CHARACTERIZATION OF THE REACTIVITIES OF SLE AND NORMAL-DERIVED HUMAN HYBRIDOMA LUPUS ANTICOAGULANT, ANTI-PHOSPHOLIPID AND ANTI-dDNA AUTOANTIBODIES WITH PLATELETS AND ENDOTHELIAL CELLS

by

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By **Qiang-hua Meng**

Short Title:

CHARACTERIZATION OF HUMAN HYBRIDOMA LUPUS AND NORMAL AUTOANTIBODIES

ABSTRACT

The mechanisms by which lupus anticoagulant and anti-phospholipid autoantibodies cause hemostatic abnormalities in patients with systemic lupus erythematosus (SLE) are poorly understood. We have approached this problem by investigating the binding and functional effects of human hybridoma lupus anticoagulant, anti-phospholipid and anti-DNA autoantibodies derived from SLE patients on platelets and endothelial cells. Most lupus anticoagulant antibodies did not bind to intact platelets and endothelial cells *in vitro*, while many anti-phospholipid and anti-DNA antibodies were reactive. A comparison of SLE and normal-derived autoantibodies demonstrated that platelet-binding autoantibodies derived from SLE patients exhibited greater antigen specificity and platelet cytotoxicity than similar antibodies derived from normal individuals. By Western blotting analysis, many SLE-derived polyspecific antibodies reacted specifically with individual platelet proteins, whereas normal-derived polyspecific antibodies did not. One SLE-derived antibody, 9604 was found to react with ADP-activated platelets but not resting platelets. The reactive components in platelets were reducible polypeptides of approximately 200,000 and 32,000 molecular weight. These data suggest that some SLE autoantibodies may be able to interact with platelets and result in cell lysis or dysfunction *in vivo*.

RÉSUMÉ

Les méchanismes par lesquels les auto-anticorps anticoagulants lupiques et anti-phospholipides causent des abnormalités hémostatiques dans les patients, avec le lupus érythémateux desséminé (LED), sont mal compris. Ce problème a été approché par l'investigation des effets de réaction et fonction des anticoagulants lupiques, anti-phospholipides et anti-ADN des hybridomes humains dérivés de patients avec LED, avec les plaquettes et cellules endothéliales. La majorité des anticorps anticoagulants lupiques n'ont pas réagit, in vitro, avec les plaquettes intactes ni avec les cellules endotheliales, pourtant, plusieurs des anticorps anti-phospholipides et anti-ADN etaient réactifs. Une comparaison d'auto-anticorps dérivés d'individus normaux et LED, a démontré que les auto-anticorps, LED-dérivés, qui reagissent avec les plaquettes, présentent une spécificite aux antigenes et une cytotoxicité plus élevée que celles des anticorps semblable d'individus normaux. Par l'analyse "Western blot", plusieurs auto-anticorps polyspécifiques, LED-dérivés, réagissent spécifiquement avec des protéines individuelles de plaquettes, tandis que les anticorps polyspécifiques, dérivés d'individus normaux, ne réagissent pas. L'anticorps, 9604, LED-derivé, a été trouvé réagissant avec des plaquettes activées avec de l'adénosinebiphosphate et non avec des plaquettes reposantes. Les composantes réactives des plaquettes sont des polypeptides réductibles de poids moléculaire d'environ 200,000 et 32,000. Les résultats suggèrent que quelques auto-anticorps, LED-dérivés, peuvent réagir avec les plaquettes, le resultat étant la lyse ou mal fonctionnement de cellules in vivo.

For my parents, and my friend, André Tsilividis

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PREFACE

In accordance with the regulations described in the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, papers which have already been published or which have been submitted for publication have been incorporated into this thesis. The Division of Experimental Medicine has approved this format for thesis submission. The following is quoted directly from the Guidelines Concerning Thesis Preparation:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what

extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

Manuscripts (**Chapters III, IV. and V**) which have already been published, accepted or submitted for publication; an individual chapter (**Chapter VI**) describing unpublished results, which is of direct interest to the subject of this thesis, but does not contain sufficient data to warrant publication at the present time; and appendices (**Chapter X**) including data of preliminary experiments and methods which have not been described in the manuscripts, have been incorporated into this thesis. The Abstract, General Introduction (**Chapter I**), Rationale, Objectives and Significance of the Present Investigation (**Chapter II**), General Discussion and Future Perspectives (**Chapter VII**) and Statement of Originality (**Chapter VIII**) are related to the research described in Chapters III, IV, V and VI.

In the thesis, each manuscript (**Chapters III, IV and V**) has its own references at the end of the chapter. The references for other chapters of the thesis are compiled in **Chapter IX** of the thesis.

The following is a list of the published, accepted or submitted manuscripts which are included in this thesis:

Chapter III. Rauch J, Meng Q-h and Tannenbaum H: Lupus anticoagulant and anti-platelet properties of human hybridoma

autoantibodies. Journal of Immunology 139:2598-604, 1987.

Chapter IV. Meng Q-h and Rauch J: Differences between human hybridoma platelet-binding autoantibodies derived from SLE patients and normal individuals. Autoimmunity 1989. (In press).

Chapter V. Meng Q-h and Rauch J: An SLE-derived human hybridoma autoantibodies reactive with antigens expressed on ADP-activated platelets. Blood 1989. (Accepted, pending revisions).

In the study described in **Chapter III**, which I co-authored with my supervisor, Dr. Joyce Rauch, and my co-supervisor, Dr. Hyman Tannenbaum, I performed all of the experiments except for the enzyme digestion experiments, which were done by Joanne Wild, a technician in Dr. Rauch's laboratory.

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ABBREVIATIONS

AA, arachidonic acid; **ADP**, adenosine diphosphate; **APTT**, activated partial thromboplastin time; **BSA**, bovine serum albumin; **BGG**, bovine gamma globulin; **C region (gene)**, constant region (gene); **CL**, cardiolipin; **^{51}Cr**, chromium-51; **DNA**, deoxyribonucleic acid; **dDNA (ssDNA)**, denatured DNA (single-stranded DNA); **nDNA (dsDNA)**, native DNA (double-stranded DNA); **DNAase**, deoxyribonuclease; **EC**, endothelial cell; **ELISA**, enzyme-linked immunosorbent assay; **GP**, glycoprotein; **H chain**, heavy chain; **HT**, hypoxanthine-thymidine; **IEF**, isoelectric focusing; **Ig**, immunoglobulin; **LA**, lupus anticoagulant; **L chain**, light chain; **M.W.**, molecular weight; **PA**, phosphatidic acid; **PBS**, phosphate-buffered saline; **PC**, phosphatidylcholine; **PE**, phosphatidylethanolamine; **PG**, phosphatidylglycerol; **PGI_2**, prostacyclin; **pI**, isoelectric point; **PI**, phosphatidylinositol; **PL**, phospholipid; **PLipase**, phospholipase; **PS**, phosphatidylserine; **RIA**, radioimmunoassay; **RNAase**, ribonuclease; **SD**, standard deviation; **SE**, standard error; **SLE**, systemic lupus erythematosus; **TBS**, Tris-buffered saline; **TXA_2**, thromboxane A_2; **VLA**, very late antigens; **VDRL**, Venereal Disease Research Laboratory; **V region (gene)**, variable region (gene); **vWF**, von Willebrand Factor.

CHAPTER I

GENERAL INTRODUCTION

A. AUTOANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

1. Multifactorial Etiology and Pathogenesis of SLE

Systemic lupus erythematosus (SLE) is a disease characterized by damage to multiple organs and tissues. Historically, Osler attributed the systemic form of the disease to vasculitis (1). Clinically, SLE is marked by the variability of its symptoms, which may include skin and mucocutaneous lesions, chronic inflammation of connective tissue, arthritis, dermatitis, vasculitis, hematologic abnormalities, myositis, nephritis, peripheral neuropathy and central nervous system disease, myocardial disease, and verrucous endocarditis (2). The laboratory discovery of the lupus erythematosus (LE) cell phenomenon by Hargraves et al. (3) in 1948, which showed that the nuclei of normal leukocytes were stained by SLE sera, and the demonstration that antinuclear antibodies in SLE sera were responsible for this reactivity (4) revealed that SLE is an autoimmune disease.

Immunologically, SLE is characterized by the presence of multiple circulating autoantibodies, in particular, antinuclear and anti-deoxyribonucleic acid (DNA) antibodies (reviewed in Section A) of this chapter), although not all patients develop high levels of these autoantibodies. The overproduction of antibodies, which is thought to be at least partially responsible for the pathogenic processes in SLE, is a result of increased numbers of activated B cells. Marked B cell hyperactivity (5), increased B cell

proliferation (6) and increased numbers of plaque-forming (antibody-secreting) cells in peripheral blood lymphocytes have been demonstrated in SLE patients (7). A strong positive correlation between the increase in numbers of immunoglobulin (Ig)-secreting cells and disease activity has also been shown (8,9).

Deposits of antigen-antibody complexes in various tissues, with subsequent complement fixation, is a primary pathogenetic mechanism causing tissue injury in SLE, and high levels of antibodies together with low C3 levels are highly predictive of the presence of active SLE disease (10-13). The damage may be caused either by immune complexes which are formed exogenously and trapped in various tissues, so called "bystander lysis", or by immune complexes formed in situ by the binding of circulating antibodies to tissue antigens. The outstanding example of the pathogenesis of DNA-containing immune complexes in SLE is lupus nephritis, in which high concentrations of high affinity anti-DNA antibodies can be eluted from kidney specimens (12-14) and tissue bound DNA and complement proteins can be detected in renal tissue by immunofluorescence techniques (15).

Cellular immunity is also abnormal in SLE patients. Although initial studies of T cell function showed that there was no difference in delayed-type hypersensitivity reaction between SLE patients and normal individuals (16), many subsequent studies have reported not only depressed delayed-type hypersensitivity reactions, but also lymphopenia and anti-T cell antibodies in patients with active disease (17-20). A reduced proliferative response to T cell mitogens was found in active SLE by a majority of investigators (21-

23). There also seemed to be a loss of T suppressor cell activity in active SLE (24-26), as well as reduced natural killer (NK) function (27) and depressed xenogeneic cell-mediated lympholysis (28).

Epidemiologically, the higher incidence of SLE in certain races and in women, the family tendency of the disease, and the relationship of disease onset to seasons (exposure to sunlight), geographical locations and infections indicate that both genetic and environmental factors are involved (2,29). It appears likely that various combinations of these factors result in the polymorphic behaviour of SLE.

2. Autoantibodies in the Sera of SLE Patients and Polyspecificity of Lupus Autoantibodies

The presence of multiple serum autoantibodies is one of the main immunological features of SLE patients (30). These lupus autoantibodies are summarized in Table 1 and include non-tissue specific antibodies reactive with cellular components of all tissues, such as antinuclear antibodies and anti-DNA antibodies, and tissue specific antibodies directed against cellular elements of certain tissues, such as anti-platelet antibodies. The presence and titers of these autoantibodies vary in different patients. Furthermore, it is difficult to determine whether the autoantibodies cause or are the result of the disease and which antibodies are involved in tissue damage.

Table 1. Autoantibodies in the Serum of SLE Patients

Autoantibodies Reported in SLE	References
Nuclear Antigens (90%)*	
Double (80%) or single-stranded DNA(90%)	10,31-34
Deoxyribonucleoproteins	35,36
Histones	37-39
Nonhistone (acidic) proteins	
Ribonucleoproteins (RNP) (30-50%)	40-44
Sm antigen (30-40%)	40,43,45
SS-A (Ro) (20-30%)	45,46
SS-B antigen (La) (12%)	43,46,47
Cytoplasmic Antigens	
RNA	48
Ribosomes	49
SS-A (Ro) (20-30%)	45,46
SS-B antigen (La) (12%)	43,46,47
Microsomes and mitochondria	50,51
Cytoskeleton	52
Cellular Antigens	
Erythrocytes (38/150)	33
Lymphocyte cell antigens (80%)	17-19
T lymphocytes	53
Neurons (75%)	54
Platelets (78%)	55-59
Endothelial Cells (45-74%)	60-62
Soluble Components	
Factors VIII, IX and XI	63,64
Phospholipids (Lupus anticoagulants) (20-30%)	65-67
IgG (rheumatoid factors) (40%)	68
Poly(ADP-ribose)	69

* The percentage (%) in parentheses indicates the % of SLE patients found to have this reactivity in the cited studies.

Until recently, the diverse serological phenomena which distinguish SLE from most other autoimmune diseases have not been well understood. However, the development of hybridoma technology has permitted cloning and precise characterization of individual autoantibodies. The initial finding that a single monoclonal antibody to DNA was reactive with multiple synthetic polynucleotides and phospholipids (PLs) (70) indicated that the heterogeneity of the serological reactions in sera from SLE patients may result not only from a diversity of autoimmune antibodies, but also from multiple reactivities of individual antibodies. In other words, the polyreactive serology of lupus sera may be due, at least in part, to the polyreactivity of individual autoantibodies. For example, the reactivity of certain hybridoma anti-DNA antibodies with PLs has been proposed to be due to binding to the phosphodiester backbone structure common to both DNA and PLs (70).

Polyspecificity to several different autoantigens was, subsequently, recognized to be a common characteristic of both mouse and human monoclonal anti-DNA autoantibodies (71-73), which have been reported to react with Ig (73), cytoskeletal proteins (74,75), platelets (76,78), Sm antigen (79) and polypeptides on cell membranes (80). Furthermore, the majority of DNA-binding monoclonal autoantibodies and purified serum anti-DNA antibodies show a wide range of cross-reactivity with synthetic polynucleotides, such as poly (dT) and poly(I), but each monoclonal autoantibody tends to have a unique binding profile (71,73,81-83).

It remains to be proven definitively, however, that

autoantibodies in SLE sera have similar properties to monoclonal hybridoma antibodies. Furthermore, questions regarding the origin of polyspecific and monospecific autoantibodies to DNA and their involvement in pathogenesis remain unanswered. There is still controversy concerning the involvement of antigen induction and polyclonal activation in the production of autoantibodies in SLE. These issues may be resolved with the cloning of genes which encode polyspecific and monospecific autoantibodies.

3. Antinuclear and Anti-DNA Autoantibodies

Considerable progress has been made in the characterization of lupus autoantibodies since the discovery of anti-DNA (31) and antinuclear (84) antibodies in the serum of lupus patients using fluorochrome-labelled antibodies in 1957. Antinuclear antibodies (ANA), which are seen in more than 90% of SLE patients, are comprised of antibodies reactive to four main groups of antigens: 1) DNA; 2) deoxyribonucleoproteins; 3) histones and 4) nonhistone (acidic) nuclear proteins (10,31-47). Of these antibodies, anti-double stranded DNA (dsDNA) antibodies are considered the most characteristic and the most specific for SLE, as approximately 85% of SLE patients are seropositive for these autoantibodies at some point in their disease (85).

The following specificities have been observed for anti-DNA antibodies: 1) reactivity to denatured or single-stranded DNA (dDNA, ssDNA) only; 2) reactivity to both dDNA and native double-stranded DNA (nDNA, dsDNA); and 3) reactivity to nDNA only. Theoretically, the heterogeneity of anti-DNA antibodies may be attributed, at least

in part, to the structural complexity of DNA molecules, which are composed of deoxyribose sugars, phosphate, and purine and pyrimidine bases and have a helical structure. Although the immunogenicity of nucleic acids has long been in question, it is well established in experimental animals that the B or native double-stranded form of nucleic acid helices, characterized by bases perpendicular to and aligned with the helix axis and a relatively narrow helical structure, is not immunogenic. On the other hand, the A form of nucleic acid helices, or Z form of poly (d(G-C)), characterized by bases tilted and offset from the helix axis and a more open helical structure, is immunogenic (reviewed in 86,87).

Cross-reactivity of some monoclonal anti-DNA antibodies with PLs suggests that phosphodiester linked groups in the backbone of DNA may be antigenic (70). Interestingly, it was indeed found that DNA-binding antibodies could be induced by immunization of normal mice with cardiolipin (CL) and that these antibodies bore a strong resemblance to CL-reactive monoclonal anti-DNA antibodies derived from autoimmune MRL/lpr mice, suggesting that PLs may be capable of inducing the production of some anti-DNA antibodies (88). In addition to the deoxyribose-phosphate backbone, base conformation might also play a role in antibody specificity (87,88). From these observations, it is apparent that the terms "anti-dsDNA antibody" and "anti-ssDNA antibody" may be not as immunologically restrictive as they seem.

The pathogenesis of anti-DNA antibodies in SLE patients has been investigated primarily in studies of the isotypes, isoelectric points (pI) and binding specificities of these antibodies. Anti-DNA

antibodies have been suggested to be responsible for SLE lupus nephritis (89). Although human lupus nephritis tends to be more severe when patients have mainly anti-DNA IgG rather than anti-DNA IgM in their serum (14,90), the relative preponderance of IgM or IgG anti-DNA antibodies does not necessarily correlate with the severity of other clinical features of lupus (91). Some investigators have found that only certain subpopulations of anti-DNA antibodies are involved in the pathogenesis of renal disease in human and mouse SLE. The pI of monoclonal anti-DNA antibodies derived from autoimmune mice and patients with SLE range from 5.5 to 9.0, but only those antibodies with pI between 8.0 and 9.0 have been eluted from kidney deposits, suggesting that anti-DNA antibodies which are positively charged may be more pathogenic to the kidney (92-94). Anti-DNA antibodies may cause damage by binding directly to tissue or cell membrane constituents, such as heparan sulfate in the glomerular basement membrane (95,96) and fibronectin, a protein in the extracellular matrix (97). Furthermore, the ability of anti-DNA antibodies to fix complement (14) and the avidity of anti-DNA antibodies may also be relevant to their pathogenicity (98).

4. Anti-Platelet and Anti-Endothelial Cell Antibodies

Anti-platelet antibodies occur frequently in patients with SLE. Karpatkin et al. (55) reported that anti-platelet antibodies could be detected in 78% of patients with SLE, although only 14% of these patients were thrombocytopenic. Efforts have been made to characterize the relationship between anti-platelet antibodies and platelet function and to identify epitopes on platelets responsible

for autoantibody binding. Kaplan et al. (58) studied the reactivities of sera from SLE patients with platelet membrane proteins derived from normal and autologous platelets using an immunoblotting technique. Two target antigens of 108,000 and 66,000 M.W. were found for some SLE sera. These antigenic determinants were not removed by proteolytic treatment of intact platelets or by reduction and were localized in the intra-cytoplasmic membranes. Another group of investigators detected elevated levels of platelet-binding Ig in serum of all 10 SLE patients with thrombocytopenia studied by ELISA and these sera consistently bound to platelet proteins of approximately 120,000 and 80,000 molecular weight (M.W.) by Western blotting analysis (59). The binding of immune complexes to platelets or an increase in IgG bound to platelets via Fc receptors was noted in some autoimmune disorders including SLE (99,100). It was also reported that all 4 SLE sera studied caused aggregation and immunofluorescent staining of normal platelets and that the active fractions in these sera contained antibodies to C1q, C1s and β_2-microglobulin (57). Weissbarth et al. (56) demonstrated that anti-platelet antibodies from patients with SLE and rheumatoid arthritis induced serotonin release from platelets. In addition, the binding of IgG-containing immune complexes to platelet Fc receptors was associated with platelet phagocytosis, which induced release of granule contents and aggregation of circulating platelets, suggesting that the formation of platelet aggregates or the destruction of platelets may be caused by cooperative effects in these patients. However, the role of platelet-binding autoantibodies in thrombosis, thrombocytopenia and

abnormal platelet function have not been as clearly defined in SLE patients as in drug-induced (101-105), homosexual-related (106,107), and idiopathic (108-117) immune thrombocytopenic purpura.

