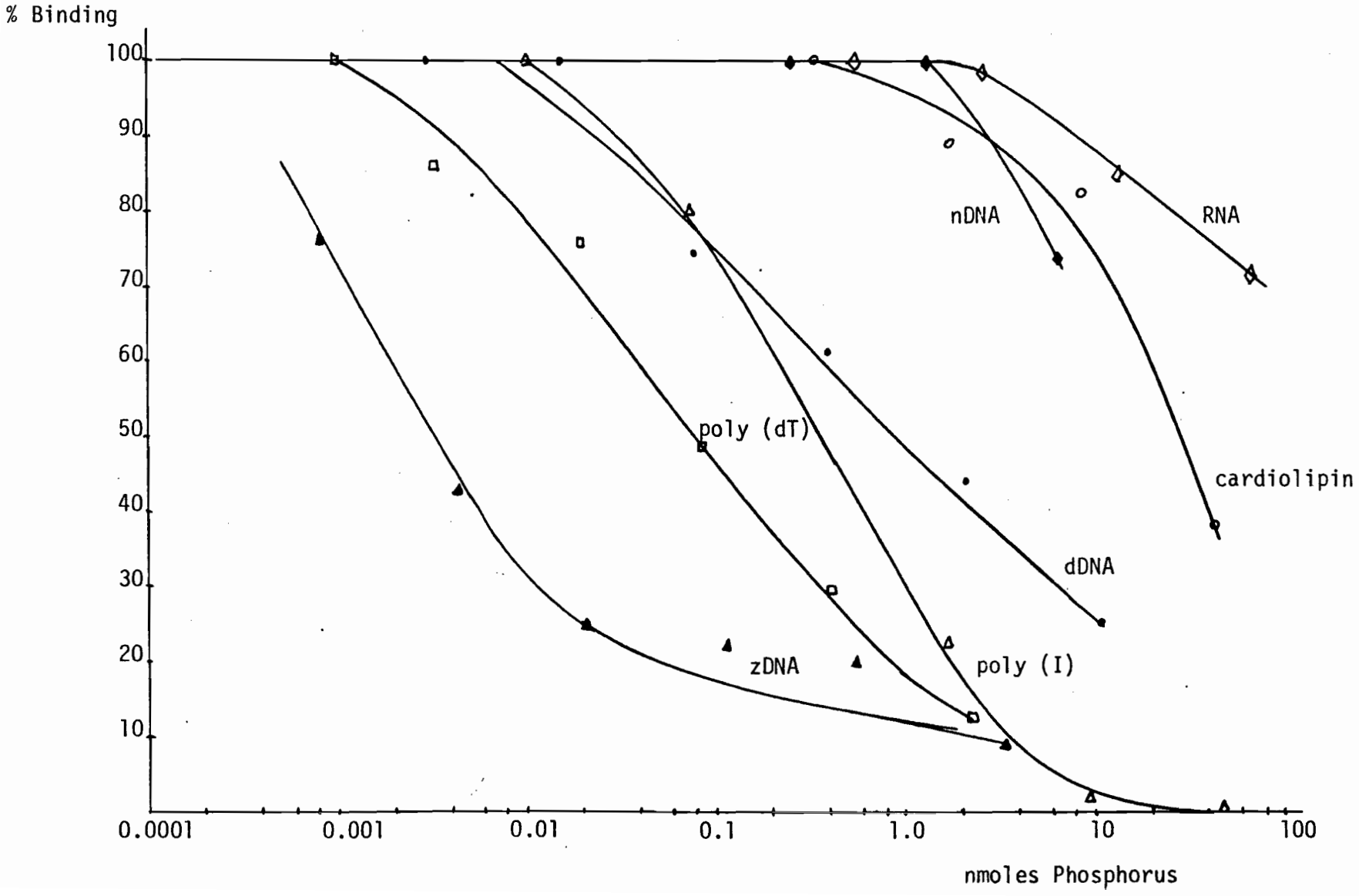


FIGURE A7. INHIBITION OF dDNA BINDING OF 1206 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

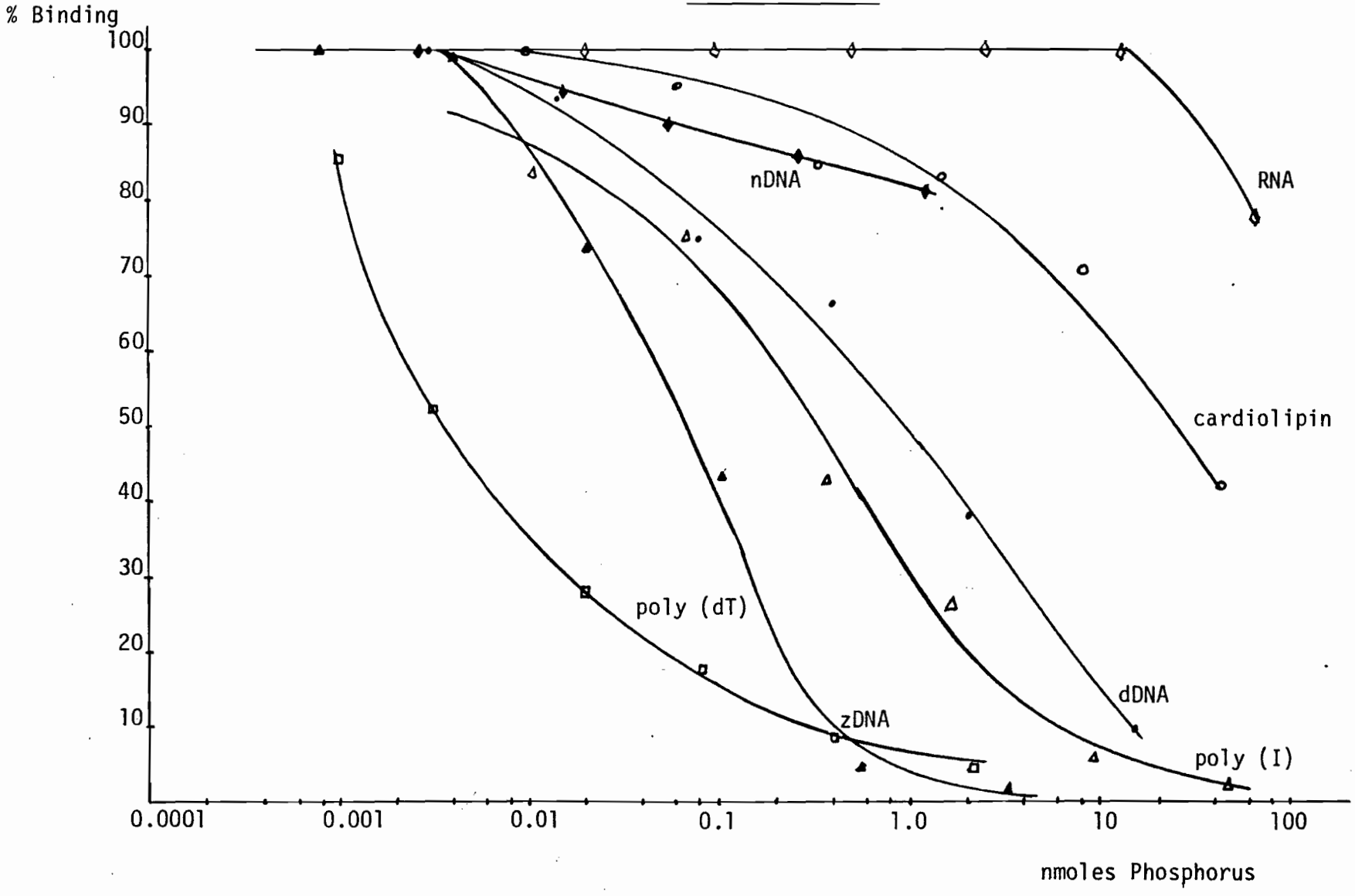


FIGURE A8. INHIBITION OF dDNA BINDING OF 1403 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

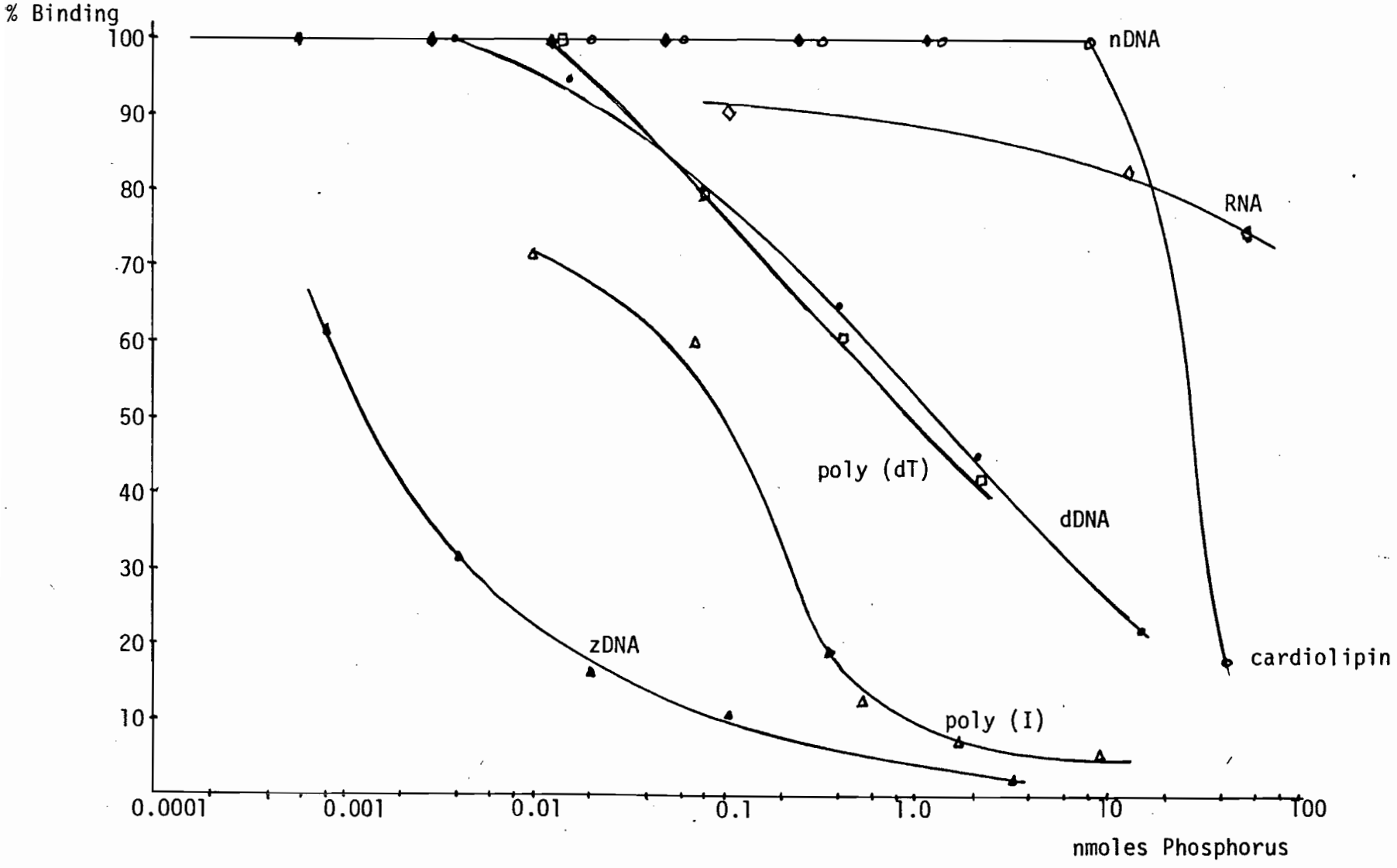


FIGURE A9. INHIBITION OF dDNA BINDING OF 134 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