There has been very little data on the reactivity of human hybridoma anti-platelet antibodies from patients with either SLE or immune thrombocytopenic purpura (76,118,119). Asano et al. (76) studied the platelet-binding properties of monoclonal hybridoma anti-DNA autoantibodies derived from SLE patients. They found that SLE-derived hybridoma autoantibodies to ssDNA crossreacted with platelets and that the epitopes did not appear to involve DNA, protein or sialic acid. Functionally, these platelet-reactive antibodies did not inhibit platelet aggregation induced by adenosine diphosphate (ADP), thrombin or ristocetin. Although some defined antigenic epitopes on the surface of platelets have been shown to serve as autoantigens, the molecular mechanisms which are involved in causing lowered platelet counts and abnormal platelet function in SLE remain unknown.

Relatively few studies have investigated the presence of anti-endothelial cell (EC) antibodies in SLE patients. This is probably due to the methodological difficulties inherent in measuring these antibodies and the fact that injury to ECs usually results in the pathogenic involvement of other cells or tissues (60-62,120,121). Anti-EC antibodies with the ability to fix complement have been reported in the sera of SLE patients (60), but recently it was found that purified anti-EC IgG from SLE sera did not induce complement-mediated cytotoxicity (61). In this study, the IgG anti-EC antibodies were not associated with either anti-CL or anti-DNA, but were

absorbed by human dermal fibroblasts and partially by erythrocytes and leukocytes. Another recent study of 51 SLE sera demonstrated that the presence of IgG and IgM anti-EC antibodies did not correlate with anti-dsDNA, anti-CL, anti-PS or lupus anticoagulant (LA) antibodies in SLE sera, although absorption with CL liposomes partially inhibited EC binding and affinity-purified anti-CL antibodies were able to react with intact ECs, suggesting a partial overlap of anti-EC and anti-CL antibodies (62). Immune injury to ECs may be an important mechanism in thrombotic complications in SLE patients, since ECs are in direct contact with circulating platelets, immune cells, antibodies, immune complexes and complement (see Section B1b of this chapter). Although it is clear that there are antibodies reactive with cultured ECs in the sera of SLE patients (60-62) and it has been shown that EC injury promotes active binding of immune complexes to these cells (122), it is uncertain whether the intact endothelium is the target of autoantibodies. Furthermore, it remains unresolved whether anti-PL antibodies form an important pathogenic subset of anti-EC antibodies.

5. Anti-Phospholipid and Lupus Anticoagulant Antibodies

a. Structure, Function and Immunogenicity of Phospholipids

Phospholipids, the main constituents of cell membranes, are extremely important in maintaining normal cell function and communication between the internal and external environments of cells. They are also necessary components of the coagulation process both in vitro and in vivo. PLs in the platelet membrane serve as storage depots for arachidonic acid (AA), the essential fatty acid

required for thromboxane (TXA_2) and hydroxy fatty acid synthesis. Phosphatidylserine (PS) and mixtures of phosphatidylinositol (PI) and PS are almost as active as the platelet surface itself in the promotion of coagulant activity (123). PLs represent 79% of lipids in whole platelets, which are mainly composed of phosphatidylcholine (PC) (38%), phosphatidylethanolamine (PE) (27%), sphingomyelin (17%) and PS (10%) (124). Most PLs in normal biological membranes exist in the bilayer phase with asymmetric transbilayer distributions. In the platelet, PS and PE, which are both important in initiating the coagulation process, are mainly localized to the interior membranes (125,126).

Isolated PLs mainly exist in three different structural configurations: lamellar (bilayer), hexagonal (H_{II}) (non-bilayer), and micellar phases (reviewed in 127). Bilayer PLs are nonimmunogenic, as repeated immunizations of rabbits or mice with liposomes produced neither a humoral nor cell-mediated immune response (128). However, the addition of a protein carrier (129,130) or a cholesterol/lecithin matrix (131-134) will render bilayer lipids immunogenic. Similar findings were noted by Rauch et al. (88) in the production of mouse monoclonal antibodies to CL which were crossreactive with DNA.

The factors responsible for inducing anti-PL antibodies in SLE patients are unknown, but there are several possibilities which have been suggested to date. When subjected to certain perturbations, natural membranes reveal non-bilayer structures (135,136), which may present foreign surface topologies compared to bilayer phase

phospholipid and might be expected to be antigenic. CL, PE and phosphatidic acid (PA) are all capable of forming hexagonal structures and non-bilayer intermediates under physiological conditions (127). The most striking example of non-bilayer PLs in animal cell membranes is unsaturated PE, which adopts the hexagonal structure in its pure isolated form, but when mixed with bilayer-forming lipids, contributes to the formation of highly structured dispersions containing lipidic particles, ridges, and isolated tubes (127). These lipids, when stabilized into the bilayer, may permit the cell to regulate intrinsic curvature (137) and permeability (138), and, thus, play a normal regulatory role in biological systems. A recent study, which supports the possibility that some non-bilayer PL structures are immunogenic, demonstrated that hexagonal phase PLs, including natural and synthetic forms of PE, were able to neutralize the LA activity of all 11 human hybridoma autoantibodies studied (139,140). In contrast, lamellar PLs, such as PC and synthetic lamellar forms of PE, had no effect on the LA activity, suggesting that these hybridoma LA antibodies are able to distinguish between different structural arrangements of PE and that the structural presentation of the PL antigen may be important in rendering it immunogenic to the host.

PL composition has also been shown to be important for reactivity of antibodies induced by liposomes. For instance, the antibodies induced by liposomes mixed with lipid A often appeared to be directed against the whole liposome, rather than against a single liposome constituent (128,141).

b. Lupus Anticoagulant and Anti-Phospholipid Antibodies in SLE

Anti-PL antibodies in SLE patients and related autoimmune disorders are a group of incompletely characterized autoantibodies (142). One of the most commonly described anti-PL antibodies in SLE is the anti-CL antibody, which is responsible for a biologically false positive result in the Venereal Disease Research Laboratory (VDRL) test for syphilis which uses CL as antigen (67,143-145). During the 1950's, several reports described a coagulation inhibitor, or "lupus anticoagulant", found in association with the biologically false positive VDRL and partially inhibitable by CL (66,146,147). Recently, many other investigators have found that anti-CL antibodies are highly correlated with the presence of LAs and with clinical events previously linked with the LA, including cardiovascular events (148), fetal distress and death (149-154), and thrombosis and thrombocytopenia (155-167). In addition, crossreactivities of anti-CL antibodies with PS and PE (166) have been described in SLE patients.

Lupus anticoagulant antibodies were first recognized in SLE patients by Conley et al. (66) in 1952. As indicated by Triplett et al. (165,168), LA represents a group of heterogeneous antibodies which may possess anti-PL activities and inhibit the assembly of the prothrombinase complex on phospholipids in vitro, but which are not specific inhibitors against any individual clotting factors. LA are defined by their ability to prolong the normal clotting time in in vitro coagulation assays, in particular, in those measuring activated partial thromboplastin times (APTT). Although the reagents and assays differ, most assays use brain cephalin, a lipid extract rich in PE

and PS.

Relatively few studies have examined the PL specificities of LA, and those which have are not in complete agreement (139,169,170). Exner et al. (169) found that LAs from SLE plasmas were most reactive with PE, PS, and PI, while Thiagarajan et al. (170) demonstrated that IgM paraproteins with LA activity reacted well with PS, PI, and PA, but not with PC and PE. Harris et al. (67,162,163) showed a strong correlation between LA activity and anti-CL binding in the sera of patients with SLE and found that serum antibodies which had been affinity-purified on CL liposomes had LA activity and were able to bind to CL, PA, PI, PS, phosphatidylglycerol (PG) and only weakly to PE. Until recently, most studies have been performed with antibodies derived from plasma or serum, and so, it is still not clear whether the LA and anti-PL antibodies are the same or different antibodies and which one is responsible for abnormal hemostasis in SLE patients.

In our laboratory, it has previously been shown that some hybridoma LA antibodies derived from patients with SLE can react with dDNA, CL, human IgG and a hexagonal form of PE (71,139) and that human hybridoma LA activity was specifically inhibited by hexagonal but not lamellar PE (139,140). The epitope responsible for these multiple cross-reactivities is unclear but more than one epitope may be involved (71-73).

B. ABNORMALITIES OF HEMOSTASIS IN SLE PATIENTS

Hemostatic disorders in SLE, whether they are manifested as a hemorrhagic tendency or hypercoagulable state, usually result from disturbances in one or more of three major mechanisms: 1) platelet function; 2) vascular/endothelial structure and integrity; and 3) the coagulation factors.

1. Cellular Components of the Hemostatic System

a. Platelets and Platelet Antigens

Platelets are extremely active, anucleate cells which normally circulate in the blood in a resting (inactive) form. Platelets undergo major functional changes which can be observed biochemically and morphologically in response to various stimuli, such as surface modification of the endothelium or exposure of the subendothelium *in vivo*, and treatment with *in vitro* activators, including ADP, collagen, epinephrine or thrombin. In response to these stimuli, the cells assume a spheroidal shape, extend pseudopodia, secrete the contents of internal granules, and participate in the formation of a hemostatic or thrombotic plug (reviewed in 171,172).

The antigenic changes on the platelet membrane that accompany platelet activation and which may be related to the biological activities of platelets have been identified mainly by a lactoperoxidase-catalyzed surface radiolabeling technique. These changes may result from the binding of exogenous proteins to the platelet surface, surface exposure of endogenous platelet proteins, or rearrangement of platelet membrane proteins and/or phospholipids. For instance, thrombospondin (glycoprotein G) (173,174), Factor V

(175), protein S (176), Factor VIII-related antigen (von Willebrand factor, vWF) (177), actin and an α-granule protein with 149,000 M.W.(178) are secreted from activated platelets and become associated with the membrane. Plasminogen (179), Factor XIa (180), Factor Xa (181), Factor XIIIa (182) and fibrinogen (173,183,184) specifically bind to activated platelets and many of these reactions may involve receptors newly exposed on the surface of activated platelets (174, 180). Fibrinogen receptors, discovered in platelets in 1979 (183,184), are composed of glycoproteins (GP) IIb and III and expressed only on activated platelets (185). Fibrinogen-induced aggregation of ADP-stimulated platelets was specifically inhibited by an antibody against a 66,000 M.W. protein, which is located on GPIII and becomes exposed upon stimulation of intact platelets (185).

It is also known that platelet activation is accompanied by a rearrangement of membrane PLs (125,186). Normally, platelet PS and most of the platelet PE are located on the cytoplasmic (interior) surface of the membrane and are not accessible to 2,4,6-trinitrobenzenesulfonate (TNBS) labeling (125,126). After platelets were activated with thrombin, increased PE but not PS labeling was detected in intact platelets, suggesting that exposure of PE on the activated platelet surface may have a critical role in platelet hemostatic function (120).

Monoclonal antibodies recognizing antigens exposed on activated platelets, but not on resting platelets, have been produced from mice immunized with thrombin activated platelets. For example, a murine monoclonal antibody, KC4, bound to an antigen expressed on the

platelet surface after activation with ADP, collagen, epinephrine or thrombin. By Western blotting analysis, this antibody reacted with a single protein of 140,000 M.W. (under both reducing and nonreducing conditions), suggesting that platelet secretion may be associated with the expression of an 140,000 M.W. integral membrane protein composed of single peptide chain. This protein may be a component of the internal granule membrane which is fused with the plasma membrane during activation (187). Another mouse monoclonal antibody, 2.28, was reported to react with a secreted lysosome-like granule protein of 53,000 M.W. on the surface of activated platelets (188). A similar phenomenon was described for human monoclonal antibody, 5E5, derived from Epstein-Barr virus transformed B lymphocytes of a patient with immune-mediated thrombocytopenic purpura (118), which recognized a neoantigen on GPIIIa expressed on platelets activated by thrombin or stored for more than 3 days. The epitope responsible for antibody 5E5 binding is a protein with an apparent M.W. of 95,000.

Platelet protein composition is tremendously complex. There are two sets of proteins important for platelet functions: membrane proteins and cytoskeletal proteins (reviewed in 189,190). Most platelet membrane proteins are integral membrane glycoproteins (totally or partially embedded in the lipid matrix), many of which serve as platelet receptors and have been well characterized (these will be discussed in detail in Section B1c of this chapter). The platelet cytoskeleton is capable of explosive reorganization during platelet activation and is composed of a group of dynamic proteins

present in the cytoplasm or associated with the plasma membrane (Table 2). The most dramatic changes occurring during platelet activation are the rapid phosphorylation and polymerization of monomeric actin (G-actin) to filamentous (F-actin) and the subsequent association of actin with other phosphorylated cytoskeletal proteins (191-196).

Table 2. Actin-Associated Proteins of the Cytoskeleton of Activated Platelets*

Proteins	M.W.	Function
Actin	45,000	Forms microfilaments; major structural protein of cytoskeleton; major role in contractility and mobility
Actin-binding protein	250,000	Crosslinks actin filaments; accelerates actin nucleation; phosphorylated
Myosin	500,000	Interacts with actin causing contraction; phorphorylated
Alpha-actin	105,000	Form gels with F-actin; promotes polymerization of actin
Tropomyosin	28,000	Binds F-actin; regulates contraction; binds filament bundles

* After Tuszynski GP, Daniel JL and Stewart G: Association of proteins with the platelet cytoskeleton. Semin Hemat 22:303-12, 1985.

b. Endothelial Cells and Immune Injury to the Endothelium

Endothelial cells are widely distributed in the body, forming the inner lining of blood and lymphatic vessels, usually in a layer one cell thick. ECs have an important role in physiological hemostasis (they present a nonthrombogenic surface to circulating blood constituents), in the permeability of blood vessels and in the supply of nutrients to the subendothelial tissue, and in the response of the blood vessels to physiological and pathological stimuli. Abnormalities in the structure and function of ECs may contribute substantially to hemostatic diseases, such as thrombosis (120,121).

There is evidence to suggest the participation of ECs in autoimmune diseases: 1) ECs share certain immune functions with monocytes in presenting antigen to lymphocytes (197,198) and in the production of interleukin-1 (199); 2) ECs play a role in regulating lymphocyte migration through vessel walls and in the activation of T cells (200); 3) ECs express HLA D/DR and EC-specific antigens on their surfaces (201,202) and Ia antigen after stimulation with T cell-derived factors (203); and 4) ECs in culture release soluble products that inhibit granulocyte aggregation in vitro and may thereby prevent granulocyte-mediated vascular injury (204).

The endothelium can be impaired by most major effectors of the immune system, including specific (antibodies and cytotoxic T cells) and nonspecific (complement, lymphokines, and inflammatory cells) immune pathways. A potential role for specific anti-EC antibodies and sensitized lymphocytes has been demonstrated in endothelial damage (205). Although the actual role of intravascular granulocyte

aggregation has not been clearly defined, it is proposed to be associated with a variety of syndromes of vascular endothelial damage (206-208). Adherence of activated leukocytes to ECs may result in damage to the endothelium through the effects of toxic oxygen metabolites (209,210) and the adhesion may be enhanced by platelet-released products, including platelet-derived growth factor (PDGF) (210,211). Complement components have frequently been reported to be active mediators in stimulating the adherence of leukocytes to the endothelium (206-209,212). Immune complex deposition on endothelium is another notable pathway by which complement components may be activated to act directly as inflammatory effectors or to enhance leukocyte adherence to the endothelium (209).

c. Interactions between Platelets and Endothelial Cells

Over the past 15 years, the development of *in vitro* EC culture techniques has resulted in significant advances in the understanding of how platelets and ECs interact (213). These are discussed below under the six major headings. 1) supportive function of platelets to the endothelium; 2) nonthrombogenic endothelial surface; 3) receptors for adhesive proteins; 4) coagulation factors; 5) arachidonic acid metabolism; and 6) effects of platelet-released substances.

1) Supportive function of platelets to the endothelium

It has been observed in experimentally induced thrombocytopenic rabbits that the endothelium exhibited marked "thin spots", fenestrations, and a reduced mean thickness, which were correlated with increased vascular permeability. The endothelial integrity was

restored when the platelet count returned to normal, suggesting that platelet counts are important for maintaining the integrity and contiguity of the endothelium and for repairing vessel injury (214-216). The supportive functions of platelets to the endothelium are, at least partially, due to the effects of platelet-derived growth factor (PDGF) or other platelet-released components, including ADP, serotonin and platelet factor 4, on ECs (216-218).

2) Nonthrombogenic endothelial surface

The most important function of the intact endothelium is to present a nonthrombogenic surface to circulating blood constituents (in particular, platelets), thereby preventing the initiation of thrombosis. This function involves almost all known natural anticoagulant mechanisms in vivo (reviewed in 219), including luminal EC surfaces covered by an ultrathin mucopolysaccharide (glycocalyx) coat, which forms a negatively charged membrane at physiologic pH and repels the negatively charged platelets; the elaboration of plasminogen activator which, in turn, initiates fibrinolysis; synthesis and expression of heparin-like molecules and antithrombin-III; the synthesis and expression of a unique cofactor, thrombomodulin, which promotes activation of the protein C - protein S system (220); and the production of prostacyclin (PGI_2), which efficiently inhibits platelet aggregation and induces vasodilation (221). Any functional change in ECs or biochemical modification of the cell surface will lead to platelet activation, adhesion and aggregation.

3) Receptors for adhesive proteins

Adhesive proteins including collagen, laminin, fibrinogen, fibronectin, thrombospondin and vWF, which mediate the attachment of cells to the extracellular matrix and cell-cell interactions, may induce the adherence of platelets to ECs, since receptors for these proteins have been identified in both cells (224). As mentioned in Section B1a of this chapter, one consequence of platelet activation is the expression of cell membrane receptors for these adhesive proteins (173,174,177,183-185). The binding of these ligands to specific platelet membrane GP receptors mediates the aggregation of platelets and adhesion of platelets to impaired endothelium or exposed subendothelium (225). It has been demonstrated that cultured ECs are able to synthesize membrane proteins which are biochemically and immunologically similar to platelet GPIIb and IIIa (226), suggesting the involvement of some adhesive proteins and their receptors in the adhesion of platelets to ECs.

Over the last few years, cell receptors for adhesive proteins have been intensively investigated and many of them have been shown to be related to the family of "very late antigens" (VLA) (reviewed in 222,223). The VLA protein family, originally defined on activated T cells, is also an important set of antigens found on platelets. VLA molecules are heterodimers composed of a unique α subunit (100-200kD) and a common β subunit (25-210kD), which gives each VLA protein its name, such as VLA-1, VLA-2, VLA-3, VLA-4 and VLA-5. One or more VLA complexes are expressed on nearly all cell types, with the exception of granulocytes and red blood cells. These molecules serve as

Table 3. Major Platelet Membrane Glycoproteins

GP	M.W. in SDS PAGE*	Proposed Functions	References
GPIa	167K	Forms receptor for collagen when complexed with GPIIa; α subunit of VLA-2	227,228
GPIb	α, 140K β, 22K	Forms receptor for vWF and thrombin when complexed with GPIX	229,230
GPIc	α, 134K β, 30 & 31K doublet	Forms receptor for fibronectin when complexed with GPIIa; α subunit of VLA-3	227,231 224
GPIIa	150K	β subunit of VLA-2 or VLA-3 found complexed with either GPIa or GPIc	227,231 224
GPIIb GPIIIa	α,130K; β,23K 114K	Forms receptor for fibrinogen, vWF and fibronectin when complexed with GPIIIa	183-185
GPIIIb (IV)	95K	Receptor for Type-1 collagen	232
GPIV	85K	Receptor for thrombospondin	174
GPV	82K	Substrate for thrombin	233
GPIX	17K & 22K	Forms complex with GPIb	230,234

* The molecular weights (M.W.) listed in this table were obtained in SDS PAGE under reducing conditions.

receptors for adhesive proteins and are responsible for cell-matrix adhesion (222,223). VLA-2 and VLA-3 have been identified in platelets as the complex of GPIa and IIa (collagen receptor), and the complex of GPIc and IIa (fibronectin receptor), respectively (227). The functions of platelet membrane GPs and their immunological relationship to the VLA family of proteins are summarized in Table 3.

4) Coagulation factors

A striking example of the dependence of platelet and EC function on coagulation factors is their relationship to the Factor VIII system (i.e. vWF and Factor VIII antigen), which is present only on ECs, platelets, and megakaryocytes in vivo. It has been shown that ECs synthesize and secrete a molecule with both Factor VIII antigen and vWF activity but without antihemophilic activity. These molecules circulate in plasma as either carriers or inactive precursors of antihemophilic factor. When Factor VIII antigen and vWF bind to subendothelial matrix components or absorb to the platelet surface, they become activated, resulting in normal platelet function and platelet "stickiness" to the subendothelium (235-237).

5) Arachidonic acid metabolism

The effects of arachidonic acid (AA) metabolites on platelets and ECs have been extensively investigated in recent years (reviewed in 238). In summary, AA can be split from the PL pool in platelet or EC membranes by phospholipase A_2, which has been activated by collagen or thrombin. Subsequently, AA is catabolized into a series of endoperoxide products by cyclooxygenase in platelets or ECs. The major cyclooxygenase product of AA in platelets is TXA_2, a potent

inducer of vasoconstriction and platelet activation. The main AA metabolite of ECs is PGI_2, which effectively inhibits platelet aggregation, and is thus necessary for platelet compatibility with ECs (221,239,240). PGI_2 probably affects platelet function by modulating the cAMP concentration in platelets, and thereby inhibiting the mobilization of fibrinogen-binding sites on human platelets *in vitro* and limiting the extent of fibrinogen-platelet interaction (241-243). The regulation of these two AA metabolites, TXA_2 and PGI_2, in platelets and ECs is important in maintaining a balance between hemostasis and thrombosis.

6) Platelet-released substances

Platelets contain several types of granules as storage-pools for active substances. The α-granules contain thrombospondin, platelet-derived growth factor, β-thromboglobulin, fibrinogen, acid hydrolases, and platelet factor 4. Dense bodies contain calcium, pyrophosphate, serotonin, and adenine nucleotides (ADP and ATP) (244, 245). Activated platelets release granule contents, such as serotonin, which can increase EC injury by causing individual cells to contract and consume more oxygen or by inducing chemotaxis (246-248).

In summary, the endothelium presents a nonthrombogenic surface to which platelets do not adhere. Any minor trauma leading to a loss of endothelial continuity or a modification of the biochemistry of ECs will permit platelets to adhere to subendothelial tissue or endothelium. Once adhesion has occurred, the release of granule components causes more platelets to aggregate and to form a platelet plug at the site of injury. A series of steps in the cascade of

coagulation is initiated, resulting in hemostasis under physiological conditions and thrombosis under pathogenic conditions.

2. Abnormal Hemostasis in SLE patients

It has long been known that clinical and laboratory abnormalities of hemostasis are a feature of SLE. One or more hematological abnormalities are present in nearly all SLE patients with active disease (68,249-251). These include abnormal platelet function and coagulation profiles (164,252-260), thrombosis and thrombocytopenia (260-266), and large vessel occlusion and gangrene (267). In a study of 112 consecutive patients with SLE, Gladman et al. (263) found that abnormalities of hemostatic function occurred frequently in SLE patients and that 96 abnormalities occurred in 64 of 112 (57%) patients studied (Table 4).

Table 4. Hemostatic Abnormalities in 112 SLE Patients*

Thrombocytopenia	18 (16.1%)
Circulating lupus anticoagulant	19 (17.0%)
Decreased antithrombin III	24 (21.4%)
Abnormalities of fibrinogen	28 (25.0%)
Abnormal platelet factors III and IV	7 (6.3%)
Total number of abnormalities	96
Number of patients with abnormalities	64 (57.1%)

* After Gladman DD, Urowitz MB, Tozman EC, and Glynn MFX: Hemostatic abnormalities in systemic lupus erythematosus. Quart J Med, New Series LII 207:424-33, 1983.

a. Abnormal Platelet Function

Abnormal platelet function is commonly seen in SLE patients (249-253). Platelet function and coagulation profiles studied in 50 SLE patients showed circulating LA antibodies in 3 patients, elevated fibrinogen degradation products in 5 patients, and isolated nonspecific abnormal findings in 7 patients (252). A qualitative platelet defect was found in 12 of 21 consecutive patients, which consisted of failure of platelets to undergo collagen-induced aggregation and impaired ADP and epinephrine-induced aggregation. It was also shown that impaired platelet function correlated with the clinical severity of disease. In another study of 18 consecutive SLE patients with LA and 59 SLE patients without LA, platelet aggregation was abnormal in most of the patients and the defect was much more pronounced if associated with LA (164). The defects in platelet aggregation in SLE patients may be partially related to a storage pool deficiency state, may be reversible, and could be mediated by plasma or platelet-associated DNA (253).

b. Thrombocytopenia and Thrombosis

Varying degrees of thrombocytopenia occur in SLE patients. Mild thrombocytopenia is present in about one third of patients, while severe thrombocytopenia with purpura occurs in only 5% (2,68,265, 266). Among 112 SLE patients studied, 18 had thrombocytopenia, and 10 of these 18 patients had platelet counts of less than 100,000/mm^3 (263). It was found that thrombocytopenia was the best diagnostic indicator of the severity of lupus nephritis (265) and decreased platelet counts correlated best with glomerular thrombosis and

subendothelial deposits (262). Renal vein thrombosis and inferior vena cava thrombosis (264), deep vein thrombosis, pulmonary embolism (261) and large vessel occlusion and gangrene (267) have also been reported in SLE patients. The incidence of thrombosis in SLE ranges from 5-14% and varies in different reports (68,164,249-251).

Specific LA, anti-PL, anti-platelet or anti-EC autoantibodies could be responsible for the thrombosis and thrombocytopenia seen in many SLE patients. A better understanding of the relationship between LA, anti-PL, anti-platelet and anti-EC autoantibodies present in patients with SLE might help to clarify the mechanisms responsible for the thrombocytopenia and thrombosis seen in these patients and in patients with other autoimmune diseases.

c. Clinical Manifestations Associated with Lupus Anticoagulants and Anti-Phospholipid Antibodies

There is a general consensus that LA and anti-CL antibodies are strongly associated with thrombosis and thrombocytopenia, clinically, but not with a tendency to bleeding (155-167). Carreras and Vermylen (268), in a review of the literature, reported that about 30% of patients with LA had arterial or venous thrombosis. A study of the prevalence of thrombosis and LA in SLE patients showed that 18 of 31 SLE patients with LA developed thrombosis, while only three of 29 SLE patients without LA had thrombosis (161). Several investigations have suggested that these antibodies may promote in vitro platelet aggregation by blocking the production of PGI_2, a potent inhibitor of platelet aggregation (221) which is implicated in the regulation of fetal circulation (269,270). Other mechanisms, such as direct

interaction of LA antibodies with PS or PE in the platelet membrane, could promote platelet activation, aggregation, and thrombocytopenia (78,166). However, there is little evidence that indicates a direct binding mechanism involving LA and live platelets. Firstly, LA in human plasma did not prolong the APTT when platelets were substituted for the PL-containing APTT reagent (170), although a recent finding shows that affinity-purified anti-CL antibodies with LA activity bound to platelets activated with thrombin and collagen, ADP, collagen, epinephrine, thrombin, or calcium ionophore, but not to resting platelets (271). Second, although the LA activity of SLE plasma could be neutralized by freeze-thawed lysed platelets, this was not true of intact platelets (165). Third, in studies from our laboratory, most human hybridoma LA antibodies were found not to bind directly to intact platelets (Chapter III). It is quite likely that more than one mechanism is operational since it is clear that LA antibodies comprise a group of antibodies with heterogeneous reactivities.

3. Rationale for the Interaction of Lupus Anticoagulant and Anti-Phospholipid Antibodies with Platelets and Endothelial Cells in Thrombosis and Thrombocytopenia

The elucidation of the role of LA antibodies in causing hemostatic abnormalities should provide a better understanding of the paradox caused by LAs. Although LAs prolong intrinsic clotting times (APTT) in vitro, these antibodies are clinically associated with hypercoagulable states (e.g. thrombosis) and thrombocytopenia, but not with a bleeding tendency (155-167). Since the coagulation process

in vitro involves the assembly of coagulation factors on a phospholipid surface (272), it is postulated that PL-reactive LA antibodies interfere with the assembly of prothrombinase on these PL surfaces (273). Interestingly, if washed platelets, ionophore-treated platelets or lysed platelets are substituted for PLs, the coagulation tests are normal (170,273,274), providing a possible explanation for the lack of clinical bleeding in these patients.

In vivo, it is possible that LA antibodies bind to PLs in the platelet and EC membranes and result in damage, activation or functional blockage of these cells which could induce thrombosis or thrombocytopenia (162,165,266). Reduced fibrinolytic capacity and increased vWF activity in SLE patients with LA may reflect endothelial damage (275). IgG with LA activity from 2 SLE patients has been reported to inhibit the function of human thrombomodulin, a unique endothelial anticoagulant cofactor in the activation of protein C by thrombin (276,277). A low functional activity of antithrombin III was detected in another patient with LA. One or all of these mechanisms combined may impair the normal in vivo anticoagulant pathways (277).

Another possible mechanism of thrombosis involves the potential interference of LA with the PGI_2 pathway. Carreras et al. (150) proposed that LA may inhibit the production or release of PGI_2 by interfering with the availability of AA from membrane PLs. The proposal was based on the finding that a LA-containing IgG fraction, isolated from a patient with a history of arterial thrombosis and multiple intrauterine deaths, reduced the release of PGI_2 from rat

aorta rings or pregnant human myometrium and the production of 6-keto-prostaglandin $F_{1\alpha}$ by bovine ECs. The inhibitory effect was abolished in the presence of AA. In a subsequent study by the same group, an inhibitory effect of plasma on PGI_2 production by vascular tissue were detected in 8 of 14 patients with LA, 6 of whom had thrombosis (268). Similar findings were obtained by other groups of investigators (278-281), suggesting that the pathogenetic role of LA may be associated with inhibition of PGI_2 formation. However, these findings require confirmation, since all of these studies were performed with plasma and none demonstrated the inhibition of PGI_2 production by all LA-containing plasmas. Furthermore, there is controversy regarding the inhibition of PGI_2 production by LA and some investigators claim that LA enhances PGI_2 production (Triplett personal communication). Others argue that the thrombosis associated with anti-PL antibodies cannot be explained by effects on EC and platelet prostanoid synthesis (282) and that the significantly increased EC procoagulant activity induced by sera from patients with SLE and LA may account for the increased incidence of thrombosis in these patients (283). These apparently contradictory data may indicate the heterogeneity of LA or that some of these observations are due to antibodies other than LA. Thus, more direct evidence is still required to explain the association of LA and anti-PL with thrombosis and thrombocytopenia.

C. THEORIES OF AUTOANTIBODY PRODUCTION

1. The Generation of Immunoglobulin Diversity

For many years, there were two theories regarding the generation of Ig diversity. The germline theory stated that the genes for the entire repertoire of the immune response are carried in the germline (sperm and egg) and passed on to each member of the species, so that each cell would have all of the variable (V) regions for heavy (H) and light (L) chains, but by some unknown mechanism, would use only one region from H and L chains. The second theory proposed the mechanism of somatic variation in which a few crucial antibody genes are transmitted through the germline and during lymphocyte differentiation, these genes undergo somatic variation, resulting in the enormous diversity. Recent advances in molecular biology have shown that, in fact, both theories are correct, but neither is complete in itself. In order to explain how the V-region genes attain a high level of diversity while the constant (C) region genes remain fairly unchanged, Dreyer and Bennett (284) proposed that V-regions and C-regions must be products of two genes. In other words, there are two genes encoding for one polypeptide chain in each antibody molecule. The discovery of genetic reorganization, in which V and C regions were proven to be encoded by two genes but read as a single gene, was made by Tonegawa and his associates (285) in a series of experiments. It is well defined now that two sets of gene segments (V and J) for the L chain and three sets of gene segments (V, D and J) for the H chain are rearranged to form functional V genes, and then join with the C genes, μ, τ, α, ϵ or δ for the H chain genes and

kappa and lambda for the L chain genes. Each set of the V gene families contains a number of different segments, which can be selected for recombination. Clearly, the tremendous diversity of Ig molecules can arise from the diverse joining of these different V gene segments, and this can be increased by variations in the combining sites between these segments (reviewed in 286,287) and be further enlarged upon by somatic mutation (288).

2. Origins of Autoantibodies

The immune system has the potential to recognize enormous numbers of antigens, including self components. Some of these are essential for a functional self-defence system and the regulation of immune functions (e.g. antibodies against mutated or old cells, and anti-idiotype antibodies), while others may be pathogenic (289). More knowledge about the origins of these non-pathogenic and pathogenic autoantibodies is crucial to the understanding of the normal immune system and autoimmunity.

The discovery of antibodies reactive with dsDNA in SLE sera (31) suggested that dsDNA may be immunogenic. However, the fact that neither anti-dsDNA antibodies nor SLE could not be induced experimentally by immunizing normal animals with dsDNA (290) indicated that mechanisms responsible for producing these autoantibodies in SLE are not simply "antigen-driven". Several hypotheses have been proposed to explain the large number of autoantibodies produced in autoimmune diseases, including: 1) genetic abnormalities; 2) loss of host tolerance; and 3) antigen-driven autoimmunity (modification of autoantigens).

a. Genetic Abnormalities

An important question in this area is whether autoantibodies are encoded in the germline or result from somatic mutations in antibodies originally directed against exogenous antigens.

1) Abnormal rearrangement of immunoglobulin germline genes

Theoretically, abnormalities in the polymorphic recombination of Ig germline V gene segments, such as higher frequencies of particular V gene segments, may contribute to or be associated with the production of autoantibodies. However, comparisons of restriction fragment length polymorphisms in DNA from all major lupus mice strains with their parental strains and with nonautoimmune strains revealed that murine lupus is not associated with a particular IgV_H haplotype and that the IgV_H loci may be essentially normal in lupus mice (291,292). This evidence strongly suggests that defects within the Ig loci are unlikely to be the primary cause of murine lupus and that associations of Ig haplotypes with lupus are weak.

The possibility that autoantibodies are encoded by specific V gene segments was also tested in mice. Autoantibodies and antibodies to exogenous antigens were found to be encoded by the same, or at least an overlapping, germline gene repertoire, although unusual V_H, V_K and D segments do appear in some autoantibodies (293-295). All 6 autoantibodies against bromelin treated red blood cells (BrRBC) (three each from NZB and CBA mice) used the same, or highly related, gene segments (V_H, D, J_H, V_K and J_K) (294). It is known that mouse lupus autoantibodies are encoded by almost all known V_H and many V_K families, but there is a certain extent of genetic restriction among

autoantibodies of different or similar specificities (295). For example, various murine autoantibodies showed restricted V_H gene usage, such as the 3'V_H gene families (7183 and Q52) (296), which rearrange early in B-cell development (297). Lyl+ B cells and hybridomas selected for 7183 V_H gene expression produced antibodies with autoreactivity at a high frequency (298). It was also reported that the V_H gene usage of 39 anti-DNA antibodies from MRL-lpr/lpr and (NZBxNZW)F_1 mice was distributed over most of the known V_H gene families but showed a predominance of J558 and 7183 V_H genes. In another analysis of 16 anti-DNA L chains originated from seven V_K families, 8 of these were encoded by V_K19 genes, but all of these antibodies were derived from one mouse (reviewed in 299).

A recent study using A/J anti-arsonate IgM antibodies addressed one aspect of this issue by testing the hypothesis that unmutated germline V genes expressed by preimmune B cells encode for autoantibodies (300). In this study, Naparstek et al.(300) analyzed the antigen-binding properties of antibodies produced during the course of the immune response of normal mice to p-azophenyl arsonate (Ars). Their results demonstrated that a high proportion of the antibodies produced by the preimmune clones reacted with autoantigens (dDNA and cytoskeletal proteins), while antibodies produced following Ars immunization showed less autoreactivity and more Ars reactivity. These investigators suggest that autoantibodies may be encoded by unmutated germline V genes and that the conversion to Ars reactivity may have been due to somatic mutation followed by specific selection of high affinity anti-Ars antibodies. However, a larger number of

autoantibodies must be analyzed to confirm this hypothesis.

Efforts have been made to probe the cellular and molecular events that determine the final outcome of class and subclass switching and their relationship to autoantibody production. The onset of autoimmune disease seems to be correlated with a switch in antibody production from predominantly IgM to IgG isotypes (14,90). In an analysis of 706 human monoclonal IgA, IgM and IgG myeloma proteins, 53% of the IgG proteins bearing the 31 idiotype bound to DNA as compared with only 7% of the IgM proteins bearing the same idiotype (301). This association of DNA binding with IgG isotype was statistically significant ($p < 0.001$) and suggests the importance of Ig isotype in determining anti-DNA antibody reactivity.

2) Somatic mutation

Autoimmunity could result from a disturbed regulation of the mechanisms responsible for somatic mutation in the maturation of the normal immune response. The finding that a single amino acid residue replacement in the first hypervariable region of the heavy chain changed the specificity of an antibody from phosphorylcholine to DNA suggests that in vitro somatic mutation can result in the generation of an anti-DNA antibody from an anti-bacterial antibody (302,303). In a series of studies on the role of somatic mutation in the immune response to phosphorylcholine (T15 system) using protein sequencing analysis of V_H regions combined with DNA sequencing analysis of germline V_H genes, several features of somatic mutation in the anti-phosphorylcholine response were demonstrated (304-306). First, 10 of 19 V_H segments analyzed had identical protein sequences (denoted

T15), whereas the remaining 9 variants were all unique and differed from the prototype T15 sequence by 1 to 8 amino acid residues. Second, in the BALB/c genome, there is one germline V_H gene encoding the T15 sequence. All of the 9 variant V_H segments appeared to be mutated from this T15 gene, implying that the entire anti-phosphorylcholine antibody system may be derived from a single germline V_H gene segment and that somatic mutation occurs frequently. Third, somatic mutation correlated with the class switch because the variant V_H regions appeared only in IgG and IgA, but not in the initially expressed IgM. A subsequent analysis, done on 2 of these variant IgA antibodies, M167 and M603 (306), revealed that somatic variation, including silent and replacement substitutions, is extensive and sharply localized in and around the rearranged V_H genes, suggesting a special hypermutational mechanism highly localized in its site of execution and highly restricted in its time of operation during B-cell development.