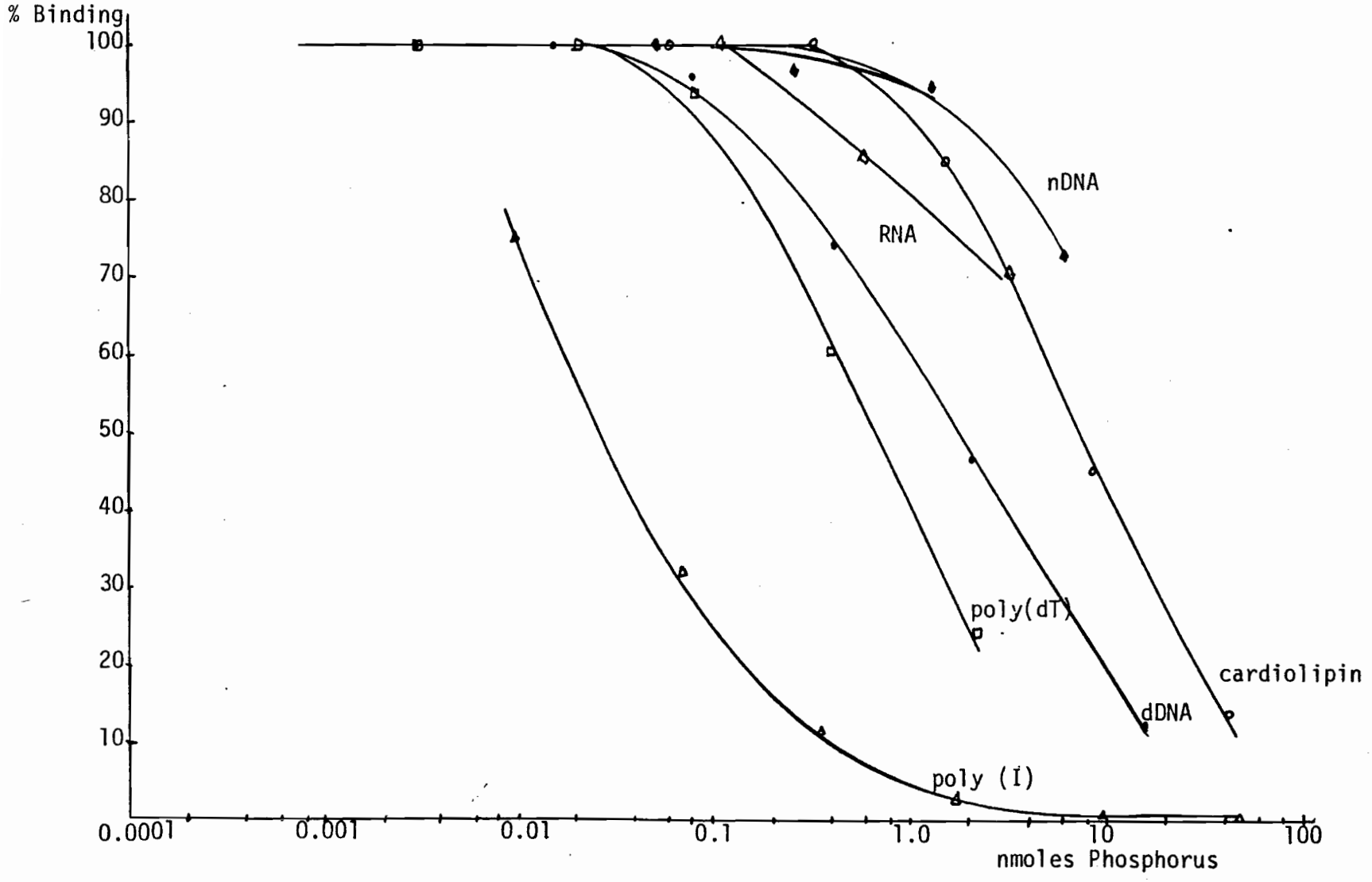


FIGURE A10. INHIBITION OF dDNA BINDING OF 1414 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

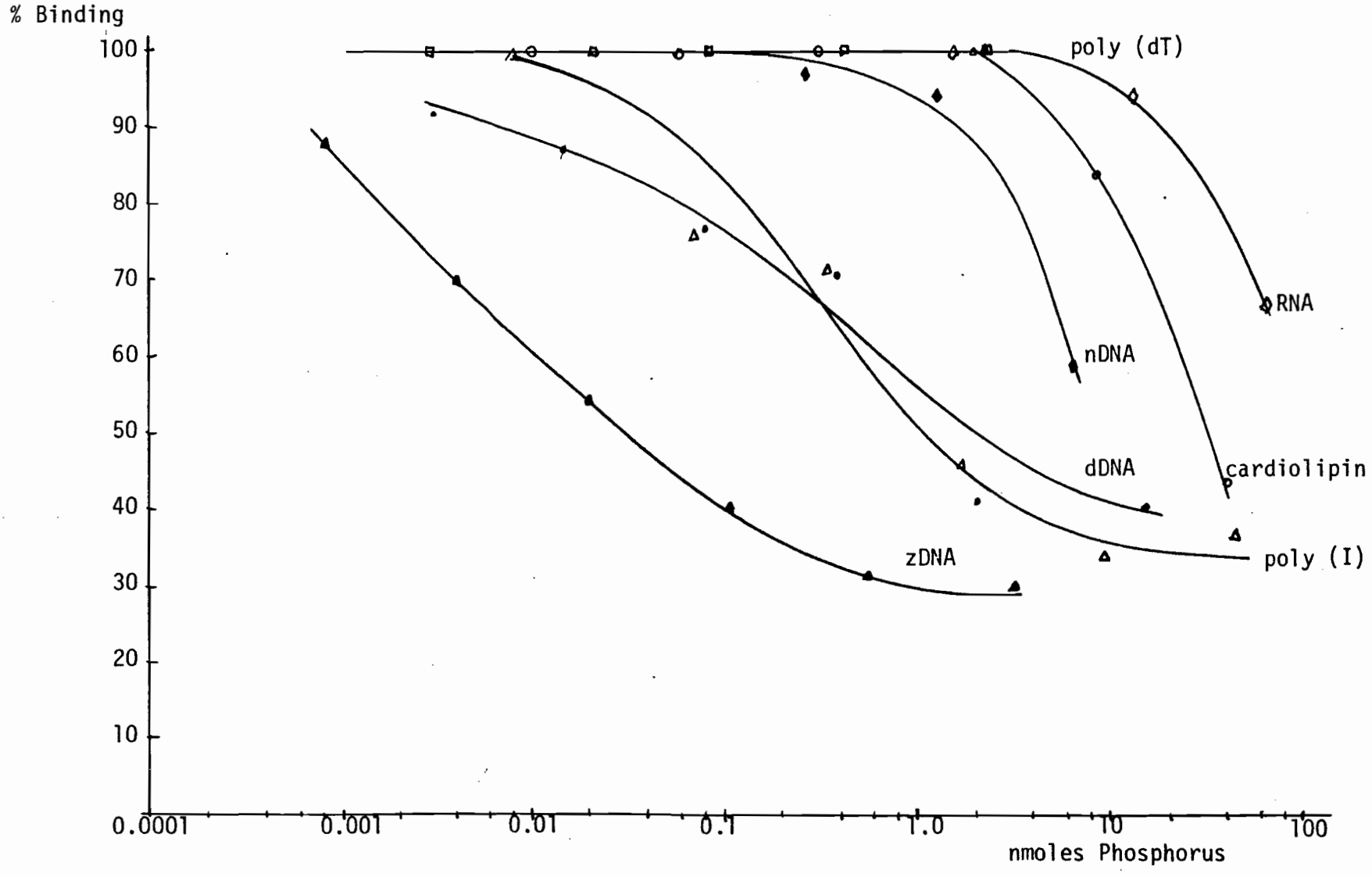


FIGURE A11. INHIBITION OF dDNA BINDING OF 2607 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

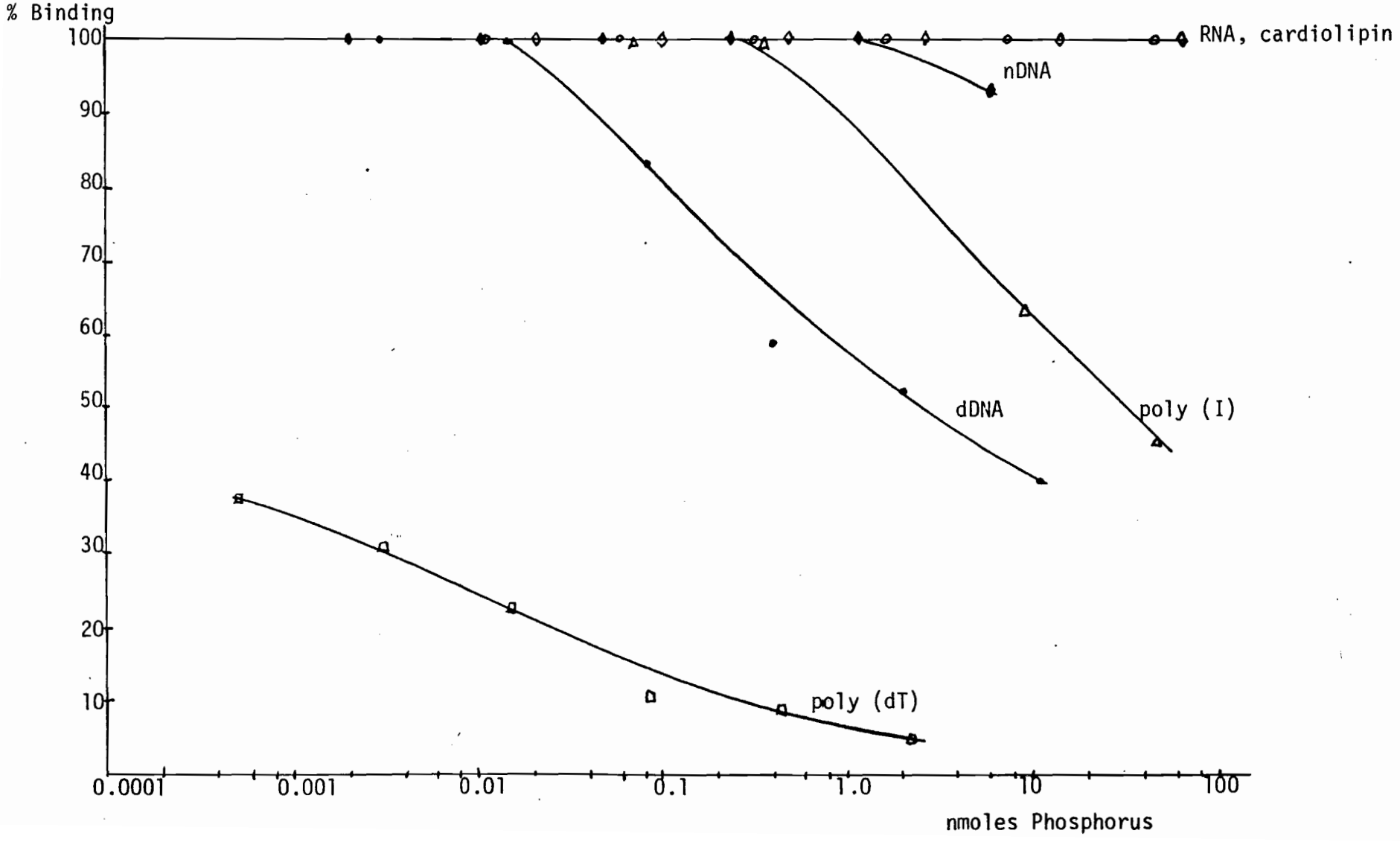


FIGURE A12. INHIBITION OF dDNA BINDING OF 2608 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

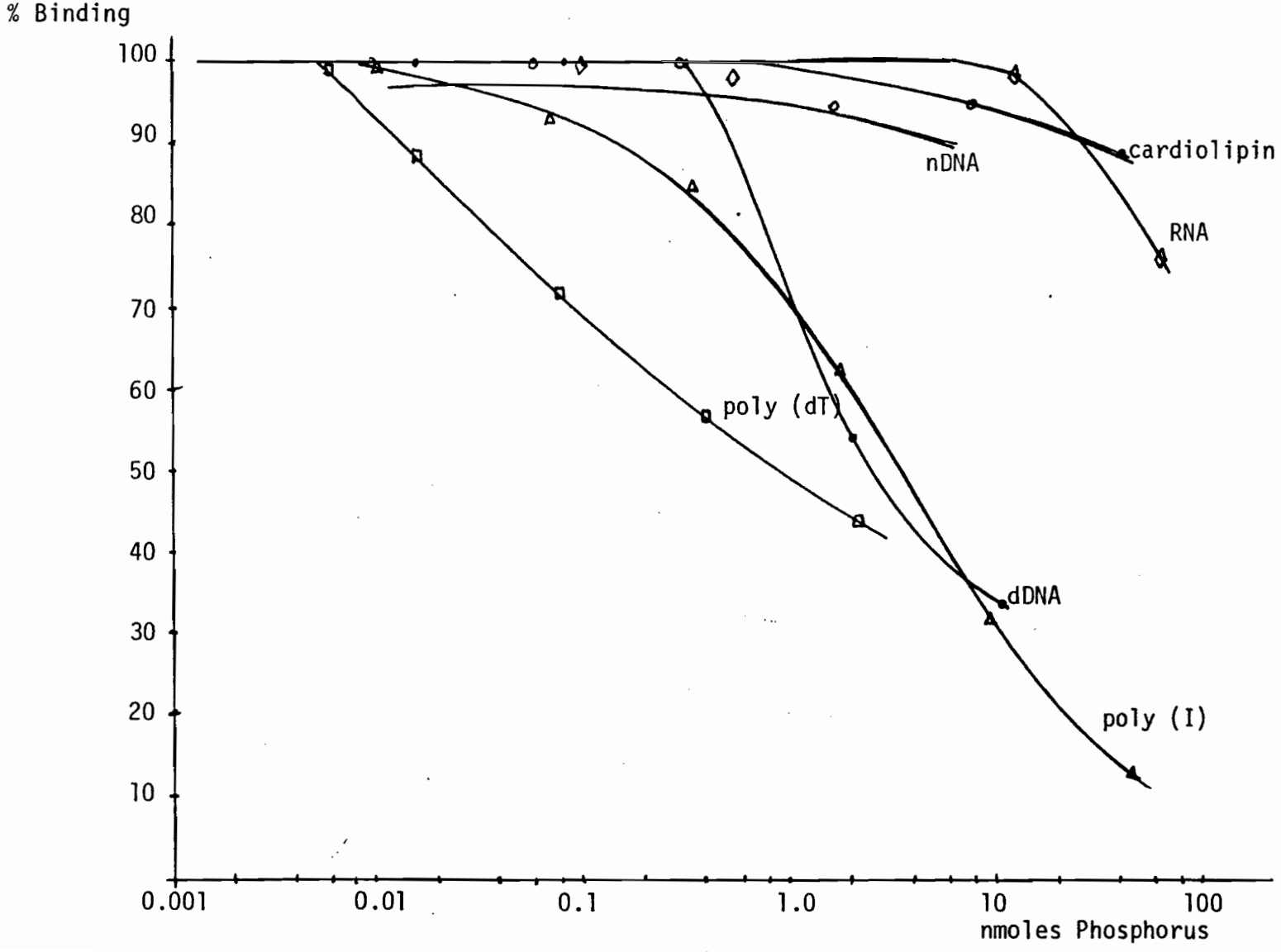


FIGURE A13. INHIBITION OF dDNA BINDING OF 1202 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

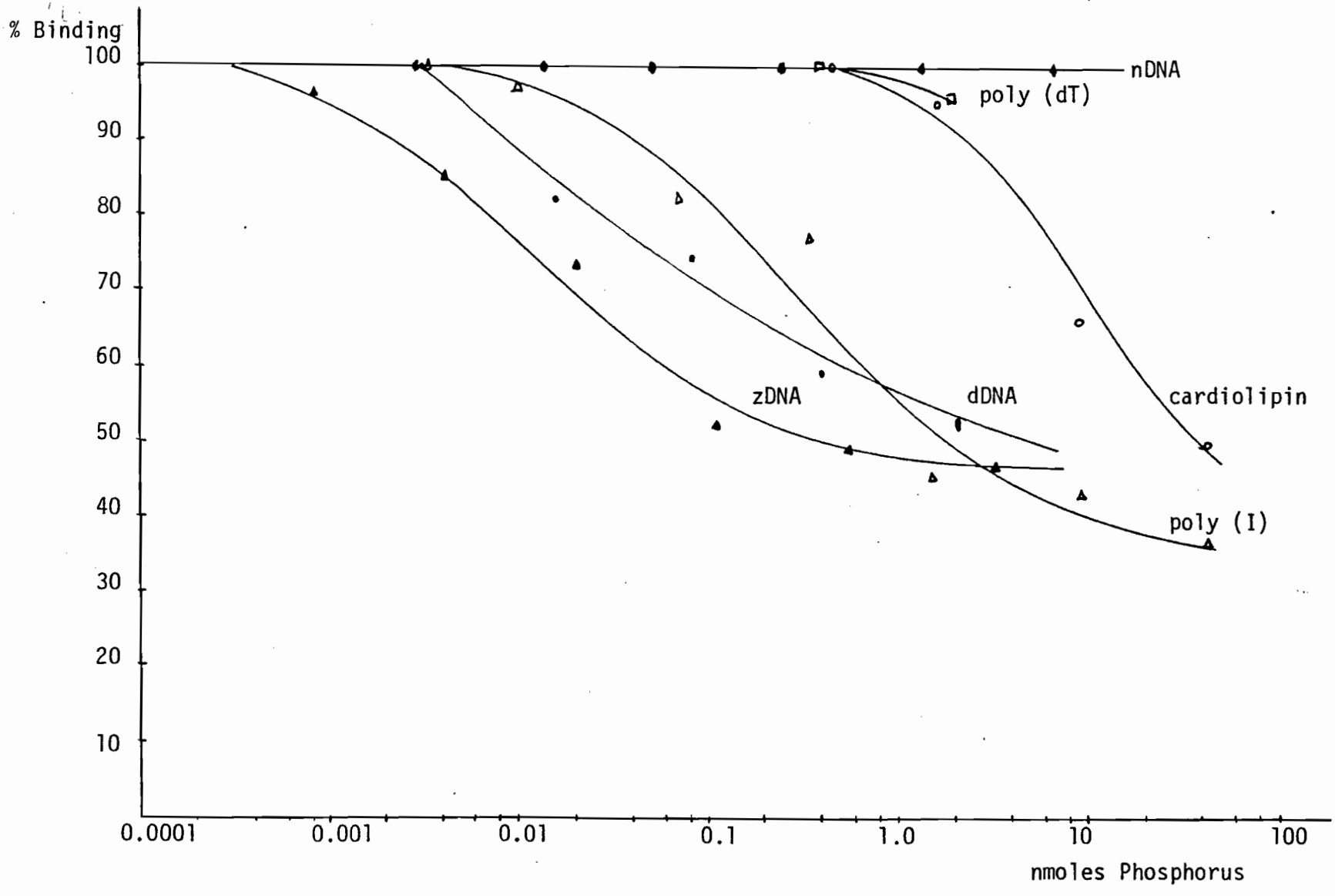


FIGURE A14. INHIBITION OF dDNA BINDING OF 100 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