If somatic mutations are present, frequency analysis of productive versus nonproductive mutations can help to determine whether the anti-self response is (auto)antigen-driven (with selection for replacement mutations) or results from polyclonal activation. Two clonally unrelated murine IgM anti-DNA antibodies (MRL-DNA10 and MRL-DNA22) and, possibly, three NZB IgM anti-BrRBC antibodies shared identical V_H gene-encoded regions, strongly suggesting that the unmutated V germline can be expressed in murine lupus autoantibodies (293-295). However, the presence of somatic mutations in J segments of some MRL-lpr/lpr autoantibodies clearly

shows that somatic mutation can occur in spontaneously produced murine lupus autoantibodies (295). Comparisons of mRNA sequences suggested that there were low levels of somatic mutation in autoantibodies from nonautoimmune mice as well. To date, the high level of autoantibody expression observed in autoimmune murine models does not appear to be caused by either a complete lack of or an abnormally high rate of somatic mutation of Ig genes.

From the data obtained until recently, some autoantibodies appear to be encoded by the germline whereas others may acquire self specificity after somatic mutation (299,302,303,307). The responses against endogenous and exogenous antigens appear to be governed by the same general principles, and self specificity, like exogenous specificities, may result from different recombinations of normal V, D, J gene segments and/or somatic mutation of the recombined heavy and light chains.

b. Loss of Immunological Tolerance

Immunological tolerance is the ability of the immune system to discriminate between "foreign" and "self". Historically, there were two series of experiments to address this subject. Medawar et al. (reviewed in 308) showed that contact with foreign antigens at a very early stage of life (fetus) allowed the immune system to tolerate a specific antigen without making a response to it. The second approach was based on the hypothesis that all antigens are foreign unless they contact the immune system before the functional differentiation of the immune system takes place. This was demonstrated by the experiments that pituitary glands removed from an embryonic frog were

rejected as "foreign" when they were re-implanted into the same frog during adulthood (reviewed in 309). It is well established that tolerance to an antigen requires that the antigen be present and come into contact with the immune system at a critical stage of development. Modern molecular and cellular biological approaches have proven that the induction of immune tolerance involves both T and B cell repertoires (310-313). BALB/c mice immunized with monoclonal anti-I-J^d antibody, which blocks T suppressor cell function, produced anti-DNA antibodies with the T15 idiotype (307). Based on this finding, it was proposed that anti-idiotype antibodies produced to anti-I-J^d antibodies may block the interaction of T suppressor cells with accessory cells necessary for T suppressor activation and so, lead to a loss of T suppressor cell function.

In summary, the mechanisms of tolerance are explained by various alternative theories (309,314), including deletion, which may involve physical (clonal) or functional (receptor blockade or aborted differentiation) deletion and suppression of clones by negative signals from veto cells and suppressor T cells to the tolerant cells. It seems clear that any factors resulting in the loss of immunological tolerance could lead to the subsequent development of autoimmune diseases.

c. Antigen-Driven Autoimmunity

Self antigens are normally not immunogenic to the immune system. However, there are many factors which could result in the production of autoantigens, which are discussed below.

1) Chemical modification

Some chemical drugs, such as quinidine, bind to the cell surface and may thereby serve as antigenic epitopes which induce antibodies against cellular components bound to the drug. Some chemicals may also modify cell antigens which could then induce autoantibodies. Examples of chemically induced autoimmunity are cases of immune thrombocytopenia induced by α-methyldoba, narcotics or quinidine (101,102,104,105) and leukopenia induced by quinidine (101).

2) Infection

Infecting microbes may act via several mechanisms to induce autoantibodies. Some bacteria or viruses may induce antibodies which are crossreactive with autoantigens. One example of such an antibody is a monoclonal myeloma Ig from a patient with Waldenstrom's macroglobulinemia who had both anti-bacterial and anti-DNA antibody reactivities (315). Infecting viruses may also alter antigen structure by absorption to the cell surface or penetration through the cell membrane. Finally, some viral genes may be incorporated into host DNA and encode for new antigens, which are seen as foreign to the host and may elicit an autoimmune reaction. Viral or bacterial products, such as endotoxin, may also result in polyclonal B cell activation and overproduction of antibodies.

3) Exposure of sequestered self-antigens

Some self-antigens, such as thyroglobulin and antigens expressed on testes, brain and eye crystalline body and humour, are not normally in physical contact with immunocompetent cells in vivo. If they are exposed to the body's immune system by trauma or infection,

these self-antigens will be recognized as "foreign" and may induce cellular and humoral immune responses, resulting in autoimmune disorders, such as autoimmune thyroiditis, autoimmune infertility and sympathetic ophthalmia (316).

3. Possible Explanations for the Polyspecificity of Monoclonal Antibodies

Monoclonal antibodies have frequently been shown to react with two or more apparently dissimilar antigens, as discussed in Section A) of this chapter. This kind of polyspecificity can be attributed to partial epitope identity on different antigens (common determinants), multiple specific binding sites on a single antibody molecule, or irrelevant interactions involving other binding interactions, such as electrostatic charge interactions between antibody and antigens. The interaction between an antigen and antibody molecule may involve many more contact points than previously believed (reviewed in 317,318).

a. Common Determinants on Antigens

Antigen-combining sites on a given Ig molecule can bind several different antigens which are structurally related to each other (share common determinants) (70,319). Examples of structurally related antigens, which may be involved in autoimmunity are the phosphodiester determinants shared by DNA and PLs (70)

b. Multiple or Flexible Binding Sites on Antibodies

A monoclonal antibody which binds distinct molecules with little or no structural similarity may represent an antibody with polyfunctional binding sites. Polyfunctional combining sites, or an antibody with more than one specific binding site, was first

demonstrated in a myeloma protein, called 460, which possessed two separate binding sites for two structurally different haptens, menadione and dinitrophenol (DNP) (320). An antibody carrying multiple combining sites may react with an antigen at all complementary epitopes, resulting in a high binding affinity, and may also crossreact with antigens bearing only some of these epitopes, thereby displaying a lower affinity interaction (see Figure 1) (317).

The dynamic nature of protein structure in solution (320,321) suggests that both the antibody and antigen may be flexible and induce in each other an optimal configuration for binding. This concept, similar to the "lock and key" theory for enzyme and substrate, proposes greater flexibility in antigen-antibody binding interactions (322).

c. Electrostatic Charge Interactions

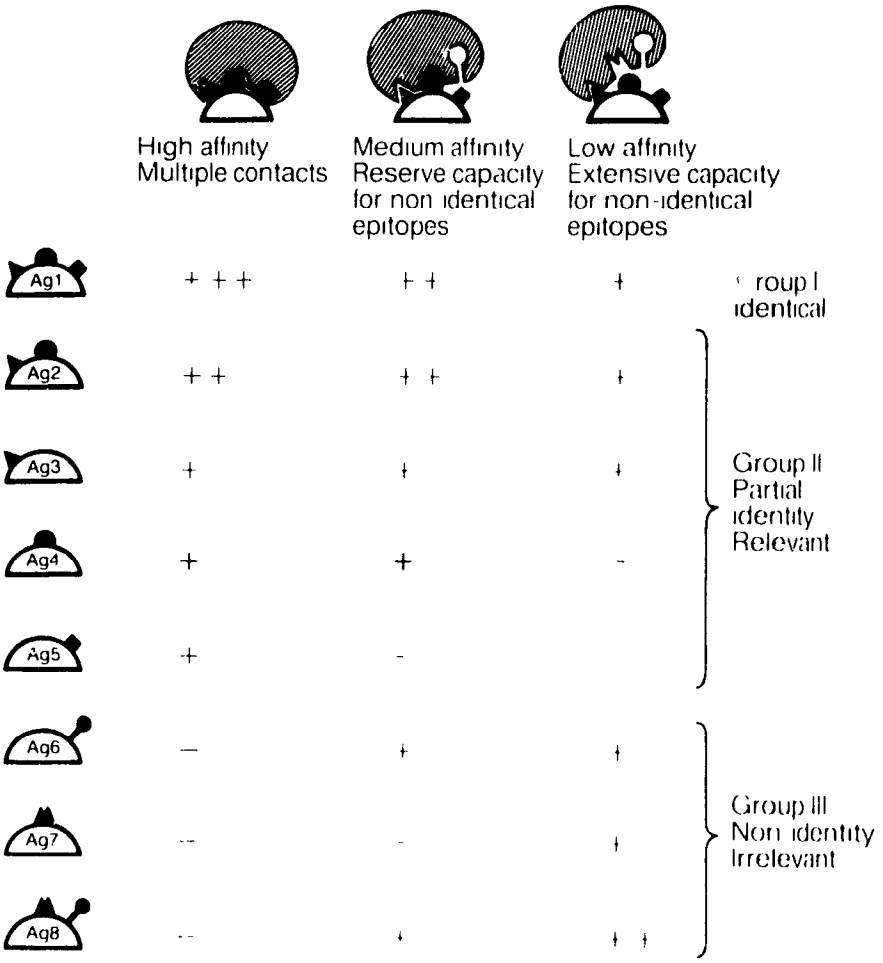
Contact between antibody and antigen may involve several forces, including hydrogen bonds, hydrophobic interactions, and ionic interactions (electrostatic charges) or combinations of all of these. Antibodies with high isoelectric points carry positive charges at physiological pH and so display binding to negatively charged molecules, such as DNA (92-94). In an analysis of 706 human myeloma Igs, the proteins focused over a wide range of pI values, but all of the DNA-binding Igs focused at a pI = 7.0, suggesting that Ig charge contributes to these antigen-antibody binding interactions (304).

Figure 1. The Relationship between the Affinity of an Antibody-Antigen Interaction and the Probability of Cross Reaction with Similar (Group II) and Dissimilar (Group III) Epitopes

The high affinity antibody on the left only shows cross reactions with antigens which have identical (Group I) or partially identical (Group II) epitopes. All cross reactions of such an antibody are therefore relevant. In contrast, the low affinity antibody on the right has spare capacity for binding to antigens which have no epitope identity (Group III antigens). In addition, its efficiency of detection of partially identical (Group II) antigens is much reduced. In the intermediate case shown in the centre, the monoclonal antibody cross reacts with most Group II antigens having partial structural identity but can also cross react with dissimilar Group III antigens. The majority of monoclonal antibody cross reactions fall into the Group III irrelevant category and do not reflect any structural identity among antigens.

5 Alter Ghosh S and Campbell AM: Multispecific monoclonal antibodies. Immunol Today 7:217-222, 1986.

Figure 1


High affinity
Multiple contacts
Medium affinity
Reserve capacity
for non identical
epitopes
Low affinity
Extensive capacity
for non-identical
epitopes
Ag1
+ + +
+ +
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roup I
identical
Ag2
+ +
+ +
+
Ag3
+
+
+
Group II
Partial
identity
Relevant
Ag4
+
+
-
Ag5
+
-
Ag6
—
+
+
Group III
Non identity
Irrelevant
Ag7
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-
+
Ag8
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+
+ +

4. Autoreactive Antibodies in Normal Individuals

Antibodies with autoreactivity have been reported to exist in the sera of normal mice and human subjects, suggesting that individuals without apparent autoimmune disease have B cells which can be activated to secrete autoantibodies (323-330). Anti-DNA antibodies occur in old age (331), in normal young mice stimulated with B cell mitogens (332), and in in vitro cultures of mouse and human lymphocytes stimulated with mitogens (25). Antibodies to other autoantigens, such as thyroglobulin and self red blood cells, were also detected from normal mouse lymphocyte cultures stimulated with mitogens (333). These autoantibodies are often characterized by extensive crossreactivity (polyspecificity) (327,328,334,335) and idiotypic connectivity (326,329,330). Antibodies reactive with a variety of lipids (PLs and glycolipids) have also been reported in normal (non-immunized) human and animal sera (142,336).

Several laboratories, including our own, have demonstrated that human hybridomas derived from normal donors can produce anti-DNA antibodies, many of which show polyspecificity (75,337-339). Anti-DNA and anti-platelet antibodies have been produced from hybridomas derived from tonsils of normal individuals (340). Some efforts have been made to compare the normal and autoimmune-derived antibodies and to study the mechanisms by which autoantibodies cause pathogenesis in autoimmune patients, but not in normal individuals. Datta et al. (341) studied the anti-DNA idiotype, $ID^{16/6}$, expressed by normal and SLE-derived lymphocytes. They found that cultured normal-derived lymphocytes produced $Id^{16/6}$ in response to pokeweed mitogen

stimulation, while SLE-derived lymphocytes produced $Id^{16/6}$ without stimulation and lymphocytes from relapsed patients spontaneously produced the highest levels of $Id^{16/6}$. The majority of $Id^{16/6}$ produced by normal lymphocytes did not bind to DNA, suggesting that antibodies expressing $Id^{16/6}$ are comprised of at least two populations with different antigenic specificities. Bell et al. (342) reported similar results in their comparison of the antigen specificity and idiotypic characteristics of normal tonsil-derived hybridoma antibodies and SLE-derived serum antibodies. One idiotype, 4.6.3, derived from a human hybridoma IgM anti-DNA antibody of tonsillar origin, is commonly expressed in the serum of SLE patients and appears to be related to clinical disease activity, while normal tonsil-derived antibodies expressing this idiotype family did not bind to DNA. Another recent study used single cells to examine the B cell repertoires of normal DBA/2, BALB/c, and C3H/HeJ mice and autoimmune-prone MRL-lpr/lpr, NZB and C3H-gld/gld mice from 1 day to 5 months of age. Although there were significantly more Ig-secreting splenic B cells in autoimmune mice than in normal adult mice, both expressed a temporally limited number of B cell clones and a sudden increase between 1 to 5 weeks of age in reactivity against many antigens, encoded by germline genes (343) (Section C2a of this chapter). It is proposed that such crossreactive antibodies increase the ability of the young animal to neutralize numerous environmental antigens using a limited B cell repertoire. The reactivities of these clones might then be diversified in response to stimulation by particular antigens in the environment, resulting in the selection of

B cells secreting antibodies with high affinity. Thus, in older mice with larger repertoires, each clone expresses a high degree of specificity to certain antigens and less cross reactivity is seen among these clones (343,344). The authors hypothesize that the similarity of the developing repertoires of normal and autoimmune mice could suggest a role for a polyclonal activator which induces the hyperproliferation of antigen-stimulated B cells in systemic autoimmune diseases in mice.

Although these studies provide some insight into the origins of autoantibodies in normal individuals, many questions remain unanswered regarding the mechanisms responsible for their production, the differences between antibodies derived from normal individuals and from patients with SLE, and the mechanisms by which autoantibodies cause pathological abnormalities in SLE patients but not in normal individuals. The use of hybridoma autoantibodies, gene cloning and DNA sequencing, and possibly, even transgenic mice, should provide the means to attain more precise information regarding the molecular genetic origins of these antibodies and their pathogenetic relevance in vivo.

CHAPTER II

RATIONALE, OBJECTIVES, AND SIGNIFICANCE OF THE PRESENT INVESTIGATION

RATIONALE

Although lupus anticoagulant, anti-phospholipid and anti-DNA autoantibodies are clinically associated with abnormalities of hemostasis in SLE, it is unclear whether these autoantibodies are the direct cause of these hemostatic disorders and by what mechanisms they act. The relationship of lupus anticoagulant, anti-phospholipid and anti-DNA antibodies to anti-platelet and anti-endothelial cell antibodies is also unknown, as are the epitopes on platelets and endothelial cells responsible for antibody binding. Thus, an investigation of the ability of individual lupus anticoagulant, anti-phospholipid and anti-DNA antibodies to bind to platelets and endothelial cells and to have functional effects on these cells should provide direct and relevant answers to these questions.

Lupus anticoagulant, anti-phospholipid and anti-DNA antibodies are also found in normal individuals without apparent autoimmune disease. It is not known what the qualitative differences between autoantibodies derived from SLE patients and normal individuals are, or why these antibodies are pathogenic in affected individuals, but not in unaffected individuals. These questions can be addressed using hybridoma autoantibodies derived from normal individuals and SLE patients in studies which compare the binding and functional properties of these two groups of antibodies.

OBJECTIVES

Our approach to these questions has involved the production of human hybridoma antibodies from normal individuals and SLE patients and the characterization of the binding and functional properties of SLE and normal-derived hybridoma lupus anticoagulant, anti-phospholipid and anti-DNA autoantibodies on platelets and endothelial cells, with the following specific objectives:

1. To analyze the relationship of platelet and endothelial cell-binding properties to lupus anticoagulant, anti-phospholipid and anti-DNA antibody reactivities;

2. To compare the binding and functional reactivities of normal and SLE-derived autoantibodies;

3. To identify the epitopes on platelets and endothelial cells responsible for autoantibody binding and functional activity.

SIGNIFICANCE

The use of monoclonal human hybridoma autoantibodies should yield useful and interpretable data about the relationship between anti-platelet and anti-endothelial cell autoantibodies, and lupus anticoagulant, anti-phospholipid and anti-DNA autoantibodies in SLE and normal individuals and the epitopes responsible for reactivity of these antibodies with platelets and endothelial cells. Furthermore, studies on the effects of individual antibodies on platelets and endothelial cells in in vitro functional assays should provide insight into autoantibody-mediated mechanisms responsible for some of the hemostatic disorders seen in patients with SLE.

CHAPTER III

LUPUS ANTICOAGULANT AND ANTIPLATELET PROPERTIES OF HUMAN HYBRIDOMA AUTOANTIBODIES

RUNNING TITLE

Lupus Anticoagulant and Antiplatelet Autoantibodies

ABSTRACT

The clinical association of lupus anticoagulant antibodies with thrombocytopenia and thrombosis was the rationale for investigating the in vitro reactivity of these human hybridoma lupus anticoagulant antibodies with platelets. Fifty human hybridoma antibodies from 13 patients with SLE, 2 women with multiple spontaneous abortions, and 4 normal individuals were analyzed for lupus anticoagulant, anti-platelet, anti-dDNA and antiphospholipid reactivities. Of the hybridoma antibodies studied, 25 had lupus anticoagulant activity, 21 had antiplatelet reactivity and 7 of these had both lupus anticoagulant and antiplatelet properties. No correlation was found between lupus anticoagulant antibody activity and antiplatelet, anti-dDNA, anticardiolipin, anti-egg phosphatidylethanolamine, anti-phosphatidylserine, antiphosphatidylinositol and antiphosphatidylcholine reactions. In contrast, antiplatelet activity was strongly correlated with antiphosphatidylethanolamine (r=0.761, $p<0.001$), anticardiolipin (r=0.748, $p<0.001$) and anti-dDNA (r=0.745, $p<0.001$) reactivities. Pretreatment of platelets with deoxyribonuclease, ribonuclease, trypsin, or phospholipases A_2 and C resulted in different effects on the binding of individual hybridoma antibodies to platelets, suggesting that antiplatelet antibodies may recognize different epitopes on the platelet membrane. Our data demonstrate that most hybridoma lupus anticoagulant antibodies did not bind directly to platelets in vitro. This suggests that additional serum factors may be required in vivo to explain the association of these antibodies with thrombocytopenia and thrombosis.

INTRODUCTION

Lupus anticoagulant antibodies belong to a broader class of antiphospholipid antibodies and are defined by their ability to prolong the clotting time in *in vitro* coagulation assays measuring the activated partial thromboplastin time (APTT). Paradoxically, the existence of lupus anticoagulants in systemic lupus erythematosus (SLE) sera has been strongly correlated with thrombosis and thrombocytopenia but not with bleeding disorders (1-7). These clinical associations may be due to platelet aggregation and/or platelet lysis, but few studies have demonstrated a direct effect of lupus anticoagulant antibodies on platelets (8,9).