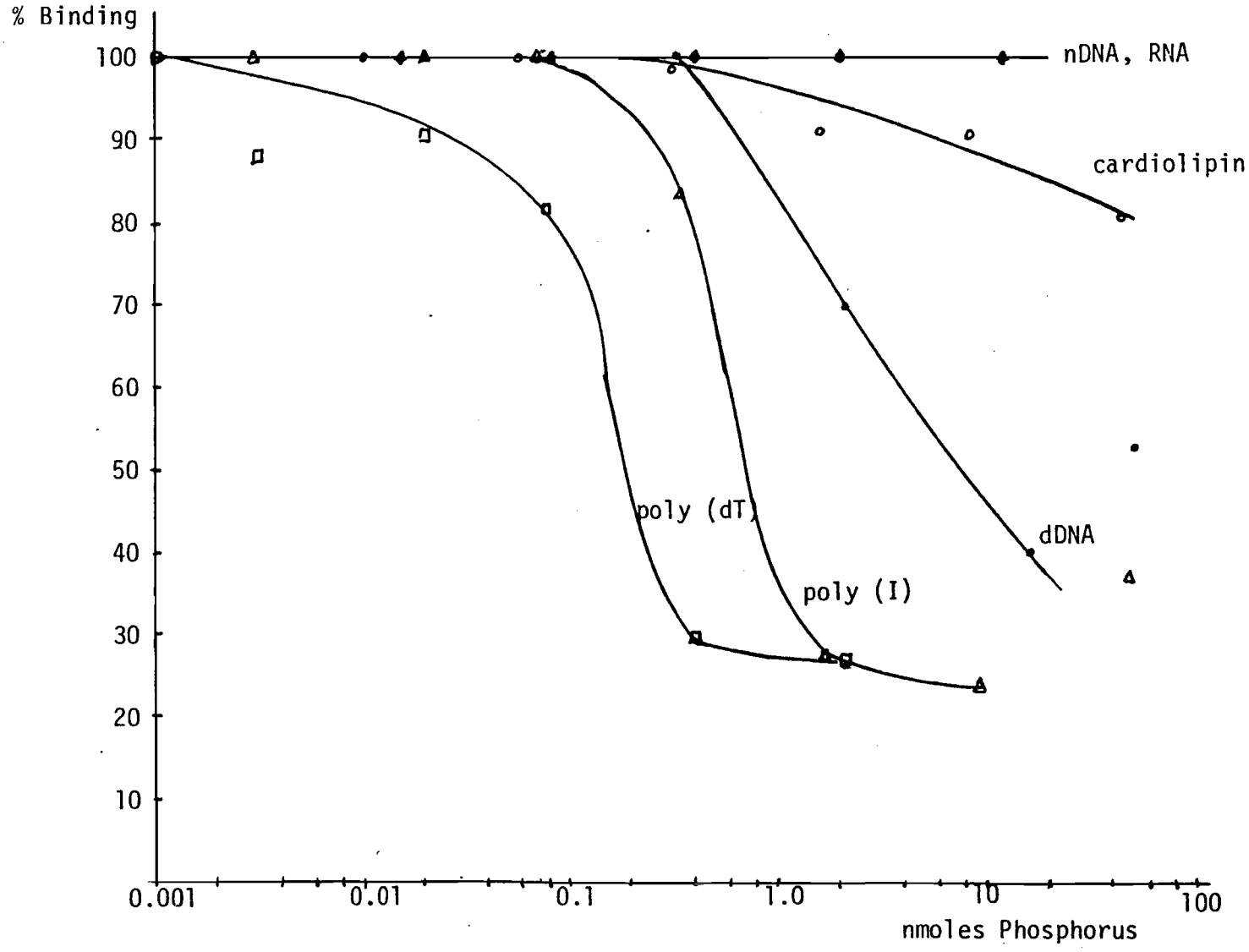


FIGURE A15. INHIBITION OF dDNA BINDING OF 600 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN

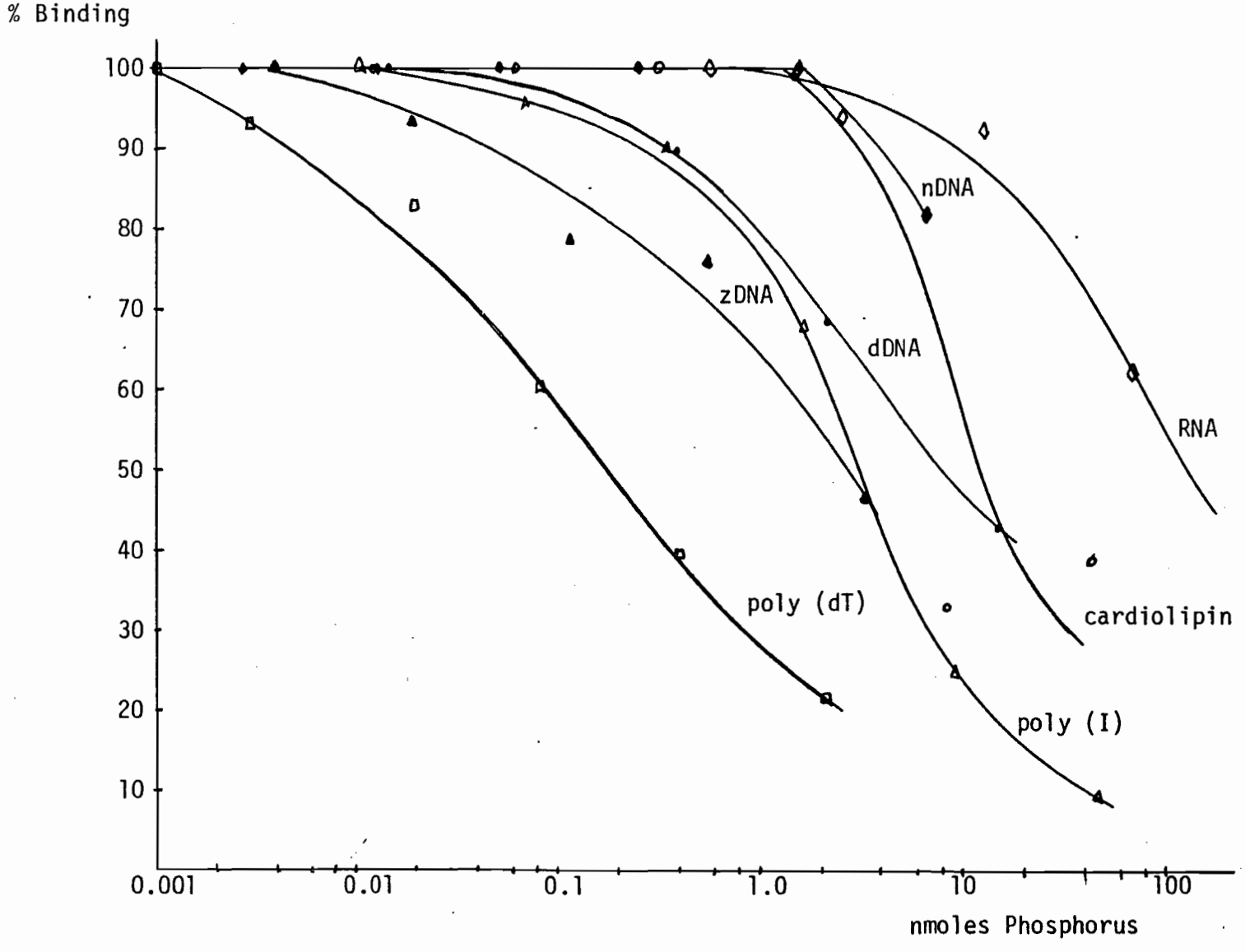
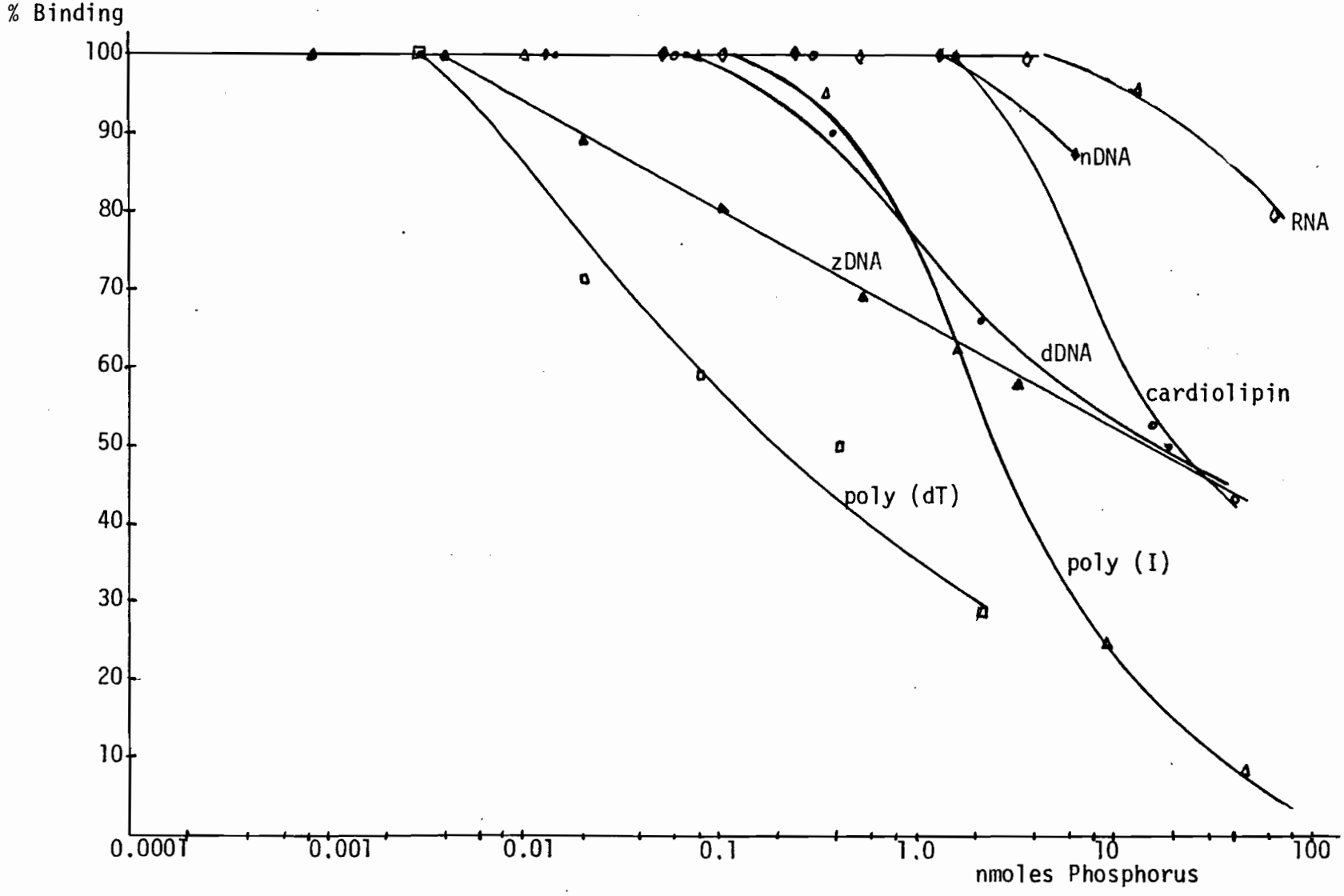


FIGURE A16. INHIBITION OF dDNA BINDING OF 604 ANTI-DNA ANTIBODY BY POLYNUCLEOTIDES AND CARDIOLIPIN



Abrams, P. G., J. A. Knost, G. Clarke, S. Wilburn, R. K. Oldam, and K. A. Foon (1983). Determination of the optimal human cell lines for development of human hybridomas. J. Immuno. 131: 1201-1204.

Agnello, V., D. Koffler, and H. G. Kunkel (1973). Immune complex systems in the nephritis of SLE. Kidney Int. 3: 90-99.

Agnello, V., A. Arbetter, G. I. deKasep, R. Powell, E. M. Tan, and F. Joslin (1980). Evidence for a subset of rheumatoid factors that cross-react with DNA-histone and have a distinct cross-idiotype. J. Exp. Med. 151: 1514-1527.

Alarcon-Segovia, D., E. Fishben and S. Estrada-Parra (1975). The heterogeneity of anti-DNA antibodies in SLE and other diseases. J. Rheum. 2: 172-177

Andrews, D. W., and J. D. Capra (1980). Structure and function of immunoglobulins, in Clinical Immunology Vol.1. by C. W. Parker. W. B. Saunders Company. Philadelphia. p. 1-18

Andrzejewski, C. A. Jr, D. B. Stollar, T. M. Lalor, and R. S. Schwartz (1980). Hybridoma autoantibodies to DNA. J. Immunol. 124: 1499-1502.

Andrzejewski, C. A. Jr, J. Rauch, E. Lafer, B. D. Stollar, and R. S. Schwartz (1981). Antigen binding diversity and idiotypic cross reactions among hybridoma autoantibodies to DNA. J. Immunol. 126: 226-231.

Andrzejewski, C.A., E. M. Lafer, J. Rauch, R. S. Schwartz and B. D. Stollar (1982). The use of hybridomas in the dissection of autoimmune phenomena in Hybridomas in cancer diagnosis and treatment by M. S. Mitchell and H. F. Oettgen, Raven press New York: pp 55-60.

Aotsuka, S., M. Okawa, K. Ikebe, and R. Yokohari (1979). Measure of anti-double stranded DNA antibodies in major immunoglobulin classes. J. Immuno. Meth. 28: 149-162.

Arana, R., and M. Seligmann (1967). Antibodies to native and denatured deoxyribonucleic acid in systemic lupus erythematosus. J. Clin. Invest. 46: 1867-1882.

Arnott, S., R. Chandrasekaram, and C. M. Martilla (1974). Structures for polyinosinic acid and polyguanylic acid. Biochem. J. 141: 537-543.

Atkinson, P. M., G. W. Lampman, B. C. Furie, Y. Naparstek, R. S. Schwartz, B. D. Stollar, and B. Furie (1985). Homology of the NH_2-terminal amino acid sequences of the heavy and light chains of human monoclonal lupus autoantibodies containing the dominant 16/6 idiotype. J. Clin. Invest. 75:

1138-1143.

Auffray, C., J. L. Sikorav, R. Ollo, and F. Fougeon (1981). Correlation between D region structure and antigen-binding specificity evidences from the comparison of closely related immunoglobulin V_H sequences. Ann. Immuno. 132D: 77-78.

Baehr, G., P. Klemperer, and A. Schifrin (1935). A diffuse disease of the peripheral circulation usually associated with lupus erythematosus and endocarditis. Trans. Ass. Amer. Physicians 50: 139-155.

Bagchi, B., D. N. Misra, S. Basu, and N. N. DasGupta (1969). Conformation of denatured and renatured DNA. Biochem. Biophys. Acta. 182: 551-561.

Bankhurst, A. D., G. Torrigrani, and A. C. Allison (1973). Lymphocytes binding human thyrogrobulin in healthy people and it relevance to tolerance for autoantigens. Lancet 1: 226-230.

Bankhurst, A. D., and R. C. Williams (1975). Identification of DNA-binding lymphocytes in patients with systemic lupus erythematosus. J. Clin. Invest. 56: 1378-1385.

Barski, G., S. Sorieul, and F. Cornefert (1961). "Hybrid" type cells in combined cultures of two different mammalian cell stains. J. Natl. Cancer Inst. 26: 1269-1291.

Bastin, J. M., J. Kirkley, and A. J. McMichael (1982). Production of monoclonal antibodies: a practical guide in Monoclonal antibodies in clinical medecine by A. J. McMichael and J. W. Fabre. Academic Press. London and New York. pp. 503-517.

Batteiger, B., V. Newhall, R. B. Jones (1982). The use of Tween 20 as a blocking agent in the immunological detection of proteins transfered to nitrocellulose membranes. J. Immuno. Meth. 55: 297-307.

Bell, C., N. Talal, and P. H. Schur (1975). Antibodies to DNA in patients with rheumatoid arthritis and juvenile rheumatoid arthritis. Arthr. Rheum. 18: 535-540.

Bell, D. A. (1979). Cell-mediated immunity in rheumatoid arthritis and systemic lupus erythematosus: too little or too much. J. Rheum. 6: 484-488

Bell, D. A., and P. J. Maddison (1980). Serologic subsets in systemic lupus erythematosus: an examination of autoantibodies in relationship to clinical features of disease and HLA antigens. Arthr. Rheum. 23: 1268-1273.

Bell, D. A. (1981). The potential role of immunoregulatory

disturbance in the pathogenesis of systemic lupus erythematosus. J. Rheum. 8: 340-344.

Berek, C. (1984). The D segment defines the T15 idiotypes: the immunoresponse of A/J mice to Pneumococcus pneumoniae. Eur. J. Immuno. 14: 1043-1048

Bona, C. A. (1981). Idiotype - general features in Idiotypes and Lymphocytes. Academic Press. New-York. p. 1-37

Briles, D. E., C. Forman, S. Hudak, and J. L. Clafin (1984). The effects of idiotype on the ability of IgG_1 anti-phosphocylcholine antibodies to protect mice from fatal infection with Streptococcus pneumoniae. Eur. J. Immuno. 14: 1027-1030

Brodin, T., L. Olsson, and H. O. Sjogren (1983). Cloning of human hybridoma, myeloma and lymphoma cell lines using enriched human monocyte as feeder layer. J. Immuno. Meth. 60: 1-7.

Burnet, F. M. (1957). A modification of Jerne's theory of antibody production using the concept of clonal selection. Austral. J. Sci. 20: 67-69.

Butler, W. T., J. T. Sharp, R. D. Rossen, M. Lidski, K. K. Mittal, and D. A. Gard (1972). Relationship of the clinical course of SLE to the presence of circulating lymphocytotoxic antibodies. Arthr. Rheum. 15: 231-238.

Cairns, E., J. Block, and D. A. Bell (1984). Anti-DNA autoantibody-producing hybridomas of normal human lymphoid cell origin. J. Clin. Invest. 74: 1-8.

Capra, J. D., and A. B. Edmundson (1977). The antibody combining site. When an antigen, or foreign substance, combines with an antibody molecule, it does so at a site that fits it precisely as a lock fits a key. The nature of this site has been worked out in considerable detail. Sci. Amer. 236:50-69.

Capra, J. D., and M. Fougereau (1983). One from column A, one from comlumn B: structural correlates of idiotypes. Immuno. Today 4:177-179.

Cathou, R. E. and C. T. O'Konski (1970). A transient electric birefringence study of the structure of specific IgG antibody. J. Mol. Biol. 48: 123-131.

Cavagnaro, J., and M. Osband (1983 abstract). Successful in vitro primary immunization of human peripheral blood mononuclear cells and subsequent fusion with human myeloma cells to produce human-human hybridomas secreting specific monoclonal antibody. Hybridoma 2: 128.

Cazenave, P. L. A. (1850-1851). Lupus Erythemateux (Erytheme centrifuge). Ann. Malad. Peau Syph. 3: 297-299.

Cepellini, R., E. Polli, and F. Celeda (1957). DNA-reacting factor in serum of a patient with lupus erythematosus diffusus. Proc. Soc. Exp. Biol. Med. 96: 572-574.

Chen, P. P., F. Goni, S. Fong, F. Jirik, J. H. Vaughan, B. Frangione, and D. A. Carson (1985). The majority of human monoclonal IgM rheumatoid factors express a "primary structure-dependent" cross-reactive idiotype. J. Immuno. 134: 3281-3285.

Claman, H. N., E. A. Chaperon, and R. F. Triplett (1966). Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. Med. 122: 1167-1174.

Claman, H. N., and D. E. Mosier (1972). Cell-cell interactions in antibody production. Progr. Allergy. 16: 40-80.

Cohen, I. R., and H. Wekerle (1977). Autoimmunity, self recognition, and blocking factors in Autoimmunity: genetic, immunologic, virologic, and clinical aspects by N. Talal. New York. Academic Press inc. pp. 231-265.

Cohen, P. L. (1980). Bone marrow as the major site of anti-erythrocyte autoantibody production in NZB mice. Arthr. Rheum. 23: 1045-1051.

Cohen, S. A., G. R. V. Hughes, G. L. Noel, and C. L. Christian (1971). Character of anti-DNA antibodies in systemic lupus erythematosus. Clin. Exp. Immuno. 8: 551-561.

Coller, H. A. and H. S. Coller (1983). Statistical analysis of repetitive subcloning by the limiting dilution technique with a view toward ensuring hybridoma monoclonality. Hybridoma. 2: 91-96.

Coons, A. H. (1951). Symposium on labelled antigens and antibodies: fluorescent antibodies as histochemical tools. Fed. Proc. 10: 558-559.

Cote, R. J., D. M. Morrissey, A. N. Houghton, E. J. Beattie Jr, H. F. Oettgen, and L. J. Old (1983). Generation of human monoclonal antibodies reactive with cellular antigens. Proc. Natl. Acad. Sci. U.S.A. 80: 2026-2030.

Cotton, R. G. H., and C. Milstein (1973). Fusion of two immunoglobulin producing myeloma cells. Nature. 244: 42-43.

Croce, C. M., A. Linnenbach, W. Hall, Z. Steplewski, and H. Koprowski (1980). Production of human hybridomas secreting antibodies to measles virus. Nature. 288:

488-489.

Datta, S. K., D. Stollar, and R. S. Schwartz (1983). Normal mice express idiotypes related to autoantibody idiotypes inherited by lupus mice. Proc. Natl. Acad. Sci. U.S.A. 80: 2723-2727.

Davidson, R. L., K. H. O'Malley, and T. B. Wheeler (1976). Polyethylene glycol-induced mammalian cell hybridization: effect of polyethylene glycol molecular weight and concentration. Som. Cell Genet. 2: 271-280.

Denis, K. A., R. Wall, and A. Saxon (1983). Human-human B cell hybridoma from in vitro stimulated lymphocytes of patients with common variable immunodeficiency. J. Immuno. 131: 2273-2278.

De Preval, C. (1982). Immunoglobulins in Immunology by J. F. Bach. John Wiley & Sons. New-York p. 163-251

Dixon, W. J., and F. J. Massey Jr (1969). Introduction to statistical analysis 3rd Ed. McGraw-Hill inc.. p. 638.

Dubois, E. L. (1974). Lupus erythematosus. A review of the current status of discoid and systemic lupus erythematosus and their variants. University of southern California Press. California. p.1-11, 164.

Eaton, R. B., G. Schneider, P. H. Schur (1983). Enzyme immuno-assay for antibodies to native DNA. Specificity and quality of antibodies. Arthr. Rheum. 26: 52-62.

Edwards, P. A. W., C. M. Smith, A. M. Neville, and M. J. O'Hara (1982). A human-human hybridoma system based on fast growing mutant of the ARH-77 plasma cell leukemia derived line. Eur. J. Immuno. 12: 641-648.

Ehrlich, P., and J. Morgenroth (1900). Uber Haemolysene. Dritte Mitthieling. Berlin Klin. Wochshi 37: 453.

Eisen H. N. (1974). Antibody structure: the immunoglobulins in Immunology - an Introduction to Molecular and Cellular Principles of the Immune Response. Harper & Row publishers. New-York. p.405-448

Eisenbarth, G. S., A. Linnenbach, R. Jackson, R. Scearce, and C. M. Croce (1982). Human hybridomas secreting anti-islet autoantibodies. Nature 300: 264-267.

Epstein W. V. (1975). Specificity of SLE serum antibody for ss and ds DNA configuration. J. Rheum. 2: 215-220

Fish, F., and M. Ziff (1981). A sensitive solid phase micro-radioimmunoassay for anti-double stranded DNA antibodies.

Arthr. Rheum. 24: 534-543.

Fish, F., and, M. Ziff (1982). The in vitro and in vivo induction of anti-double-stranded DNA antibodies in normal and autoimmune mice. J. Immuno. 128: 409-414.

Fishbach, M., J. R. Roubinian, and N. Talal (1978). Lypopolysaccharide induction of IgM antibodies to polyadenylic acid in normal mice. J. Immuno. 120: 1856-1861.