Hybridoma technology has permitted the cloning of monoclonal lupus anticoagulant antibodies. We have previously shown that hybridoma lupus anticoagulant antibodies derived from patients with SLE can react with dDNA, cardiolipin, human IgG, and the hexagonal form of phosphatidylethanolamine (PE) (10,11). The epitope(s) responsible for these multiple cross-reactivities remains unclear but evidence suggests that more than one epitope may be involved (9).

Several different phospholipid reactivities have been described for lupus anticoagulant antibodies. Thiagarajan et al. (12) reported the reactivity of monoclonal lupus anticoagulants with phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA), but not with phosphatidylcholine (PC) or PE, whereas Exner (13) suggested the PE may be the most reactive phospholipid. Harris et al. (14) showed a strong correlation between lupus anticoagulant activity and anticardiolipin binding in the sera of patients with SLE and

found that serum antibodies which had been affinity-purified on cardiolipin liposomes had lupus anticoagulant activity and were able to bind to cardiolipin, PA, PI, PS, phosphatidylglycerol (PG), and only weakly to PE (15). It is still not clear, however, whether the lupus anticoagulant and anticardiolipin antibodies are one and the same.

In the present investigation, we have studied the in vitro reactivity of human hybridoma antibodies with platelets and the association of this reactivity with lupus anticoagulant, antiphospholipid and anti-denatured DNA (dDNA) antibody activities. Our results demonstrate that some human hybridoma lupus anticoagulant antibodies can react with both live and fixed platelets and that these interactions are due in part to dDNA, protein, and phospholipid epitopes. No significant correlations were found between the lupus anticoagulant activity and the antiplatelet, anti-dDNA, and antiphospholipid activities of a given hybridoma lupus anticoagulant antibody, but strong positive correlations were seen between antiplatelet activity, anti-dDNA, and antiphospholipid reactivities.

MATERIALS AND METHODS

Production and Selection of Human-Human Hybridoma Lupus Anticoagulants

Venous blood (50 ml) was obtained from 13 patients who satisfied the revised American Rheumatism Association criteria for the classification of SLE (16), 2 apparently healthy women with multiple spontaneous abortions, but with completely normal laboratory tests and serology, and 4 healthy individuals. Of the SLE patients, 7 of 13

had a lupus anticoagulant, 9 of 13 had anti-dDNA antibodies (> 30% binding in a Farr radioimmunoassay (RIA)), 6 of 12 tested had a false positive Venereal Disease Research Laboratory (VDRL) serological test for syphilis, and 5 of 10 tested had a positive anticardiolipin test ($OD_{410} \geq 0.40$ by enzyme-linked immunosorbent assay (ELISA)). Of the 6 healthy subjects (2 with multiple spontaneous abortions), 0 of 6 had a lupus anticoagulant, 0 of 3 tested had anti-dDNA antibodies, 0 of 3 tested had a false positive VDRL, and 0 of 6 had a positive anticardiolipin test. The peripheral blood lymphocytes were isolated on a Ficoll-Hypaque density gradient and were fused with the GM 4672 human lymphoblastoid cell line (IgG-producing), obtained from the Cell Repository Institute of Medical Research, Camden, NJ, at a cell ratio of 1:1, using 44.4% polyethylene glycol. The fusion, plating, and cloning procedures have been described elsewhere in detail (17). The hybridoma clones were screened and selected for lupus anticoagulant and anti-dDNA activities. Nine hybridoma IgM antibodies which had neither of these activities were selected as negative controls. All hybridoma antibodies except one (9702, an IgG) were IgM immunoglobulins, as determined by a solid phase RIA (18).

Detection of Hybridoma Lupus Anticoagulants

Hybridoma supernatants were tested for the production of lupus anticoagulants using a modified activated partial thromboplastin time (APTT) assay (10). Clotting times were determined using the General Diagnostics Coag-a-mate Iso single channel instrument (Warner-Lambert Co., Morris Plains, NJ). Fifty microliters of hybridoma culture supernatant was diluted with an equal volume of freshly reconstituted Verify Normal Citrate (pooled normal human plasma) (General

Diagnostics, Scarborough, Ontario, Canada) in a cuvet in a disposable circular test tray (General Diagnostics). One hundred microliters of a 1/32 dilution of APTT reagent (General Diagnostics) in 0.025 M Tris-buffered saline, pH 7.4, which had been prewarmed to 37°C, were then added and the mixture incubated for 5 min at 37°C. After mixing, 100 µl of 0.025 M $CaCl_2$ were added to the cuvet through the reagent incubation arm to initiate the clotting sequence. The clotting time was displayed on the Coag-a-mate digital timer and recorded. All samples were tested in duplicate.

In a previous study, 53 of 67 hybridoma antibody supernatants gave APTT values below or equal (± 1.2 sec.) to that of the GM 4672 control medium (mean APTT = 60 sec). An antibody was defined as having anticoagulant activity only if its APTT exceeded the APTT of the GM 4672 control by greater than 5 SD (6 sec) (10). A total of 27 antibodies satisfied this criterion, but only 24 were available in sufficient quantities to be tested in all assays.

The prolongation of the APTT assay by all hybridoma lupus anticoagulant antibodies was inhibited by hexagonal but not lamellar PF (10). In similar inhibition studies with plasma samples, hexagonal PF inhibited the anticoagulant activity of plasmas with known lupus anticoagulant activity, but not plasmas with anti-Factor VIII or factor deficiencies (J. Rauch and A. S. Janoff, unpublished results).

Detection of Antiplatelet Antibody by FLISA

Following the procedure of Asano et al. (19), polystyrene Immulon-2 (Dynatech) 96-well plates were coated with 100 µl/well of platelets (10^8/ml) isolated from normal individuals and fixed with

2.5% glutaraldehyde. The plates were stored at -70°C for up to 2 mo and washed 5 times with TBS before using. Then 75 µl of hybridoma supernatant was added to each well in duplicate and incubated for 90 min at room temperature. After washing five times, the plates were incubated with alkaline phosphatase conjugated anti-human polyvalent immunoglobulin (Sigma Chemical Co., St. Louis, MO) for 90 min at room temperature. The colour was developed by adding 100 µl of p-nitrophenyl phosphate disodium (Sigma) and the OD values were read at 410 nm using a Dynatech MR600 ELISA reader.

Detection of Antiplatelet Antibody by RIA

Platelets were isolated from citrated blood of normal donors by centrifugation of the blood at 180 x g for 15 min to obtain platelet-rich plasma, followed by centrifugation of the platelet-rich plasma on a Percoll (Pharmacia, Upsalla, Sweden) gradient 300 x g for 20 min. The platelet-containing layer was removed and washed 3 times with Hanks' balanced salt solution containing 4 mM EDTA, and the washed platelets were resuspended in the same buffer and counted. Fifty microliters of platelets (3×10^9 platelets/ml) in assay buffer (Hanks' - 0.1% bovine serum albumin (BSA) - 1% bovine gamma globulin (BGG), pH 7.2) was placed into 12 x 75 mm glass tubes. The platelets were then preincubated with 50 µl assay buffer for 30 min at 37°C. Fifty microliters of hybridoma antibody were added to each tube and incubated for 1 hr at 37°C. The platelets were then washed twice with 0.5 ml of assay buffer, and centrifuged for 10 min at 190 x g in an IEC clinical centrifuge (IEC, Needham Heights, MA). Aliquots of 100 µl of assay buffer and 100 µl of ^{125}I-labelled rabbit anti-human

IgM were added and incubated for 16 to 20 hr at 4°C. The next day, the platelets were washed three times with 0.5 ml assay buffer and centrifuged for 10 min at 190 x g in an IEC clinical centrifuge. The pellets were counted for 1 minute in a Beckman Gamma counter.

Pretreatment of Platelets with DNA and Enzymes

Pretreatment of the platelets with dDNA (Millipore Corporation, Freehold, NJ) followed by buffer, DNAase (Cooper Biomedical, Malvern, PA), or RNAase (Cooper Biomedical) was performed in a two step procedure as follows. Fifty microliters of platelets (3×10^8 platelets/ml) in platelet RIA buffer were incubated with 100 µl of dDNA (0.5 mg/ml in saline sodium citrate buffer, pH 7.0) or 100 µl of assay buffer for 45 min at 37°C. After the incubation, the platelets were washed with 0.5 ml of assay buffer by centrifugation for 10 min at 190 x g. The supernatants were aspirated and 100 µl of DNAase (1 mg/ml in Hanks' solution + 6.25 mM $MgSO_4$ + 5 mM $CaCl_2$) or 100 µl RNAase (1 mg/ml in Hanks' solution) was incubated with the platelets for 45 min at 37°C. The platelets were then washed twice with 0.5 ml assay buffer by centrifugation for 10 min at 190 x g and subjected to the platelet-binding assay described above.

In the pretreatment of platelets with enzymes only, the platelets were incubated for 45 min at 37°C with 100 µl of assay buffer or enzyme (DNAase, 1 mg/ml + 6.25 mM $MgSO_4$ + 5 mM $CaCl_2$; RNAase, 1 mg/ml; phospholipase C (Cooper Biomedical), 0.5 mg/ml + 40 mM $CaCl_2$; phospholipase A_2 (Boehringer Mannheim, Dorval, Quebec), 0.5 mg/ml + 5 mM $CaCl_2$; protease (Cooper Biomedical), 0.5 mg/ml + 5 mM $CaCl_2$, or trypsin (Cooper Biomedical), 0.5 mg/ml). The platelets

were then washed twice with assay buffer by centrifugation for 10 min at 190 x g and subjected to the platelet-binding assay described above.

Detection of Antiphospholipid Antibodies

Cardiolipin, egg PE, PS, PI, and egg PC were purchased from Avanti Polar Lipids (Birmingham, AL), dissolved in chloroform, and stored at -70°C. For coating of ELISA plates, the chloroform was evaporated using nitrogen gas and the phospholipids were suspended in phosphate buffered saline (PBS) (pH 7.3) at a concentration of 90 µg/ml and hydrated above their transition temperatures for 1 hour. Fifty microliters of the phospholipid suspension was added to polystyrene Immulon-2 plates well (Dynatech) and dried for 16 to 20 hr at 37°C. To control for nonspecific antibody binding, one half of each plate was coated with phospholipid while the other half was coated with PBS. The coated plates were washed 3 times with PBS containing 1 mM EDTA and 0.3% gelatin (prewarmed to 37°C). A 5 min incubation was used between each wash to block any free binding sites. Test samples (50 µl/well) were incubated in duplicate on phospholipid and PBS coated wells for 3 hr at room temperature. The plates were then washed three times with PBS containing 0.4% BSA, and 75 µl of alkaline phosphatase-conjugated polyvalent goat anti-human Ig (Sigma) diluted in PBS-0.4% BSA was added and the plates incubated for 16 to 20 hr at room temperature. The plates were then washed 3 times with PBS-0.4% BSA; and 100 µl of p-nitrophenyl phosphate disodium (Sigma) in 50 mM carbonate buffer, pH 9.5, containing 2 mM $MgCl_2$ were added and incubated for 1 hr at 37°C. The resulting

colour change was recorded at 410 nm using a Dynatech MR600 ELISA reader.

Anti-dDNA RIA

RIA for anti-dDNA antibody activity was performed as previously described (18).

Statistics

The negative ranges of the ELISA and RIA results were expressed by 99% confidence intervals (means ± 2 SE) which were calculated from five known negative samples in four repeated assays.

The correlation coefficients of the different antibody activities were calculated using Pearson's (r) or Spearman's rank (ρ) correlation coefficients, depending on their dot plots, and their significance was estimated from correlation tables (20,21). Association was assessed using a two-tailed Fisher's exact test.

RESULTS

Production and Selection of Human-Human Hybridoma Autoantibodies

Fifty hybridoma clones were selected for study from 19 human fusions of peripheral blood lymphocytes from 13 SLE patients, 2 apparently healthy women with recurrent spontaneous abortions, and 4 normal individuals. The hybridoma clones were initially screened and selected for lupus anticoagulant and anti-dDNA activities. A total of 22 SLE-derived antibodies and 19 normal-derived antibodies with either one or both of these activities and 6 SLE-derived and 3 normal-derived antibodies with neither of these activities were selected for this study. Thus, of the 50 hybridoma antibodies, 9 had

both lupus anticoagulant and anti-dDNA activities, 16 had lupus anticoagulant activity only, 16 antibodies had anti-dDNA activity only, and 9 did not have either lupus anticoagulant or anti-dDNA activity. All of the hybridoma antibodies except one were IgM immunoglobulins. This single IgG hybridoma antibody had lupus anticoagulant activity only and showed similar reactivities to the IgM lupus anticoagulant antibodies. Supernatants were not available from all of the 50 hybridoma clones to be studied in each of the assays.

Comparison of the Direct Binding of Hybridoma Antibodies to Live and Glutaraldehyde Fixed Platelets

Of the 50 hybridoma antibodies studied, 32 antibodies were tested in direct binding assays to compare their binding to live and glutaraldehyde fixed platelets by RIA and ELISA, respectively (Table I). Five antibodies known to be negative in all assays were tested in each assay and had a mean value of 572 ± 295 (mean ± 2 SE) cpm in the RIA and 0.02 ± 0.05 (mean ± 2 SE) OD_{410} in the ELISA. A positive antiplatelet antibody by RIA was defined as having greater than 900 cpm and 17 of 32 hybridoma supernatant fulfilled this criterion.

Table I. Hybridoma Antibody Binding to Live and Fixed Platelets

Binding to Fixed Platelets by ELISA	Binding to Live Platelets by RIA	
	Positive	Negative
Positive	15	1
Negative	2	14

Table II. Association of Lupus Anticoagulant Activity with Anti-platelet, Antiphospholipid, and Anti-dDNA Reactivities

Antibody Binding Reactivity	APTT Activity[a]		Correlation Coefficient[b]	Association[c]
	Positive	Negative		
Anti-Platelet Positive[d]	7	14	r=-0.174 NS	0.05
Anti-Platelet Negative	18	9		
Anti-dDNA Positive[e]	9	16	r=-0.076 NS	< 0.05
Anti-dDNA Negative	16	8		
Anti-CL Positive[d]	3	9	r=-0.145 NS	0.05
Anti-CL Negative	22	15		
Anti-PE Positive[d]	6	13	r=-0.168 NS	NS
Anti-PE Negative	18	11		
Anti-PS Positive[d]	2	4	r=-0.049 NS	NS
Anti-PS Negative	17	14		
Anti-PI Positive[d]	2	5	r=-0.004 NS	NS
Anti-PI Negative	17	11		
Anti-PC Positive[d]	1	4	r=-0.033 NS	NS
Anti-PC Negative	20	14		

[a] A positive APTT was defined as greater than 66.0 sec, where the mean ± SE of the negative hybridoma antibodies was 61.7 ± 4.2.

[b] Pearson's correlation coefficient (r) was determined from the numerical data and was not based on the assignment of positive or negative assay results. NS = not significant.

[c] The association, as measured by a Fisher's exact test, is shown as a p value.

[d] A positive ELISA value was greater than 0.08 OD_{410} units for antiplatelet reactivity, greater than or equal to 0.12 OD_{410} units for anti-PE, and greater than or equal to 0.10 OD_{410} units for anticardiolipin (CL), anti-PS, anti-PI, and anti-PC.

[e] A positive dDNA binding was greater than or equal to 750 cpm, where the mean ± 2 SE of the negative controls was 302 ± 178 cpm.

A positive antiplatelet ELISA result was defined as an OD_{410} value greater than 0.08 and 16 of 32 hybridoma antibodies were positive by ELISA. Similar results of hybridoma antibody binding to live and fixed platelets were observed with 29 of the 32 antibodies and the kappa value was 0.81, which denotes excellent agreement between the assays (Table I). As the results of the ELISA and RIA were very comparable, the antiplatelet ELISA was used for all further assays, except for the dDNA and enzyme pretreatment studies. In the latter studies, live platelets are required for dDNA binding and enzymatic modification of the platelet membrane.

Association of Lupus Anticoagulant Activity with Antiplatelet, Anti-phospholipid, and Anti-DNA Reactivities

Fifty hybridoma antibodies were tested to study the relationship of their lupus anticoagulant activity to their antiplatelet, anti-phospholipid and anti-dDNA properties (Table II). Sufficient supernatant was not available for simultaneous testing in each assay and, thus, only 48 of the 50 antibodies were tested in the antiplatelet and anti-dDNA assays, and between 42 and 50 antibodies were analyzed in the various antiphospholipid assays. No significant correlations were found between the lupus anticoagulant activity of the hybridoma antibodies and their anti-egg PE, anti-PS, anti-PI, and anti-PC reactions. Surprisingly, significant negative associations were demonstrated for lupus anticoagulant activity and antiplatelet reactivity ($p < 0.05$), anti-dDNA reactivity (p 0.05), and anticardiolipin reactivity ($p = 0.05$).

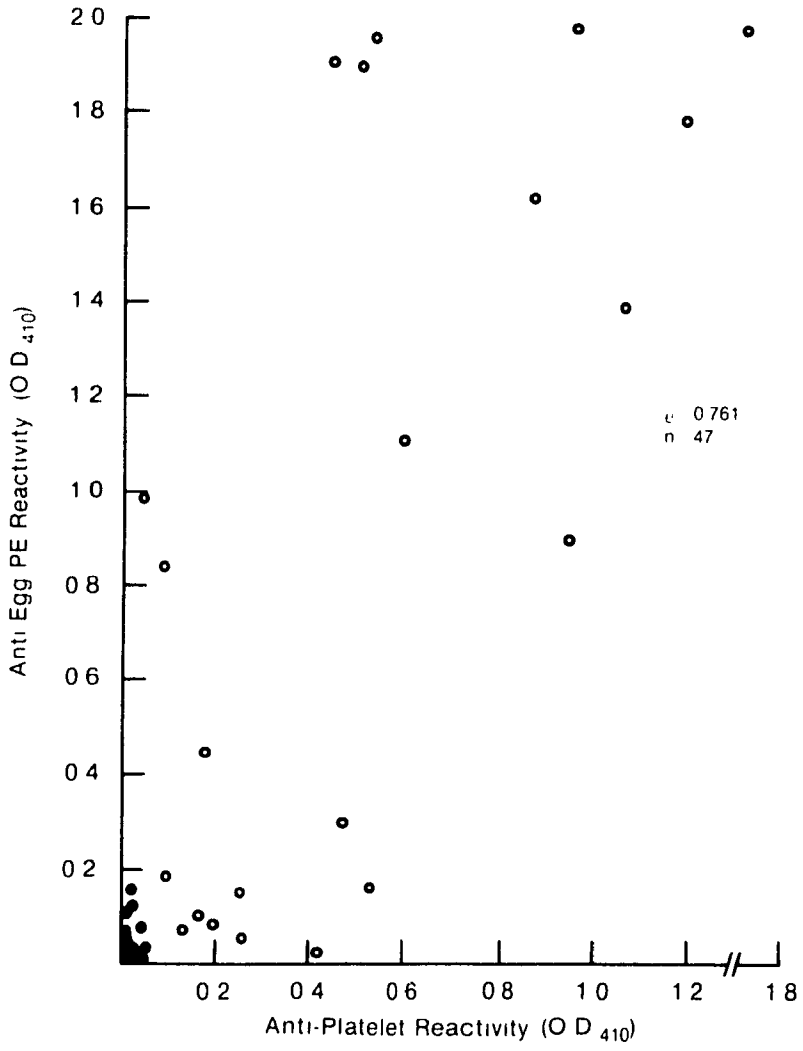


Figure 1. Correlation of Anti-Egg Phosphatidylethanolamine Reactivity with Antiplatelet Reactivity

Anti-egg PE and antiplatelet reactivities were measured on 47 hybridoma antibodies in ELISA assays. Each point represents the mean of duplicate samples. The Spearman's rank correlation coefficient (ρ) was 0.761 ($p < 0.001$).

Table III. Correlation of Antiplatelet Reactivity with Antiphospholipid and Anti-dDNA Reactivities

Antibody Binding Reactivity[a]	Binding to Fixed platelets		Correlation Coefficient[b]	Association[c]
	Positive	Negative		
Anti-dDNA Positive	20	1	0.745	< 0.00001
Anti-dDNA Negative	4	23		
Anti-CL Positive	11	0	0.748	0.00002
Anti-CL Negative	10	27		
Anti-PE Positive	16	2	0.761	0.00001
Anti-PE Negative	5	24		
Anti-PS Positive	6	0	0.636	< 0.01
Anti-PS Negative	11	19		
Anti-PI Positive	7	0	0.655	0.01
Anti-PI Negative	10	17		
Anti-PC Positive	5	0	0.556	0.05
Anti-PC Negative	12	20		

[a] Positive and negative values were as defined in Table II.

[b] Spearman's rank correlation coefficient (ρ) was determined by ranking the numerical data and was not based on the assignment of positive or negative assay results.

[c] The association, as measured by a Fisher's exact test, is shown as a p value.

Correlation of Antiplatelet with Antiphospholipid and Anti-dDNA Reactivities

Figure 1 and Table III demonstrate a significant positive correlation of antiplatelet reactivity with anti-egg PE (r = 0.761, p < 0.001) and anticardiolipin activity (r = 0.748, p < 0.001). There were also various degrees of correlation found between the antiplatelet reactivity and the anti-PC (r = 0.556, p < 0.001), anti-PI (r = 0.655, p < 0.001), and anti-PS (r = 0.636, p < 0.001) reactivities of the hybridoma antibodies (Table III). The p values for the association of antiplatelet reactivity with the different antiphospholipid reactivities were completely consistent with the correlation coefficients.

Figure 2 and Table III demonstrate a significant correlation between the antiplatelet and anti-dDNA reactivities of 48 hybridoma antibodies (r = 0.745, p < 0.001). Of the 22 anti-dDNA antibodies, all reacted with platelets. All anti-dDNA negative antibodies (n = 26), with one exception, were antiplatelet negative. The association of the antiplatelet and anti-dDNA reactivities of the hybridoma antibodies was highly significant (p < 0.00001).

Effects of Pretreatment of Platelets with dDNA and Enzymes

The effect of the pretreatment of platelets with DNAase and RNAase is shown for two antiplatelet hybridoma antibodies in Figure 3. For both antibodies 1403 and 1400, DNAase treatment resulted in a small decrease (18 and 25%, respectively) in antiplatelet antibody binding, while RNAase treatment had no effect on the binding to platelets. In contrast, preincubation of the platelets with dDNA

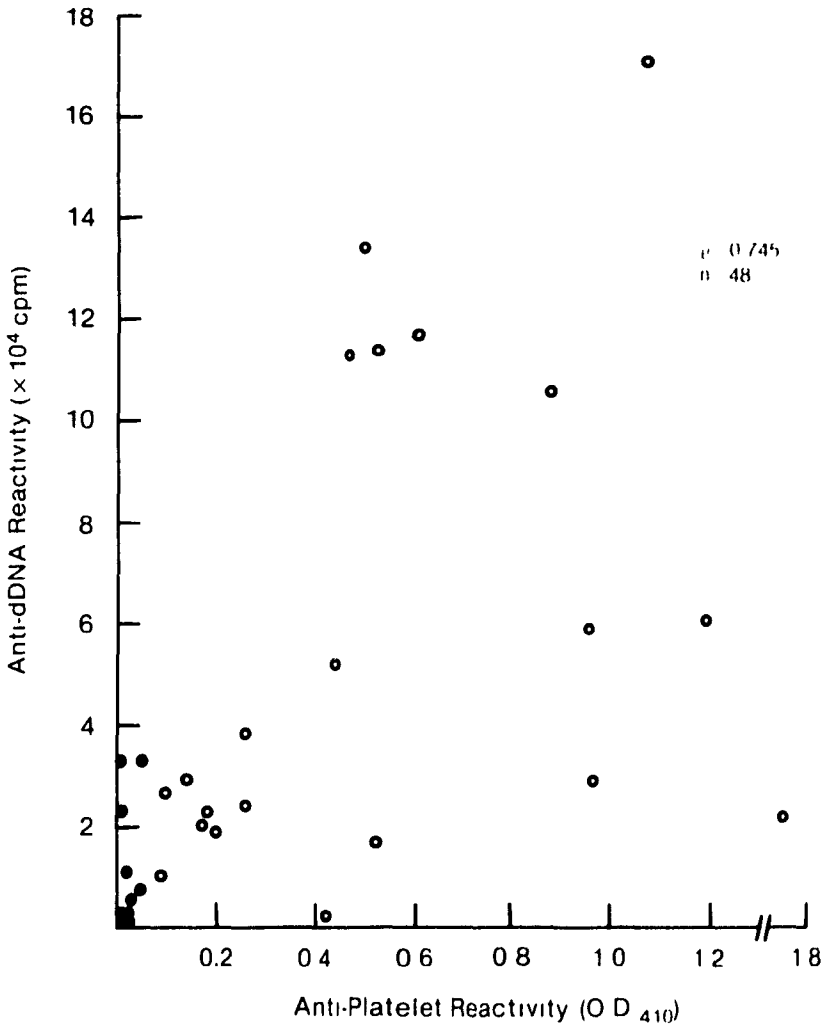


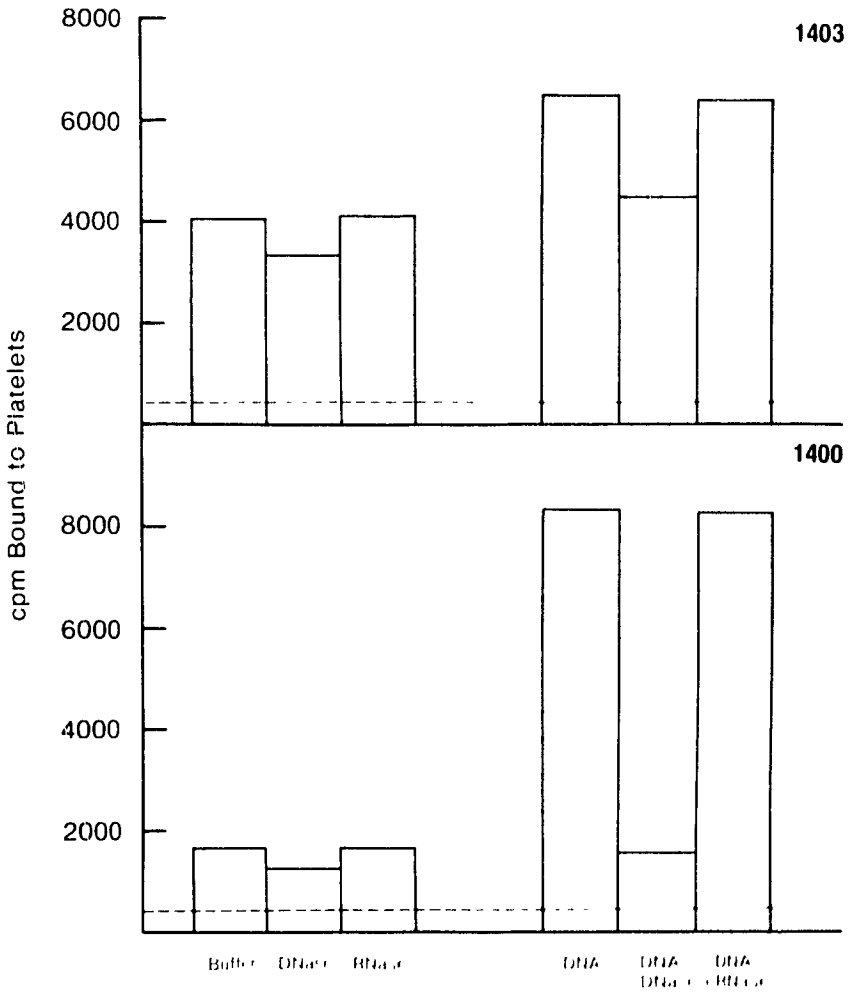
Figure 2. Correlation of Anti-dDNA Reactivity with Antiplatelet Reactivity

Anti-dDNA and antiplatelet reactivities were measured on 48 hybridoma antibodies by RIA and ELISA assays, respectively. Each point represents the mean of duplicate samples. The Spearman's rank correlation coefficient (r) was 0.745 ($p < 0.001$).

Figure 3. Effects of Pretreatment of Platelets with DNAase, RNAase, and DNA on Platelet Binding of Hybridoma Antibodies 1403 and 1400

In addition to their antiplatelet reactivity, both 1403 and 1400 had strong anti-dDNA and weak anticardiolipin reactivity. Antibody 1403 had lupus anticoagulant activity, while 1400 did not. The bars on the left side of the figure denote cpm bound to platelets pretreated with buffer, DNAase, or RNAase. Bars on the right side of the figure denote cpm bound to platelets pretreated first with DNA and then with buffer, DNAase, or RNAase. Each bar represents the mean of duplicate samples. The dotted line shows the mean cpm bound by a negative control hybridoma antibody.

Figure 3


8000
6000
4000
2000
1403
1400
cpm Bound to Platelets
DNA
DNA
DNA

resulted in enhanced binding (160% for 1403 and 495% for 1400). This enhancement was completely abolished by incubation of the DNA-treated platelets with DNAase but not RNAase.

The effects of pretreatment of platelets with different enzymes is shown in Figure 4. The platelet binding of two hybridoma antibodies (9200, 9700) in duplicate experiments (the means ± SE are shown) to these enzyme-treated platelets further demonstrates the different binding specificities of the hybridoma antibodies. The binding of antibody 9200 was decreased 20% by phospholipase A_2, but increased approximately 20% by pretreatment with phospholipase C. Protease caused a 16% decrease in the binding of antibody 9200, whereas DNAase and trypsin had little effect on this antibody's binding. In contrast, the binding of antibody 9700 to platelets was decreased by approximately 28% and 43% after treatment of the platelets with DNAase and trypsin, respectively, but unaffected by RNAase, phospholipases A_2 and C, and protease, suggesting that this antibody had a specificity for an epitope on the platelet membrane composed of both dDNA and protein.

DISCUSSION

Strong clinical associations between lupus anticoagulant antibodies (or anticardiolipin antibodies) and thrombocytopenia, thrombosis, and multiple spontaneous abortions in SLE suggest that the lupus anticoagulant antibodies may be important in the pathogenesis of these clinical manifestations. Reactivity of the lupus anticoagulant antibodies with platelet and/or endothelial cell

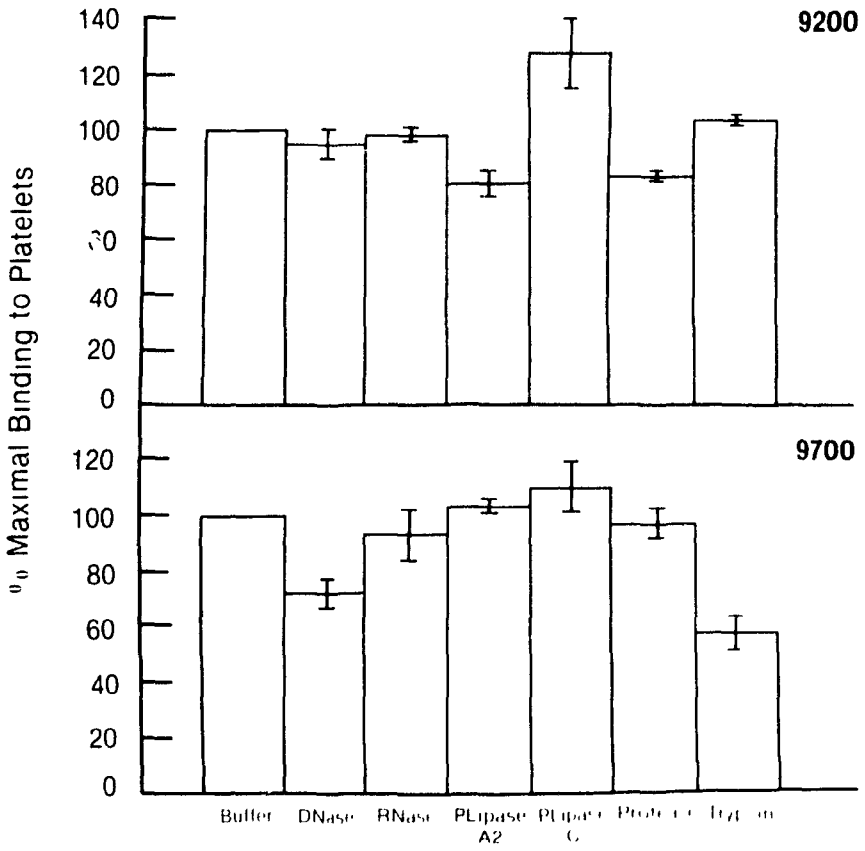


Figure 4. Effects of Pretreatment of Platelets with DNAase, RNAase, Phospholipase A_2 (PLipase A_2), Phospholipase C (PLipase C), Protease, and Trypsin on Hybridoma Antibodies 9200 (Top), and 9700 (Bottom)

In addition to its antiplatelet reactivity, antibody 9200 had strong anti-dDNA, weak anticardiolipin, and no lupus anticoagulant activity. In contrast, antibody 9700 had weak anti-dDNA, no anticardiolipin, and fairly strong lupus anticoagulant activity. The bars denote percent (%) maximal binding of ^{125}I-labelled anti-human IgM to platelets after incubation with the indicated hybridoma antibody and represent the means of duplicate experiments, with SE, as shown.

membrane phospholipids is a likely mechanism. In this study, hybridoma lupus anticoagulant activity did not correlate with antiplatelet or antiphospholipid reactivities and, in fact, showed a negative association with antiplatelet, anti-dDNA, and anti-cardiolipin reactivities. However, the antiplatelet reactivity of the hybridoma antibodies was strongly correlated with anti-phospholipid and anti-dDNA reactivities. Further evaluation of the platelet membrane determinants recognized by the hybridoma antibodies suggested that protein and DNA or protein and phospholipid may form two of the possible platelet determinants.

The possibility that platelet reactivity may be due to phospholipids on the platelet membrane led us to investigate the phospholipid specificities of these antibodies. The phospholipid composition of the human platelet membrane is 17% sphingomyelin, 38% PC, 10% PS, 5% PI, 27% PE, 1% lysolecithin, and 0.5% cardiolipin (22). These phospholipids are distributed asymmetrically between the interior and exterior half of the platelet cell membrane (23,24), and approximately 91% of the sphingomyelin, 40% of the PC, 34% of the PE, and less than 6% of the PS (+ PI) forms the outer half of the surface membrane (23). The initiation of clotting requires the exposure of "inner-monolayer" lipids such as PE and the negatively charged lipid PS on the outer monolayer of platelets (24).

The hybridoma antibodies were tested for reactivity with cardiolipin, egg PE, PS, PI, and egg PC in ELISA, and a close correlation was demonstrated between antiplatelet and anticardiolipin reactivities ($r = 0.748$; $p < 0.001$), and antiplatelet and anti-PE

(r = 0.761; $p < 0.001$) reactivities. A similar correlation was noted with platelet and dDNA-binding reactivities (r = 0.745; $p < 0.001$). It was found that DNAase, but not RNase, diminished the binding of some antiplatelet hybridoma antibodies by 10-30%. DNA pretreatment of platelets greatly enhanced the binding of the hybridoma antibodies to platelets, and this enhancement was completely abolished by DNAase but not RNAase digestion, indicating that DNA could bind to the platelets and thereby augment hybridoma antiplatelet antibody binding and that this DNA could be removed by DNAase enzyme digestion. However, endogenously bound DNA may not be an important epitope, as DNAase digestion of untreated platelets often had a minimal effect on the binding of antiplatelet antibodies to platelets.

Pretreatment of platelets with other enzymes, including two phospholipases, protease and trypsin, affected the binding of some of the hybridoma antibodies. The most dramatic effects were seen on antibody 9700, where DNAase and trypsin decreased the antibody binding by 28% and 43%, respectively, suggesting that antibody 9700 was binding to a platelet determinant which was composed of both protein and DNA. The binding of another hybridoma antibody (9200) was affected after treatment of platelets with phospholipase A_2, phospholipase C and protease but not by DNAase or trypsin. The binding of this hybridoma antibody to the platelet membrane appears to involve a phospholipid and protein determinant. Decreased binding after treatment with phospholipase A_2 suggests that the fatty acid in the 2-position of a membrane phospholipid may be an important site for antibody binding.

Studies with phospholipases on red cell membranes have shown that the ability of the enzymes to attack membrane phospholipids is dependent on multiple variables, including the substrate specificity of the enzyme and the sidedness of the phospholipids in the membrane (25). Thus, complete abrogation of a phospholipid determinant may not be achieved in an intact cell. Our data suggests that phospholipase may partially expose or destroy epitopes important for some antiplatelet antibodies (e.g. antibody 9200). The fact that phospholipase C treatment resulted in enhanced antibody binding may be due to exposure of cryptic determinants or reaction of the hybridoma antibodies with enzyme bound to the platelet surface. Based on solid phase RIA in which enzymes were coated to polystyrene test tubes, antibody 9200 did not show significant binding to either phospholipase C or A_2, suggesting that the enhanced binding to phospholipase C treated platelets may be due to exposure of a new phospholipid epitope.

Several reports have demonstrated that anti-DNA autoantibodies can bind to platelets (19,26,27). Asano et al. (19) found that DNA inhibited the binding of their hybridoma anti-DNA antibodies to platelets and suggested that these antibodies interact with platelets through the same site responsible for DNA binding. However, enzymatic digestion studies suggested that the platelet epitope did not appear to involve DNA, protein, or sialic acid. A mouse monoclonal anti-DNA autoantibody (PME77) derived from autoimmune B/W mice has also been found to react with platelets and other cell types (27). This antibody recognized identical protein(s) in platelets,

erythrocytes, glomeruli, T and B cells, and neuronal tissue, and, in further studies, the target antigens were shown to be sensitive to proteinase K, papain and trypsin (28).

Although DNA receptors have been described on human platelets (29), our study and those of others (19,27) suggest that DNA is not the major antigen responsible for antibody binding to the platelet surface. Recently, Bennett et al. (30) demonstrated that the majority of SLE patients had serum antibodies which could induce a DNA-receptor defect in normal leukocytes, consistent with the hypothesis that these antibodies may react with the DNA receptor or an associated antigen. It is tempting to speculate that some of our platelet-reactive hybridoma antibodies may react with the DNA receptor on platelets and be able to induce platelet aggregation and serotonin release in a manner analogous to DNA (31). Our data suggest that the antiplatelet reactivity of our hybridoma antibodies is independent of the lupus anticoagulant activity of these antibodies, and that although both reactivities appear to involve phospholipid epitopes, these epitopes are probably not identical and, in the case of the platelet-reactive determinant, may require protein.