Foung, S. K. H., S. Perkins, A. Raubitschek, J. Larrick, G. Lizak, D. Fishwild, E. G. Engleman and F. C. Grumet (1984). Rescue of human monoclonal antibody production from an EBV-transformed B cell line by fusion to an human-mouse hybridoma. J. Immuno. Meth. 70: 83-90.

Friou, G. J. (1957 abstract). Clinical application of lupus serum-nucleoprotein reaction using fluorescent antibody technique. J. Clin. Invest. 36: 890.

Friou, G. J, S. C. Finch, K. D. Detre (1958a). Interaction of nuclei with globulin from lupus erythematosus serum demonstrated with fluorescent antibody. J. Immuno. 80: 324-329.

Friou, G. J. (1958b). Identification of the nuclear component of the interaction of lupus erythmatosus globulin and nuclei. J. Immuno. 80: 476-481.

Friou, G. J. (1958c). Improved method for measuring lupus globulin nucleoprotein interaction. Proc. Natl. Acad. Sci. U.S.A. 94: 738-740.

Gefter, M. L., D. H. Margulies, and M. D. Scharff (1977). A simple mithod for polyethylene glycol-promoted hybridization of mouse myeloma cells. Som. Cell Genet. 3: 231-238.

Geha, R. S. (1981). Regulation of the immune response by idiotypic-antiidiotypic interactions. N. Engl. J. Med. 305: 25-28.

Geha, R. S. (1982). The presence of auto-anti-idiotypic antibody during the normal human immune response to tetanus toxoid antigen. J. Immuno. 129: 139-144.

Geltner, D., E. G. Franklin, and B. Frangione (1980). Anti-idiotypic activity in the IgM fractions of mixed cryoglobulins. J. Immuno. 125: 1530-1535.

Gilliam, A. C., D. Lang, and J. J. LoSpaluto (1980). Antibodies to double-stranded DNA: purification and characterization of binding specificities. J. Immuno. 125: 874-885.

Glassy, M. C., H. H. Handley, H. Hagivara, and I. Royston

(1983). UC-729-6, a human lymphoblastoid B cell line useful for generating antibody-secreting human-human hybridomas. Proc. Natl. Acad. Sci. U.S.A. 80: 6327-6331.

Gorer, P. A., and P. O'Gorman (1956). The cytotoxic activity of isoantibodies in mice. Transpl. Bull. 3: 142-148.

Hahn, B. H., G. C. Sharp, W. S. Irwin, O. S. Kantor, C. A. Gardner, M. Bagby, J. M. Peny Jr, and C. K. Osterland (1972). Immune responses to hydralazine and nuclear antigens in hydralazine-induced lupus erythematosus. Am. J. Med. 76: 365-374.

Hahn, B. H. (1975). Cell-mediated immunity in systemic lupus erythematosus. Clin. Rheum. Dis. 1: 497-504.

Hahn, B. H. (1980). Systemic lupus erythematosus in Clinical Immunology Vol.1 by C. W. Parker, W. B. Saunder Company. Toronto. Chapter 19 pp 583-631.

Hahn, B. H. (1984). Suppression of autoimmune diseases with an idiotypic antibodies. Murine lupus nephritis as a model. Spring. Semin. Immunopatho. 7: 25-32.

Hahn, B. H., E. M. Ebling (1984). Suppression of murine lupus nephritis by administration of an anti-idiotypic antibody to anti-DNA. J. Immuno. 132: 187-190.

Handley, H. H., and I. Royston (1982). A human lymphoblastoid B cell line useful for generating immunoglobulin-secreting human hybridomas in Hybridomas in cancer diagnosis and treatment by M. S. Mitchell and H. F. Oettgen, Raven Press, New-York. pp 125-132.

Hargraves, M. M., H. Richmond, and R. Morton (1948). Presentation of two bone marrow elements : the "tart" cell and "L. E."cell. Proc. Staff. Meet. Mayo. Clin. 23: 25-28.

Harris, E. N., A. E. Gharavi, M. L. Boey, B. M. Patel, C. G. Mackwoth-Young, S. Loizou, and G. R. V. Hughes (1983). Anti-cardiolipin antibodies: detection by radioimmunoassay and association with trombosis in SLE. Lancet 2: 1211-1214.

Harris, H., and J. F. Watkins (1965). Hybrid cells derived from mouse and man : artificial heterokaryons of mammalian cells from different species. Nature 205: 640-646.

Harris, H. (1970). Cell fusion, the Dunham lectures. Oxford Univ. Press, London and New-York. pp. 108.

Harris, N. S. (1974) Plasma cell surface antigen on human blood lymphocytes. Nature 250: 507-509.

Hasselbacher, P., and E. Carwile LeRoy (1975). Serum DNA

binding activity in healthy subjects and in rheumatic desease. Arthr. Rheum. 17: 63-71.

Houghton, A. N., N. Brooks, R. J. Cote, M. C. Tasmina, H. F. Oettgen, and L. J. Old (1983). Detection of cell surface and intracellular antigens by human monoclonal antibodies. Hybrid cell lines derived from lymphocytes of patients with malignant melanoma. J. Exp. Med. 158: 53-65.

Horibata, K., and A. W. Harris (1970). Mouse myelomas and lymphomas in culture. Exp. Cell. Res. 60: 61-77.

Isenberg, D. A., Y. Shoenfeld, M. P. Madaio, J. Rauch, M. Reichlin, B. D. Stollar and R. S. Schwartz (1984). Anti-DNA antibody idiotypes in systemic lupus erythemathosus. Lancet 2: 417-422.

Izui, S., P. H. Lambert, G. J. Fournie, H. Turler, and P. A. Miescher (1977). Features of systemic lupus erythematosus in mice injected with bacterial lipopoly-saccharides. Identification of circulating DNA and renal localization of DNA-anti-DNA complexes. J. Exp. Med. 145: 1115-1130.

Jerne, N. K. (1974). Toward a network theory of the immune system. Ann. Immuno. (Paris) 125c: 373-389.

Jerne, N. K. (1984). Idiotypic networks and other preconceived ideas. Ann. Rev. 79: 5-24.

Jeske, D. J., J. Jarvis, C. Milstein and J. D. Capra (1984). Junctional diversity is essential to antibody activity. J. Immuno. 133: 1090-1092

Jorgensen, T., G. Gaudernack, and K. Hannestad (1977). Production of Balb/c anti-idiotypic antibodies against the Balb/c myeloma protein 315 does not require an intact ligand-binding site. Scand. J. Immuno. 6: 311-318.

Ju, S. T., T. J. Kipps, J. Theze, B. Benacerraf, and M. E. Dorf (1978). Idiotypic analysis of anti-GAT antibodies. I. Presence of common idiotypic specificities in both responder and nonresponder mice. J. Immuno. 121: 1034-1039.

Juszczak, E., R. I. Neau, M. L. Gefter, and M. N. Margolies (1984). Complete heavy and light chain variable region sequence of anti-arsonate monoclonal antibodies from Balb/c and A/J mice sharing the 36-60 idiotype are highly homologous. J. Immuno. 133: 2603-2609.

Kabat, E. A., T. T. Wu, and H. Bilofsky (1978). Variable region genes for the immunoglobulin framework are assembled from small segments of DNA. A hypothesis. Proc. Natl. Acad.

Sci. U.S.A. 75: 2429-2433.

Kabat, E. A., T. T. Wu, and H. Bilofsky (1979). Evidence supporting somatic assembly of the DNA segments (minigenes), coding for the framework and complementarity-determining segments of immunoglobulin variable regions. J. Exp. Med. 150: 1299-1313.

Kanai, Y., M. Tauchi, S. I. Aotsuka, and R. Yokohari (1982). A simple and rapid microenzyme-linked immunosorbent assay for antibodies to poly (ADP-ribose) in systemic lupus erythematosus. J. Immuno. Meth. 53: 355-365.

Kaplan, H. S., and L. Olsson (1982). Human-human hybridomas in diagnosis and treatment in Hybridomas in cancer diagnosis and treatment by M. S. Mitchell, and H. F. Oettgen. Raven Press, New-York. p. 113-124.

Kaplan, M. H., and M. Meyeserian (1962). An immunological cross-reaction between group A-streptococcal cells and human heart tissus. Lancet 1: 706-710.

Kaplan, M. H., and K. H. Svec (1964). Immunologic relation of streptococcal and tissue antigens. III. Presence in human sera of streptococcal antibody cross-reactive with heart tissus. Association with streptococcal infection, rheumatic fever, and glomerulonephritis. J. Exp. Med. 119: 651-665.

Kaposi, M. (1872). New Reports on knowledge of lupus erythematosus (orig. in german). Arch. Dermat. U. Syph. 4: 36-78.

Karol, B. R., M. Reichlin, and R. W. Noble (1977). Evolution of an idiotypic determinant anti-Val. J. Exp. Med. 146: 435-444.

Kearney, J. F. (1984). Hybridomas and monoclonal antibodies in Fundamental Immunology by W. E. Paul, Raven Press, New York pp. 751-766.

Klaus, G. G. B. (1978). Antigen-antibody complexes elicit anti-idiotypic antibodies to self-idiotypes. Nature 272: 265-271.

Klemperer, P., A. D. Pollack, and G. Baehr (1941). Pathology of disseminated lupus erythematosus. Arch. Path. (Chicago). 32: 569-631.

Klinman, N. R., and R. B. Taylor (1969). General methods for the study of cells and serum during the immune response : the response to dinitrophenyl in mice. Clin. Exp. Immuno. 4: 473-487.

Klinman, N. R., N. H. Sigal, E. S. Metcalf, S. K. Pierce, and

P. J. Gearhart (1976). The interplay of evolution and environment in B-cell diversification. Cold Spring Harbor Symp. Quant. Biol. 41: 23-32.

Klotz, J. L., R. M. Minamiz, and R. L. Teplitz (1979). An enzyme-linked immunosorbent assay for antibodies to native and denatured DNA. J. Immuno. Meth. 29: 155-165.

Koffler, D., R. Carr, V. Agnello, R. Thoburn, and H. G. Kunkel (1971). Antibodies to polynucleotides in human sera : antigenic specificity and relation to disease. J. Exp. Med. 134: 294-312.

Koffler, D., V. Agnello, R. Winchester and H. G. Kunkel (1973). The occurrence of single-stranded DNA in serum of patients with systemic lupus erythematosus and other diseases. J. Clin. Invest. 52: 198-204

Koffler, D., V. Agnello, and H. G. Kunkel (1974). Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. Am. J. Patho. 74: 109-122.

Koffler, D. (1980). Systemic lupus erythematosus : a rheumatic disease with varied symptoms, SLE is a disorder of the immune system. A patient makes autoantibodies that bind to such antigens as DNA, forming immune complexes that can injure tissus. Sci. Am. 243: 40-49.

Kofler, R., D. J. Noonan, D. E. Levy, M. C. Wilson, N. P. H. Moller, F. J. Dixon, and A. N. Theofilopoulos ((1985). Genetic elements used for a murine lupus anti-DNA autoantibody are closely related to those for antibodies to exogenous antigens. J. Exp. Med. 161: 805-815.