Studies of antiplatelet antibodies using sera from patients with immune thrombocytopenic purpura (ITP) have identified specific reactivities of antibodies with glycoproteins IIIa (32), IIb/IIIa (33), and Ib (34). Harris et al. (35) have reported the presence of anticardiolipin antibodies in 30 of 96 patients with chronic autoimmune thrombocytopenic purpura but no overt manifestations of

SLE. However, the levels of platelet-associated antibody did not correlate with either IgG or IgM anticardiolipin antibodies. More recently, the same group has reported that anticardiolipin antibody activity in the sera of five patients with antiphospholipid syndrome could be partially inhibited by freeze-thawed platelets (36). To date, it appears that the antiplatelet antibodies in ITP are directed primarily at specific platelet glycoproteins, whereas the antibodies reactive with platelets in patients with SLE and antiphospholipid syndrome may be more heterogeneous and react with proteins, phospholipids, or DNA on the platelet suface. It will be important to determine whether the proteins responsible for antiplatelet reactivity in SLE are similar or different to those in ITP. The nature of the platelet reactivity of antibodies from SLE patients may be crossreactive, as opposed to the apparently specific reactivity to platelet glycoproteins seen in ITP patients. Hybridoma monoclonal anti-dDNA and antiphospholipid autoantibodies from patients with SLE have been demonstrated to react with DNA, phospholipids, and proteins, including human immunoglobulins and cytoskeletal proteins (9-11,37).

Our findings that antiplatelet reactivity correlated strongly with anti-dDNA and antiphospholipid reactivity and may be due to determinants composed of dDNA and protein, or phospholipid and protein, are in agreement with previously reported cross-reactivities of these antibodies. The lack of correlation, however, of lupus anticoagulant activity with antiplatelet reactivity suggests that additional factors may be required to explain the association of

lupus anticoagulant antibodies with thrombocytopenia and thrombosis.

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REFERENCES

1. **Schleider, M.A., R.L. Nachman, E.A. Jaffee, and M. Coleman.** 1976. A clinical study of the lupus anticoagulants. Blood 48: 499.

2. **Lechner, K.** 1974. Acquired inhibitors in nonhemophilic patients. Haemostasis 3: 65.

3. **Mueh, J.R., K.D. Herbst, and S.I. Rapaport.** 1980. Thrombosis in patients with the lupus anticoagulant. Ann. Int. Med. 92: 156.

4. **Bowie, E.J.W., J.H. Thompson, C.A. Pascuzzi, and C.A. Owen.** 1963. Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. J. Lab. Clin. Med. 62: 416.

5. **Carreras, L.O., and J.G. Vermylen.** 1982. "Lupus" anticoagulant and thrombosis - possible role of inhibition of prostacyclin formation. Thromb. Haemostas. 48: 38.

6. **Boey, M.L., C.B. Colaco, A. Gharavi, K. Elkon, S. Loizou, and G.R.V. Hughes.** 1983. Thrombosis in systemic lupus erythematosus: Striking association with the presence of circulating lupus anticoagulant. Br. Med. J. 287: 1021.

7. **Jungers, P., S. Liote, M.D. Dautzenberg, C. Gazengel, M. Dougados, F. Tron, and J.F. Bach.** 1984. Lupus anticoagulant and thrombosis in systemic lupus erythematosus. Lancet 1: 574.

8. **Dahlbäck, B., I.M. Nilsson, and B. Frohm.** 1983. Inhibition of platelet prothrombinase activity by a lupus anticoagulant. Blood 62: 218.

9. **Rauch, J., H. Tannenbaum, J.-L. Senécal, A.S. Janoff, P.R. Cullis, and M.M. Frojmovic.** 1987 Polyfunctional properties of hybridoma lupus anticoagulant. J. Rheum. (In Press).

10. **Rauch, J., M. Tannenbaum, H. Tannenbaum, H. Ramelson, P.R. Cullis, C.P.S. Tilcock, M.J. Hope, and A.S. Janoff.** 1986. Human hybridoma lupus anticoagulants distinguish between lamellar and hexagonal phase lipid systems. J. Biol. Chem. 261: 9672.

11. **Janoff, A.S., and J. Rauch.** 1986. The structural specificity of anti-phospholipid antibodies in autoimmune disease. Chem. Phys. Lipids 40: 315.

12. **Thiagarajan, P., S.S. Shapiro, and L. de Marco.** 1980. Monoclonal immunoglobulin M lambda coagulation inhibitor with phospholipid specificity. Mechanism of lupus anticoagulant. J. Clin. Invest. 66: 397.

13. **Exner, T., K.A. Richard, and H. Kronenberg.** 1975. Studies on phospholipids in the action of a lupus coagulation inhibitor. Pathology 7: 319.

14. **Harris, E.N., A.E. Gharavi, M.L. Boey, B.M. Patel, C.G. Mackworth-Young, S. Loizou, and G.R.V. Hughes.** 1983. Anticardiolipin antibodies: Detection by radioimmunoassay and

association with thrombosis in systemic lupus erythematosus. Lancet 2: 1211.

15. **Harris, E.N., A.E. Gharavi, A. Tincani, J.K.H. Chan, H. Englert, P. Mantelli, F. Allegro, G. Ballestrieri, and G.R.V. Hughes.** 1985. Affinity purified anticardiolipin and anti-DNA antibodies. J. Lab. Clin. Immunol. **17**: 155.

16. **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 erythematosus. Arthritis Rheum. **25**: 1271.

17. **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.

18. **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.

19. **Asano, T., B.C. Furie, and B. Furie.** 1985. Platelet binding properties of monoclonal lupus autoantibodies produced by human hybridomas. Blood **66**: 1254.

20. **Milton, J.S., and J.O. Tsokos.** Statistical methods in the Biological and Health Sciences. McGraw-Hill Inc., New York, 1983, p. 351.

21. **Pipkin, F.B.** Medical Statistics Made Easy. Churchill

Livingstone, New York, 1984, pp. 94-96 and p. 134.

22. **Marcus, A.J., H.L. Ullman, and L.B. Safier.** 1969. Lipid composition of subcellular particles of human blood platelets. J. Lipid Res. **10**: 108.

23. **Chap, H.J., R.F.A. Zwaal, and L.L.M. Van Deenen.** 1977. Action of highly purified phospholipases on blood platelets. Evidence for an asymmetric distribution of phospholipids in the surface membrane. Biochim. Biophys. Acta. **467**: 146.

24. **Zwaal, R.F.A.** 1978. Membrane and lipid involvement in blood coagulation. Biochem. Biophys. Acta. **515**: 163.

25. **Zwaal, R.F.A., B. Roelofsen, P. Comfurius, and L.L.M. Van Deenen.** 1975. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. Biochim. Biophys. Acta **406**: 83.

26. **Shoenfeld, Y., S.C. Hsu-Lin, J.E. Gabriels, L.E. Silberstein, B.C. Furie, B. Furie, B.D. Stollar, and R.S. Schwartz.** 1980. Production of autoantibodies by human-human hybridomas. J. Clin. Invest. **70**: 205.

27. **Jacob, L., M.-A. Lety, D. Louvard, D., and J.-F. Bach.** 1985. Binding of a monoclonal anti-DNA autoantibody to identical protein(s) present at the surface of several human cell types involved in lupus pathogenesis. J. Clin. Invest. **75**: 315.

28. **Jacob, L., M.-A. Lety, J.-F. Bach, J.-F., and D. Louvard.** 1986. Human systemic lupus erythematosus sera contain antibodies against cell-surface protein(s) that share(s) epitope(s) with DNA. Proc. Natl. Acad. Sci. USA **83**: 6970.

29. **Clejan, L., and M. Menahem.** 1977. Binding of deoxyribonucleic acid to the surface of human platelets. Acta Haematol. (Basel) 58: 84.

30. **Bennett, R.M., J.S. Peller, and M.M. Merritt.** 1986. Defective DNA-receptor function in systemic lupus erythematosus and related diseases: Evidence for an autoantibody influencing cell physiology. Lancet 1: 186.

31. **Fiedel, B.A., J.S. Schoenberger, and H. Gewurz, H.** 1979. Modulation of platelet activation by native DNA. J. Immunol. 123: 2479.

32. **Beardsley, D.S., J.E. Spiegel, M.M. Jacobs, R.I. Handin, and S.E. Lux IV.** 1984. Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J. Clin. Invest. **74**: 1701.

33. **Woods, V.L., E.H. Oh, D. Mason, and R. McMillan.** 1984. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. Blood **63**: 368.

34. **Woods, V.L., Y. Kurata, R.R. Montgomery, P. Tani, D. Mason, E.H. Oh, and R. McMillan.** 1984. Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood **64**: 156.

35. **Harris, E.N., A.E. Gharavi, U. Hegde, G. Denu, S.H. Morgan, H. Englert, J.K.H. Chan, R.A. Asherton, and G.R.V. Hughes.** 1985. Anticardiolipin antibodies in autoimmune thrombocytopenic purpura. Br. J. Haematol. **59**: 231.

36. **Khamashta, M.A., E.N. Harris, A.E. Gharavi, L. Morillas, and**

G.R.V. Hughes. 1987. Towards an immune mediated mechanism for thrombosis-anti-phospholipid (αPL) antibody binding to platelet membranes. Arth. Rheum. **30**: S53 (abstr.).

37. **André-Schwartz, J., S.K. Datta, Y. Shoenfeld, D.A. Isenberg, B.D. Stollar, and R.S. Schwartz.** 1984. Binding of cytoskeletal proteins to monoclonal anti-dDNA lupus autoantibodies. Clin. Immunol. Immunopathol. **31**: 261.

CHAPTER IV

DIFFERENCES BETWEEN HUMAN HYBRIDOMA PLATELET-BINDING ANTIBODIES DERIVED FROM SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND NORMAL INDIVIDUALS

RUNNING TITLE

Platelet-Binding Autoantibodies

KEY WORDS

SLE, normal, hybridoma autoantibodies, platelet-binding, anti-phospholipid, polyspecificity

ABSTRACT

The binding and functional activities of platelet-binding hybridoma autoantibodies from SLE patients were compared with those derived from normal individuals. Twenty-nine SLE-derived hybridoma antibodies and 20 normal-derived hybridoma antibodies were analyzed for binding to glutaraldehyde fixed platelets, dDNA and phospholipids, and for lupus anticoagulant activity. Twenty-four of the 29 SLE-derived antibodies and 9 of the 20 normal-derived antibodies showed one or more activities in these assays. Of the 24 SLE-derived antibodies, 8 (33.3%) were reactive in only one assay (monospecific), while the other 16 (66.7%) had more than one of these activities (polyspecific). In contrast, none (0%) of the 9 normal-derived antibodies with known activities were monospecific, while all 9 (100%) showed polyspecificity. Statistical analyses demonstrated that there was no correlation of anti-dDNA activity with anti-platelet and most anti-phospholipid activities for the SLE-derived antibodies, and strong positive correlations between these reactivities for the normal-derived antibodies. Differences were observed in Western blotting analyses; SLE-derived antibodies bound more specifically to individual platelet proteins than normal-derived antibodies. In ^{51}Cr release assays, all of the SLE-derived platelet-binding antibodies were cytotoxic to platelets, while none of the normal-derived antibodies showed significant cytotoxicity. Our results suggest that hybridoma platelet-binding autoantibodies derived from SLE patients exhibit greater antigen specificity and functional activity than similar antibodies derived from normal individuals.

INTRODUCTION

Antibodies with autoreactivity have been reported to exist in the sera of normal mice and human subjects, suggesting that individuals without apparent autoimmune disease have B cells which can be activated to secrete autoantibodies[1-8]. These autoantibodies are often characterized by extensive crossreactivity (polyspecificity)[5,6] and idiotypic connectivity[4,7,8]. The production of autoantibodies by normal individuals raises several important questions regarding the differences between the autoantibodies derived from patients with systemic lupus erythematosus (SLE) and normal individuals, and the mechanisms by which autoantibodies cause pathological abnormalities in patients but not in normal individuals. Although individual studies have examined the characteristics of either normal-derived[1,3,7,11] or autoimmune-derived[12-20] antibodies with autoreactivity, few laboratories[4,21,22] have compared autoantibodies from normal and autoimmune subjects. In particular, it has been difficult to produce human hybridoma antibodies from peripheral blood lymphocytes of normal subjects to compare with similarly derived antibodies of SLE patients.

In our laboratory, we have produced human hybridoma platelet-binding antibodies from both SLE patients and normal individuals[23]. In a previous study, we evaluated the lupus anticoagulant, anti-phospholipid and anti-platelet properties of a group of hybridoma antibodies derived from both SLE patients and normal individuals[23]. Statistical analyses performed on the SLE and normal-derived antibody pool showed no correlation between lupus anticoagulant antibody

activity and anti-platelet, anti-dDNA, or anti-phospholipid antibody reactivity. In contrast, anti-platelet reactivity of these hybridoma antibodies was found to correlate strongly with anti-dDNA and several anti-phospholipid reactivities. In the present study, we divided the hybridoma antibodies into two groups according to their origin from either SLE or normal individuals and analyzed the correlations between anti-platelet, anti-dDNA and anti-phospholipid reactivities, as well as differences and similarities between the SLE and normal-derived hybridoma antibodies in their binding and functional characteristics. The results demonstrate that many of the platelet-binding antibodies derived from either SLE patients or normal individuals are polyspecific, but that the polyspecific antibodies derived from SLE patients differ from normal-derived antibodies in their binding and functional activities. Our data show that most SLE-derived autoantibodies exhibit greater antigen specificity and functional activity than autoreactive antibodies derived from normal individuals, suggesting that these differences in reactivity may be relevant to differences in pathogenicity in vivo.

MATERIALS AND METHODS

Production, Selection, and Purification of Human Hybridoma Antibodies

Human hybridomas were produced from fusions of the GM 4672 lymphoblastoid cell line (IgG-producing) with peripheral blood lymphocytes from 13 patients with SLE and 4 healthy normal individuals, as previously described[15]. All of the 13 patients fulfilled the American Rheumatism Association revised criteria for

the classification of SLE[24]. The hybridoma clones were screened and selected for anti-dDNA, anti-platelet and lupus anticoagulant activities. Antibodies positive for any of these 3 reactivities were tested for binding to different phospholipids. Hybridoma clones were screened for IgM and IgG production by solid phase radioimmunoassay (RIA)[25]. All hybridoma antibodies used in this study were IgM immunoglobulins, with the exception of 9702, derived from fusion 97, which was an IgG.

The purification of hybridoma antibodies was performed by affinity chromatography using a rabbit anti-human IgM or IgG antibody conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) column[26]. Bound immunoglobulin was eluted from the columns using 0.1 M glycine-HCl buffer, pH 2.3, and dialyzed against phosphate-buffered saline, pH 7.3 (PBS).

Detection of Antibody Binding to Phospholipids and Denatured DNA

The enzyme-linked immunoassay (ELISA) for phospholipid binding, and the radioimmunoassay (RIA) for binding to denatured DNA (dDNA) have been previously described in detail[15,23]. Phospholipids used in binding assays, including cardiolipin (CL), egg phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS), were purchased from Avanti Polar Lipids (Birmingham, AL). DNA was purchased from Worthington (Freehold, New Jersey). Briefly, Immulon-2 plates (Dynatech, Alexandria, Virginia) (for the ELISA) or polystyrene tubes (for the RIA) were coated with antigen, incubated with hybridoma culture supernatant, and then with

alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo) or ^{125}I-labelled anti-human polyvalent immunoglobulin (Tago, Burlingame, CA). ELISA plates were developed with p-nitrophenyl phosphate disodium (1 mg/ml) and read at 410nm using a Dynatech MR600 ELISA reader, and radioactive tubes were counted for 1 minute in a Beckman gamma-counter.

Detection of Antibody Binding to Platelets

Following the procedure of Asano et al.[18], polystyrene Immulon-2 (Dynatech) 96-well plates were coated with 100 µl/well of platelets (10^8/ml) isolated from normal individuals and fixed with 2.5% glutaraldehyde. The plates were blocked with 0.5% gelatin, stored in 60% glycerol at -70°C for up to 2 months, and washed five times with Tris-buffered saline, pH 7.4, before using. Then 75 µl of hybridoma supernatant were added to each well in duplicate and incubated for 90 min at room temperature. After washing five times, the plates were incubated with alkaline phosphatase-conjugated anti-human polyvalent immunoglobulin (Sigma Chemical Co.) for 90 min at room temperature. The colour was developed and read as above.

Activated Partial Thromboplastin Time (APTT) Assay

The dilute APTT assay was performed by the method of Rauch et al.[19] as previously described. Briefly, hybridoma supernatants were incubated with an equal volume of freshly reconstituted Verify Normal Citrate (pooled normal human plasma) (General Diagnostics, Scarborough, Ontario), and a 1/32 dilution of APTT reagent (General Diagnostics) in cuvets in a disposable circular test tray for 5 min at 37°C. Then, 100 µl of 0.025 M $CaCl_2$ was added to initiate the

clotting sequence. The clotting time was displayed on the General Diagnostics Coag-a-mate Iso single channel instrument (Warner-Lambert Co., Morris Plains, NJ) digital timer. All samples were tested in duplicate and an antibody was defined as having lupus anticoagulant activity if its APTT exceeded the APTT of the GM 4672 control by greater than 6 seconds[19].

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting

Platelets isolated from normal individuals were solubilized in SDS sample buffer at 2 x 10^9 cells/ml and the proteins were electrophoretically separated in an 8-12% SDS polyacrylamide gradient gel and then transferred onto 0.45 µm nitrocellulose membrane (Bio Rad, Richmond, CA) at 60 volts for 17 hours at 4°C. The nitrocellulose membrane was cut into strips and incubated with hybridoma antibody for 3 hours at 37°C, followed by incubation with peroxidase-conjugated goat anti-human IgM (µ specific) antibody (Tago, Burlingame, CA) for 1 hour at 37°C. The colour was developed using diaminobenzidine.

Cytotoxicity of Hybridoma Antibodies to Platelets and Lymphocytes.

Platelets or lymphocytes isolated from citrated normal blood were incubated with ^{51}Cr sodium chromate (Frosst, Kirkland, Quebec) and washed to remove free radioactivity. The ^{51}Cr-labeled cell suspension was plated in V-bottom microtiter wells at 5 x 10^6 cells per well and incubated with 150 µl of hybridoma culture supernatant or affinity-purified antibody diluted to 5 µg/ml in PBS-A buffer, containing 2.8 mM KH_2PO_4, 12.6 mM Na_2HPO_4, 0.12% citric acid, 0.33%

sodium citrate, 0.05% EDTA, 20 mM glucose, 7.2 mM NaOH and 0.5% bovine serum albumin, at 37°C for 1 hour. Then, 50 µl of rabbit complement (GIBCO, Grand Island, NY) were added and the plates incubated for 30 minutes at 37°C. The supernatants were harvested and counted for 1 minute in a Beckman gamma-radiation counter to measure ^{51}Cr-release from the cells (cell lysis). The spontaneous release was the ^{51}Cr counts released from cells incubated with only PBS-A buffer or medium and the total release was obtained by Triton X100 lysis of the ^{51}Cr-labeled and washed platelets. The percentage of ^{51}Cr-release was calculated by dividing the ^{51}Cr release for a sample by the total ^{51}Cr-release.