Kohler, G, and C. Milstein (1975). Continuous culture of a fused cell secreting antibody of predefined specificity. Nature 256: 495-497.

Koike, T., H. Tomioka, and A. Kumagai (1982). Antibodies that cross-react with DNA and cardiolipin in patients with systemic lupus erythematosus. Clin. Exp. Immuno. 50: 298-302.

Koike, T., N. Maruyama, H. Funaki, H. Tomioka, and S. Yoshida (1984). Specificity of mouse hybridoma to DNA. II. Phospholipid reactivity and biological false positive serological test for syphilix. Clin. Exp. Immuno. 57: 345-350.

Kozbor, D., D. Dexter, and J. C. Roder (1983). A comparative analysis of the phenotypic characteristics of available fusion partners for the construction of human hybridomas. Hybridoma 2: 7-16.

Kozbor, D., and J. C. Roder (1983). The production of monoclonal antibodies from human lymphocyte. Immuno. Today 1: 72-79.

Kranz, D. M., E. W. Voss Jr (1981). Restricted reassociation of heavy and light chains from hapten-specific monoclonal antibodies. Proc. Natl. Aca. Sci. U.S.A. 78: 5807-5811.

Kunkel, H. G., V. Agnello, F. G. Joslin, and J. D. Capra (1973). Cross-idiotypic specificity among monoclonal IgM-proteins with anti-gamma-globulin activity. J. Exp. Med. 137: 331-342.

Kuntz, M. M., J. B. Irnes, and M. E. Weksler (1979). The cellular basis of the impaired autologous mixed lymphocyte reaction in patients with systemic lupus erythematosus. J. Clin. Invest. 63: 151-153.

Lafer, E. M., A. Moller, A. Nordheim, B. D. Stollar, and A. Rich (1981a). Antibodies specific for left-handed-z-DNA. Proc. Natl. Acad. Sci. U.S.A. 78: 3546-3550.

Lafer, E. M., J. Rauch, C. Adrzejewski, D. Mudd, B. Furie, B. Furie, R. S. Schwartz, and B. D. Stollar (1981b). Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J. Exp. Med. 153: 897-909.

Lafer, E. M., R. P. C. Valle, A. Moller, A. Nordheim, P. H. Schur, A. Rich, and B. D. Stollar (1983). Z-DNA-specific antibodies in human systemic lupus erythematosus. J. Clin. Invest. 71: 314-321.

Lane, R. D., R. S. Crissman, and M. F. Lachman (1984). Comparison of polyethylene glycols as fusogens for producing lymphocyte-myeloma hybrids. J. Immuno. Meth. 72: 71-76.

Lange, A., I. M. Roitt, D. Doniach (1976). A double antibody solid phase assay for DNA autoantibodies for clinical use. Clin. Exp. Immuno. 25: 191-198.

Larrick, J. W., K. E. Truitt, A. A. Raubitschek, G. Senyk, and J. C. N. Wang (1983). Characterization of human hybridomas secreting antibody to tetanus toxoid. Proc. Natl. Acad. Sci. U.S.A. 80: 6376-6380.

Lefvert, A. K., R. W. James, C. Alliod, and B. W. Fulpius (1982). A monoclonal anti-idiotypic antibody against the anti-receptor antibodies from myasthenic sera. Eur. J. Immuno. 12: 790-792.

Legrain, P., and G. Buttin (1985). The V_H gene expressed by Balb/c ABPC48 cross-reactive idiotypes induced by anti-idiotypic immunization is identical to that of Balb/c anti-oxazolone and A/J anti-arsonate antibodies. J. Immuno. 134: 3468-3473.

Littlefield, J. W. (1964). Selection of hybrids from mating of fibroblast in vitro and their presumed recombinants. Science 145: 709-710.

Littman, B. H., A. V. Muchmore, A. D. Steinberg, and W. C. Greene (1983). Monoclonal lupus autoantibody secretion by human-human hybridomas. Selection of hybrids by conventional and novel techniques. J. Clin. Invest. 72: 1987-1994.

Locker, J. D., M. E. Medof, R. M. Bennet and S. Sukhupunyaraksa (1977). Characterization of DNA used to assay sera for anti-DNA antibodies; determination of the specificities of anti-DNA antibody in SLE and non-SLE rheumatic disease states. J. Immuno. 118: 694-701

Manser, T., L. J. Wysocki, T. Gridley, R. I. Near, and M. L. Gefter (1985). The molecular evolution of the immune response. Immuno. Today 6: 94-101.

Margolies, M. N., L. J. Wysocki, and V. L. Sato (1983). Immunoglobulin idiotype and anti-anti-idiotype utilize the same variable region genes irrespective of antigen specificity. J. Immuno. 130: 515-517.

Marion, T. N., A. R. Lawton, J. F. Kearney, and D. E. Briles (1982). Anti-DNA autoantibodies in (NZBxNXW)F_1 mice are clonally heterogeneous, but the majority share a common idiotype. J. Immuno. 128: 668-674.

Massicotte, H., J. Rauch, Y. Shoenfeld, and H. Tannenbaum (1984). Influence of fusion cell ratio and cell plating density on production of human-human hybridomas secreting anti-DNA autoantibodies from patients with systemic lupus erythematosus. Hybridoma 3: 215-222.

McConahey, P. H., and D. J. Dixon (1966). A method for trace iodination of proteins for immunologic studies. Int. Arch. Allergy. Appl. Immuno. 29: 185-189.

Meyer, O., J. Margulis, and M. F. Kahn (1982). Lupus erythemateux disséminé in Maladies dites systémiques by M. F. Kahn and A. P. Peltier. Flammarion medecine sciences, Paris. Chapter 8, pp. 202-295.

Miller, R. A., D. G. Maloney, R. Warnke, R. Levy (1982). Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. N. Engl. J. Med. 306: 517-522.

Morrow, W. J. W., P. Y. Ouinou, D. A. Isenberg, and M. L. Snaith (1983). Systemic lupus erythematosus: 25 years of treatment related to immunopathology. Lancet 2: 206-210.

Mushinsky, E. B., and M. Potter (1977). Idiotypes on galactan binding myeloma proteins and anti-galactan antibodies in mice.

J. Immuno. 119: 1888-1893.

Nakamura, R. M., and E. M. Tan (1978). Recent progress in the study of autoantibodies to nuclear antigens. Hum. Pathol. 9: 85-91.

Norwood, T. H., C. J. Zeigler, and G. M. Martin (1976). Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. Som. Cell Genet. 2: 263-270.

Norton, F. L., P. R. Morrow, C. Y. Leung, and E. Benjamine (1985). The idiotypic characterization of the immune response to a defined epitope of a protein antigen and the specific in vivo suppression of the immune response to this epitope by anti-idiotypic antibodies. J. Immuno. 134: 3226-3232.

Nossal, G. J., B. L. Pike, J. W. Stocker, J. E. Layton, and J. W. Goding (1977). Hapten-specific B lymphocytes: enrichment, cloning, receptor analysis, and tolerance induction. Cold Spring Harbor Symp. Quant. Biol. 41: 237-243.

Okada, Y., and F. Murayama (1965). Multinucleated giant cell formation by fusion between cells of two different strains. Exp. Cell. Res. 40: 154-158.

Olsson, L., and H. S. Kaplan (1980). Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. Proc. Natl. Acad. Sci. U.S.A. 77: 5429-5431.

Olsson, L., H. Kronstom, A. Cambon-DeMouzon, C. Honsik, T. Brodin, and B. Jakobeen (1983). Antibody producing human-human hybridomas. I. Technical aspects. J. Immuno. Meth. 61: 17-32.

Olsson, L., R. B. Andreasen, A. Ost, B. Christensen, and P. Biberfeld (1984). Antibody producing human-human hybridomas. II. Derivation and characterization of an antibody specific for human leukemia cells. J. Exp. Med. 159: 537-550.

Page, N., N. Murray, G. Perruisseau, and A. J. Steck (1985). A monoclonal anti-idiotypic antibody against a human monoclonal IgM with specificity for myelin-associated glycoprotein. J. Immuno. 134: 3094-3099.

Papalian, M., E. Lafer, R. Wong, and B. D. Stollar (1980). Reaction of systemic lupus erythematosus anti-native DNA antibodies with native DNA fragments from 20 to 1200 base pairs. J. Clin. Invest. 65: 469-477.

Paul, W. E., and C. Bona (1982). Regulatory idiotypes and immune networks: a hypothesis. Immuno. Today 3: 230-234.

Pettengill, O. S., and G. D. Sorenson (1967). Murine myeloma cells in suspension culture. Exp. Cell. Res. 47: 608-613.

Pesce, A. J., N. Mendoza, I. Boreisha, M. A. Gaizutis, and V. E. Pollak (1974). Use of enzyme-linked antibodies to measure serum anti-DNA antibody in systemic lupus erythematosus. Clin. Chem. 20: 353-359.

Pesce, A. J., K. S. Kant, B. S. Ooi, and V. E. Pollak (1978). The application of enzyme immunoassay in the diagnosis of autoimmune diseases. Scand. J. Immuno. 8(suppl.7): 91-96.

Pickering, J. W., and F. B. Gelder (1982). A human myeloma cell line that does not express immunoglobulin but yields a high frequency of antibody-secreting hybridomas. J. Immuno. 129: 406-412.

Pisetsky, D. S., and K. F. Semper (1984). Influence of assay conditions on ELISA determinations of anti-DNA antibodies. J. Immuno. Meth. 74: 217-227.

Pontecorvo, G. (1976). Production of indefinetely multiplying mammalian somatic cell hybrids by polyethylene glycol (PEG) treatment. Som. Cell Genet. 1: 397-400.

Poole, A. R., J. I. Howell, and J. A. Lucy (1970). Lysolecithin in cell fusion. Nature 227: 810-814.

Potter, M. (1972). Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol. Rev. 52: 631-719.

Rauch, J., E. Murphy, J. B. Roth, B. D. Stollar, and R. S. Schwartz (1982). A high frequency idiotypic marker of anti-DNA autoantibodies in MRL/lpr/lpr mice. J. Immuno. 129: 236-241.

Rauch, J., H. Tannenbaum, B. D. Stollar, and R. S. Schwartz (1984). Monoclonal anti-cardiolipin antibodies to DNA. Eur. J. Immunol. 14: 529-534.

Rauch, J., H. Massicotte, and H. Tannenbaum (1985) Hybridoma anti-DNA autoantibodies from patients with rheumatoid arthritis and systemic lupus erythematosus demonstrate similar nucleic acid binding characteristics. J. Immunol. 134: 180-186.

Rauch, J., H Tannenbaum, K. Straaton, H. Massicotte, and J. Wild. (1986). Human-human autoantibodies with both anti-DNA and rheumatoid factor activities. J. Clin. Invest. 77: 107-112.

Reif, A. E. (1969). Batch preparation of rabbit G globulin with DEAE cellulose. Immunochem. 6: 723-731.

Ringertz, N. R., and R. E. Savage (1976). Cell Hybrids. Academic Press, New-York.

Rocca-Serra, J., C. Tonnelle, and M. Fougereau (1983). Two monoclonal antibodies against different antigens using the same V_H germ-line gene. Nature 304: 353-355.

Rodkey, L. S. (1974). Production and characterization of auto-antiidiotypic antisera. J. Exp. Med. 139: 712-720.

Rudikoff, S., A. M. Giusti, W. D. Cook, and M. D. Scharff (1982). Single amino acid substitution altering antigen-binding specificity. Proc. Natl. Acad. Sci. U.S.A. 79: 1979-1983.

Rudikoff, S. (1983). Immunoglobulin structure function correlates: antigen binding and idiotypes. Cont. Top. in Molec. Immuno. 9: 169-209.

Sakane, T., A. D. Steinberg, and I. Green (1978). Failure of autologous mixed lymphocyte reactions between T and non-T cells in patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. U.S.A. 75: 3464-3468.

Satoh, J., B. S. Prabhakar, M. V. Haspel, F. Ginsberg-Fellner, and A. L. Notkins (1983). Human monoclonal autoantibodies that react with multiple endocrine organs. N. Engl. J. Med. 309: 217-220.

Schilling, J., B. Clevinger, J. M. Davie, and L. Hood (1980). Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangments in heavy chain V-region gene segments. Nature 283: 35-40.

Schneider, J., W. Chin, G. J. Friou, S. M. Cooper, B. Harding, R. L. Hill, and F. P. Quismorio (1975). Reduced antibody-dependent cell-mediated cytotoxicity in systemic lupus erythematosus. Clin. Exp. Immuno. 20: 187-192.

Schwaber, J., and E. P. Cohen (1974). Pattern of immunoglobulin synthesis and assembly on a human-mouse somatic cell hybrid clone. Proc. Natl. Acad. Sci. U.S.A. 71: 2203-2207.

Schwaber, J. F., M. R. Posner, S. F. Schlossman, and H. Lazarus (1984). Human-human hybrids secreting pneumoccoccal antibodies. Human Immuno. 9: 137-143.

Seligmann, M. (1957). Evidence in the serum of patients with systemic lupus erythematosus of a substance producing a precipitation reaction with DNA (orig. in French) C. R. Soc. Biol. Paris 245: 243-245.

Sharp G. C., W. S. Irvin, R. L. LaRoque, C. Velez, V. Daily, A. D. Kaiser, H. R. Holman (1971). Association of autoantibody to different nuclear antigens with clinical patterns of rheumatic disease and responsiveness to therapy. J. Clin. Invest. 50: 350-359

Shishido, K., and T. Ando (1972). Estimation of double-helical content in various single-stranded nucleic acids by treatment with a single strand-specific nuclease. Biochim. Biophys. Acta. 287: 477-484.

Shoenfeld, Y., S. C. Hsu-Lin, J. E. Gabriels, L. E. Silverstein, B. C. Furie, B. Furie, B. D. Stollar, and R. S. Schwartz (1982). Production of autoantibodies by human-human hybridomas. J. Clin. Invest. 70: 205-208.

Shoenfeld, Y., J. Rauch, H. Massicotte, S. K. Datta, J. Andre-Schwartz, B. D. Stollar, and R. S. Schwartz (1983). Poly-specificity of monoclonal lupus autoantibodies produced by human-human hybridomas. N. Engl. J. Med. 308: 414-420.

Shoenfeld, Y., D. A. Isenberg, J. Rauch, M. P. Madaio, B. D. Stollar, and R. S. Schwartz (1983b). Idiotypic cross-reaction of monoclonal human lupus autoantibodies. J. Exp. Med. 158: 718-730.

Sibley, J. T., J. S. Lee, and W. E. Decoteau (1984). Left-handed "z"DNA antibodies in rheumatoid arthritis and systemic lupus erythematosus. J. Rheum. 11: 633-640.

Sikora, K., and T. Alderson (1982). Human hybridomas from malignant gliomas. Lancet 322: 11-17.

Solomon, G., J. Schiffenbauer, H. D. Keiser, and B. Diamond (1983). Use of monoclonal antibody to identify shared idiotypes on human antibody to nDNA from patients wuth SLE. Proc. Natl. Acad. Sci. U.S.A. 80: 850-854.

Stanworth, D. R., and M. W. Turner (1973). Immunochemical analysis of immunoglobulins and their subunits in Hardbook for experimental immunology. D. M. Weir. ed. Blackwell scientific publications, Philadelphia. p. 10-13.

Steinman, C. R., V. Decsomchok, and H. Spiera (1978). Detection of anti-DNA antibody using synthetic antigens. Characterization and clinical significance of binding of poly (deoxyadenylate-deoxythymidilate) by serum. J. Clin. Invest. 57: 1330-1341.

Stocker, J. W., and G. V. H. Nossal (1976). Pathways to immunological tolerance: an approach through the study of maturing B lymphocytes. Contemp. Topics Immunobiol. 5: 191-210.

Stollar, B. D. (1975). Crit. Rev. Biochem. 3: 45-69.

Stollar, B. D., E. DeClercq, J. L. Drocourt, and M. N. Thang (1978). Immunochemical measurement of conformational heterogeneity of poly-inosinic acid. Eur. J. Biochem. 82: 339-346.

Stollar, B. D., and M. Papalian (1980). Secondary structure in denaturated DNA is responsible for its reaction with anti-native DNA antibodies of systemic lupus erythematosus. J. Clin. Invest. 66: 210-219.

Strike, L. E., B. H. Devens, and R. L. Lundak (1984). Production of human-human hybridomas secreting antibodies to sheep erythocytes after in vitro immunization. J. Immuno. 132: 1798-1803.

Suzan, M., C. Boyer, C. Schiff, J. Trucy, M. Milele, and C. de Preval (1982). Monoclonal antibodies reveal three types of idiotypic determinants on MOPC 173: H-L-specific private and public idiotypes, and V_H-specific public idiotype(s). Molec. Immuno. 19: 1051-1062.

Tachovsky, T. G., M. Sandber-Wollheim, and L. G. Baird (1982). Rabbit anti-human CSF IgG. Characterization of anti-idiotype antibodies procuced against MS CSF and detection of cross-reactive idiotypes in several MS CSF. J. Immuno. 129: 764-770.

Takahashi, N., T. Noma, and T. Honjo (1984). Rearranged immunoglobulin heavy region (V_H) pseudogene that deletes the second complementarity-determining region. Proc. Natl. Acad. Sci. U.S.A. 81: 5194-5198.

Talal N. (1975). Antibodies to nucleic acids in human and murine lupus. J. Rheum. 2: 130-134.

Talal, N. (1978). Autoimmunity and the immunology Network. Arthr. Rheum. 25: 853-861.

Talal, N. (1980). Tolerance and autoimmunity in Clinical Immunology vol. 1 by C. W. Parker, W. B. Saunders Company. Toronto pp. 86-114.

Tan, E. M., W. V. Epstein (1973). A solid phase immunoassay for antibody to DNA and RNA. J. Lab. Clin. Med. 81: 122-132.

Tan, E.M. (1976a). Sunlight as a potential aeteological factor in systemic lupus erythematosus in Modern topics in Rheumatology by G. R. Hughes, Chicago: Year Book Medical Publishers. pp. 99-106.

Tan, E.M. (1976b). Immunopathology and the pathogenesis of cutaneous involvement in systemic lupus erythematosus. J. Invest. Dermatol. 67: 360-365.

Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester (1982). The 1982 revised criteria for the classification of systemic lupus eryhthematosus. Arthr. Rheum. 25: 1271-1277.

Tan, E. M. (1982). Special antibody for the study of SLE. An analysis. Arthr. Rheum. 25: 753-756

Taylor, R. B. (1969). Cellular cooperation in the antibody response to two serum albumins. Specific function of thymus cells. Transpl. Rev. 1: 114-149

Teitelbaum, D., J. Rauch, B. D. Stollar, R. S. Schwartz (1984). Effects of antibodies against a high frequency idiotype of anti-DNA antibodies in MRL mice. J. Immuno. 132: 1282-1285.

Thorbeeke, G. J., B. S. Bhogal, and G. W. Siskind (1984). Possible mechanism for down-regulation of autoantibody production by auto-anti-idiotype. Immuno. Today 3: 92-93.

Tom, B. H. (1983). Somatic cell hybrids and hybridomas in Hybridomas and cellular immortality by B. H. Tom, and J. P. Allison. Plenum. Press, New York. pp. 3-11.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature 302: 575-581.

Tron, F., C. Guern, R. A. Cazenave, and J. F. Bach (1982). Intrastrain recurrent idiotypes among anti-DNA antibodies $(NZB \times NZW)F_1$ hybrid mice. Eur. J. Immuno. 12: 761-766.

Tron, F., L. Jacob, and J. F. Bach (1983). Murine monoclonal anti-DNA antibodies with an absolute specificity for DNA have a large amount of idiotypic diversity. Proc. Natl. Acad. Sci. U.S.A. 80: 6024-6027.

Tucker, E. M., A. R. Dain, S. W. Clarke and R. A. Donker (1984). Specific bovine monoclonal antibody produced by a re-fused mouse/calf hybridoma. Hybridoma 3: 171-176

Urbain, J., and C. Wuilmart (1982). Some thoughts on idiotypic networks and immunoregulation. Immuno. Today 3: 88-92.

Vaitukaitis, J., J. B. Robbins, E. Nieschlag, and G. T. Ross (1971). A method for producing specific antisera with small doses of immunogen. J. Clin. Endocrino. 33: 988-991.

Valentine, R. D. and N. M. Green (1967). Electron microscopy of an antibody-hapten complex. J. Mol. Biol. 27: 615-617

Wang, A. H. J., G. J. Quigley, F. J. Kolpak, J. L. Crawford, J. H. Van Boom, G. Van Der Marel, and A. Rich (1979). Molecular structure of a left-handed double helical DNA fragment at atomic resolution. Nature 282: 680-686.

Watson, J. D., F. H. C. Crick (1953). Molecular structure of nucleic acids. A structure for deoxyribose nucleic acid. Nature 171: 737-738.

Werner, T. C., J. R. Bunting and R. E. Cathou (1972). The shape of immunoglobulin G molecules in solution. Proc. Natl. Acad. Sci. U.S.A. 69: 795-799

Williams, R. Jr, H. G. Kunkel, and J. D. Capra (1968). Antigenic specificities related to the cold agglutinin activity of gamma M globulins. Science 161: 379-381.

Wu, T. T., and E. A. Kabat (1982). Fourteen nucleotides in the second complementarity-determining region of a human heavy-chain variable region gene are identical with a sequence in a human D minigen. Proc. Natl. Acad. Sci. U.S.A. 79: 5031-5039.

Zanetti, M. (1983). Anti-idiotypic antibodies and auto-antibodies. Ann. N. Y. Acad. Sci. 418: 363-378.

Zola, H. and D. Brooks (1982). Technics for the production and characterization of monoclonal hybridoma antibodies in Monoclonal hybridoma antibodies: technics and applications by J. G. H. Hurrel, CRC Press Inc. U.S.A. p. 1-57

Zouali, M., and A. Eyquen (1984). Idiotype restriction in human autoantibody to DNA in SLE. Immuno. Letters 7: 187-190.