In these experiments, MCA 167 (Serotec, Blackthorn, England), a commercial monoclonal antibody which specifically binds to Zw^a, a platelet and megakaryocyte alloantigen present in 98% of individuals, was used as a positive control.

Statistical Methods

The negative ranges used in all assays were expressed by 99% confidence intervals, that is, the mean plus or minus 2 standard deviations (mean ± 2 SD). The following values, which were considered positive in each assay, were calculated from the mean plus 2 SD of 5 known negative samples in repeated assays: cpm > 750 in the anti-dDNA radioimmunoassay; OD_{410} > 0.08 in the anti-platelet ELISA; OD_{410} ≥ 0.10 in the anti-phospholipid ELISAs (except for anti-egg PE); OD_{410} ≥ 0.12 in the anti-egg PE ELISA; and an APTT ≥ 6 seconds above the negative control in the APTT assay.

The correlations between different antibody activities were

analyzed using Pearson's correlation coefficients and their significance was estimated from correlation tables[27]. Association was assessed using a two-tailed Fisher's exact test[28].

RESULTS

Platelet Binding Reactivities and Lupus Anticoagulant Activity of Hybridoma Antibodies Derived from SLE Patients and Normal Individuals

Hybridoma antibodies used in this study were produced from fusions between the parental lymphoblastoid cell line GM 4672, which secretes trace amounts of IgG (0.01-0.1 µg/ml), and peripheral blood lymphocytes isolated from 13 SLE patients and 4 normal individuals. Serological tests done previous to or at the time of fusion indicated that of the 13 SLE patients, 9 had anti-DNA antibodies by a Farr RIA, 7 had lupus anticoagulant antibodies by APTT assay, and 9 had either anti-CL antibodies or a false positive Venereal Disease Research Laboratory (VDRL) test (Table 1). All of the normal donors for fusion had completely normal serology and were negative for all of these antibodies. The hybridoma antibodies produced from these fusions were screened for anti-dDNA, anti-platelet and lupus anticoagulant activities. The hybridoma antibody reactivities often did not reflect the donor's serology, as positive hybridoma antibodies were obtained from both SLE and normal donors who were serologically negative (Table 1). All of the hybridoma antibodies used in this study were IgM, with the exception of 9702, which was an IgG. The average immunoglobulin concentrations in the hybridoma supernatants were 10-20 µg/ml for 9702 IgG, and 2.0-10.0 µg/ml for the IgM-secreting hybridomas.

Legend to Table 1

[a] This number represents the total number of clones analyzed in the present study and not the total number of clones derived from each fusion. All hybridoma clones in this table secreted IgM, with the exception of 9702 (derived from fusion 97), which secreted IgG.

[b] Activities shown are binding to dDNA by RIA, binding to fixed platelets by ELISA, and lupus anticoagulant (LA) activity by APTT assay.

[c] Patients with anti-DNA antibodies in their serum, as measured by a Farr RIA.

[d] Patients with lupus anticoagulant antibodies in their plasma, as detected by an APTT assay.

e Patients with serum anti-CL antibodies or a false positive VDRL test.

[f] This patient had a low platelet count at the time of fusion. Anti-platelet antibodies were not measured in the serum of any of the patients.

Table 1. Activities of Human Hybridoma Antibodies from SLE Patients and Normal Individuals

Fusion Code #	Donors (Age/Sex)	Total Clones[a]	Clones with activities[b]			Specificity		
			DNA	Platelet	LA	Mono	Poly	None
SLE								
1	23/F^{c}	2	2	2	0	0	2	0
6	26/F^{c,e}	1	1	1	0	0	1	0
8	38/F^{d,e}	1	0	0	0	0	0	1
11	44/F^{c,d,e}	2	0	0	2	2	0	0
12	26/F^{c,e}	4	2	2	2	1	2	1
13	38/F^{c,d,e}	1	1	1	1	0	1	0
14	36/M	4	2	2	2	2	2	0
15	53/F^{d,e}	1	0	0	0	0	0	1
26	23/M^{c,d}	1	1	0	1	0	1	0
92	29/F^{c,e,f}	1	1	1	1	0	1	0
95	69/F^{c}	2	1	1	1	0	2	0
96	56/F^{d,e}	3	1	0	2	1	1	1
97	40/F^{c,d,e}	6	3	3	4	2	3	1
Total	13	29	15	13	16	8	16	5
Normal								
40	31/F	1	1	1	1	0	1	0
100	27/F	9	0	0	0	0	0	9
B1	30/M	9	8	8	0	0	8	1
B6	29/M	1	0	0	0	0	0	1
Total	4	20	9	9	1	0	9	11

Solid phase immunoassays were employed to test the reactivities of hybridoma antibodies with dDNA and platelets and the APTT assay was used to identify hybridoma lupus anticoagulant antibodies. Five known activities were used as negative controls. Table 1 shows the origin and distribution of the hybridoma antibodies analyzed. Among 29 SLE-derived hybridoma antibodies, 15 antibodies with dDNA-binding activity were derived from 10 patients, 13 antibodies with platelet-binding activity were from 8 patients and 16 antibodies with lupus anticoagulant activity were from 9 patients. Among 20 normal-derived antibodies, 9 antibodies derived from 2 donors bound to both dDNA and platelets, while only one of these antibodies had lupus anticoagulant activity.

An antibody showing reactivity in only one of the assays for anti-dDNA, anti-platelet, and lupus anticoagulant activity was defined as monospecific, while an antibody positive for more than one of these activities was defined as polyspecific. Eight (33.3%) of the 24 SLE-derived hybridoma antibodies with known activities were monospecific and 16 (66.7%) of them were polyspecific. Five of the 29 SLE-derived antibodies had no reactivity. In contrast, none of the normal-derived antibodies were monospecific. All 9 (100%) of the normal-derived antibodies with known reactivity were polyspecific and were reactive with both dDNA and platelets, while 11 antibodies had no known activity. These results indicate that although polyspecific hybridoma antibodies can be produced from both normal donors and SLE patients, the frequency of monospecific and lupus anticoagulant antibodies in the total number of active antibodies was much higher

among antibodies derived from SLE patients than from normal individuals. In contrast, the frequency of polyspecific antibodies was higher in normal-derived antibodies.

Polyspecific antibodies which were positive for both anti-dDNA and anti-platelet assays were tested for their phospholipid-binding properties. Table 2 summarizes the reactivities of 12 representative SLE and normal-derived polyspecific platelet-binding antibodies and shows that there were significantly different patterns of reactivity between the polyspecific antibodies derived from SLE patients and normal individuals. Since 8 of the 9 normal-derived polyspecific antibodies were derived from 1 donor and showed very similar binding reactivities, three representative antibodies (4003, B122 and B135) derived from 2 donors are shown in Table 2. These 3 antibodies showed strongly positive reactions to all phospholipids tested in solid phase immunoassays. However, these three antibodies did not bind to control antigens, which included bovine serum albumin, gelatin, and Tween 20 blocked polystyrene wells or tubes. In contrast, the 9 polyspecific antibodies from 7 SLE patients demonstrated diverse reactivities to the different antigens, with the exception of antibody 103, which reacted strongly with all of the phospholipids tested. Furthermore, lupus anticoagulant activity was found in only 1 of the 3 normal-derived antibodies compared with 7 of 9 of the SLE-derived antibodies.

Legend to Table 2

[a] HyAb = hybridoma antibody, designated by its code number. The IgM concentrations of these supernatants ranged between 2.0 to 10.0 μg/ml.

[b] A positive value for dDNA binding was greater than 750 cpm (see Materials and Methods).

[c] Positive ELISA values were defined as follows: greater than 0.08 OD_{410} units for anti-platelet, greater than or equal to 0.12 OD_{410} units for anti-PE, and greater than or equal to 0.10 OD_{410} units for anti-CL, anti-PS, anti-PG, anti-PI, and anti-PC.

[d] A positive APTT was defined as greater than or equal to 66.0 sec.

[e] Values for RIA and ELISA results represent the means calculated from 4 to 6 repeated assays on duplicate samples. Positive values for all assays are indicated in bold print.

Table 2. Activities of 12 Representative Polyspecific Hybridoma Autoantibodies from SLE Patients and Normal Donors

HyAb[a]	Anti-dDNA RIA[b] (cpm)	Anti-Platelet ELISA[c] (OD_{410})	Anti-Phospholipid ELISA (OD_{410})[c]						APTT[d]
			CL	PE	PS	PG	PI	PC	
SLE-Derived:									
9200	**11711**[e]	**0.60**	**0.13**	**1.11**	0.05	0.06	0.04	0.02	+
1305	**2186**	**1.74**	**1.00**	**1.96**	0.03	**0.51**	**0.21**	**1.04**	+
9700	**2004**	**0.09**	0.05	**0.84**	0	0.02	0.02	0.01	+
9703	**5932**	**0.95**	**0.40**	**0.90**	**0.46**	0.05	**0.20**	**1.21**	+
103	**2884**	**0.96**	**1.93**	**1.98**	**1.86**	**1.23**	**1.54**	**1.23**	-
600	**2664**	**0.10**	0.04	**0.18**	0.03	0.03	0.03	0.04	+
1206	**13449**	**0.50**	**0.13**	**1.90**	0.02	0.03	0.05	0.01	+
1400	**11276**	**0.47**	**0.19**	**0.30**	0.02	0.04	0.02	0	-
1407	**11425**	**0.53**	**1.25**	**1.96**	**0.47**	0.03	**0.10**	0.01	+
Normal-Derived:									
4003	**5206**	**0.44**	**1.81**	**1.91**	**1.71**	**1.56**	**0.75**	**0.50**	+
B122	**10589**	**0.87**	**1.32**	**1.62**	**1.73**	**1.08**	**0.32**	**0.60**	-
B135	**6071**	**1.19**	**1.11**	**1.78**	**1.90**	**1.55**	**0.36**	**0.58**	-

Statistical Comparison of the Reactivities of Hybridoma Antibodies Derived from SLE Patients and Normal Individuals

When the hybridoma antibodies were grouped according to their origin from SLE patients or healthy normal donors, statistical analyses revealed an interesting pattern. Figure 1 shows the dot plots for the correlation of anti-dDNA activity with platelet-binding activity for SLE and normal-derived hybridoma antibodies. There was a striking difference between the SLE-derived and normal-derived antibodies. SLE-derived antibodies showed no correlation between these two reactivities ($r = 0.35$, $p > 0.05$), while normal-derived antibodies demonstrated a highly significant positive correlation ($r = 0.89$, $p < 0.001$). This finding, taken together with the data shown in Table 2, suggest that the reactivities of normal and SLE-derived hybridoma antibodies with platelets differ and may be due to binding to different epitopes, where an epitope is defined as an antigenic determinant.

Statistical analyses of the relationships between anti-dDNA and anti-phospholipid reactivities were done to further analyze the differences and similarities between SLE and normal-derived hybridoma antibodies (Table 3). As sufficient supernatant was not available for simultaneous testing of the antibodies in all of the phospholipid binding assays, the sample numbers varied for different assays. For the normal-derived hybridoma antibodies, dDNA binding activity was strongly correlated with reactivity to platelets ($r = 0.89$, $p < 0.001$), PI ($r = 0.69$, $p < 0.01$), PG ($r = 0.81$, $p < 0.01$), PC ($r = 0.84$, $p < 0.001$), PS ($r = 0.92$, $p < 0.001$), CL ($r = 0.80$, $p < 0.001$)

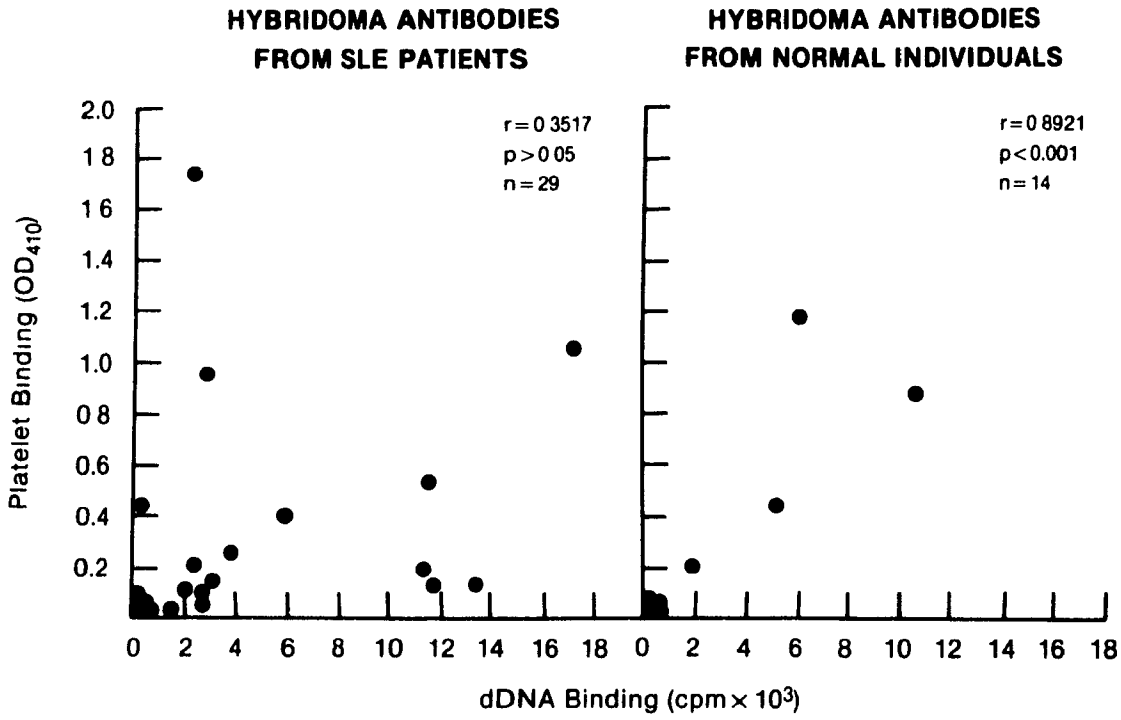

Figure 1. Correlation of Anti-dDNA Reactivity with Anti-Platelet Reactivity of Hybridoma Autoantibodies Derived from SLE Patients and Normal Individuals

For SLE-derived antibodies, there was no correlation of anti-dDNA reactivity with anti-platelet reactivity, while normal-derived antibodies showed a strong positive correlation of these two reactivities. Each value on the dot plot represents the mean of duplicate samples repeated in 4 separate immunoassays. n = total number of hybridoma antibodies tested. r = Pearson's correlation coefficient.

In contrast, the antibodies derived from SLE patients showed no correlation of anti-dDNA reactivity with anti-platelet ($r = 0.3517$, $p > 0.05$), anti-PI ($r = 0.0238$, $p > 0.10$), anti-PG ($r = 0.0259$, $p > 0.10$), anti-PC ($r = 0.0622$, $p > 0.10$), anti-PS ($r = 0.1400$, $p > 0.10$) and anti-CL ($r = 0.3292$, $p > 0.05$) reactivities (Table 3 and Figure 2A). The only positive correlation among the SLE-derived hybridoma antibodies was found between anti-dDNA and anti-PF reactivities ($r = 0.6163$, $p < 0.001$) and for this correlation, there was little difference between SLE-derived and normal-derived hybridoma antibodies (Figure 2B). These data indicate that most antibodies from normal donors showed either positive or negative reactions to all of the antigens tested in solid phase immunoassay systems, while most antibodies from SLE patients showed more restricted specificity to these antigens.

There were less significant differences seen between the normal and SLE-derived antibodies in correlations of anti-platelet reactivity with anti-phospholipid reactivities. Although the normal-derived antibodies showed consistently higher correlation coefficients (Table 4), both groups of antibodies had significant positive correlations for all of the phospholipids tested. However, correlation coefficients above 0.7 were observed only for anti-platelet and anti-CL, anti-PE, and anti-PC for the SLE-derived antibodies and for anti-PS, anti-PG, anti-CL, anti-PE, and anti-PC for the normal-derived antibodies.

Table 3. Correlation of Anti-dDNA Reactivity with Anti-Platelet and Anti-Phospholipid Reactivities of Hybridoma Antibodies Derived from SLE Patients and Normal Individuals

Correlation of Anti dDNA Activity with:	Correlation Coefficient (r)*	
	Antibodies from SLE	Antibodies from Normals
Anti-Platelet	n=29, r=0.35, p>0.05	n=14, r=0.89, p<0.001
Anti-PI	n=25, r=0.02, p>0.10	n=8, r=0.69, p<0.01
Anti-PG	n=27, r=0.03, p>0.10	n=7, r=0.81, p<0.01
Anti-PC	n=26, r=0.06, p>0.10	n=11, r=0.84, p<0.001
Anti-PS	n=27, r=0.14, p>0.10	n=9, r=0.92, p<0.001
Anti-CL	n=29, r=0.33, p>0.05	n=20, r=0.80, p<0.001
Anti-PE	n=28, r=0.62, p<0.001	n=18, r=0.86, p<0.001

* Pearson's correlation coefficients (r) were determined from the numerical data obtained in repeated assays (part of which is shown in Table 2), and not based on the assignment of positive or negative assay results. The n represents the number of individual antibodies in the particular category indicated.

Figure 2. Correlation of Anti-dDNA Reactivity with Anti-Cardiolipin (panel A) and Anti-Phosphatidylethanolamine (panel B) Reactivities of Hybridoma Autoantibodies Derived from SLE Patients and Normal Individuals

For SLE-derived antibodies, there was no correlation of anti-dDNA with anti-CL reactivity, but there was a significant positive correlation between anti-dDNA and anti-PE reactivities. Normal-derived antibodies showed a strong positive correlation of anti-dDNA reactivity with both anti-CL and anti-PE reactivities. Each value on the dot plot represents the mean of duplicate samples repeated in 4 separate immunoassays. n = total number of hybridoma antibodies tested. r = Pearson's correlation coefficient.

Figure 2A

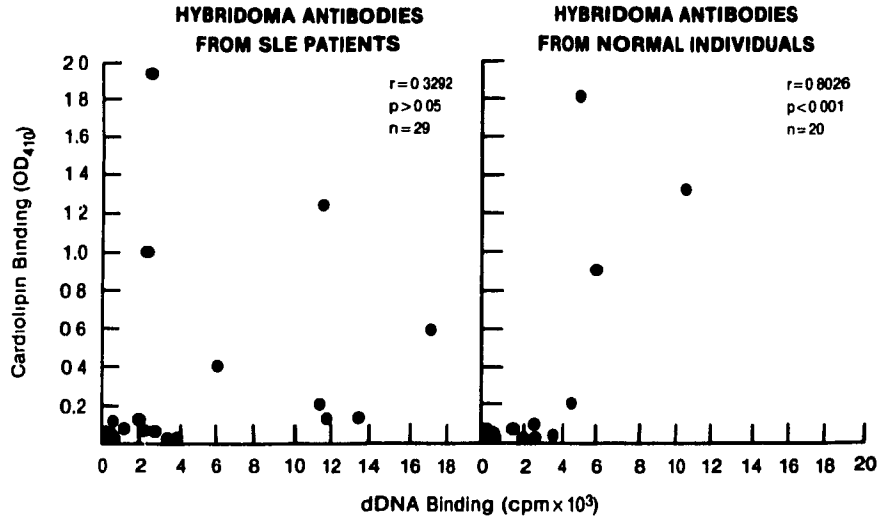

Figure 2B

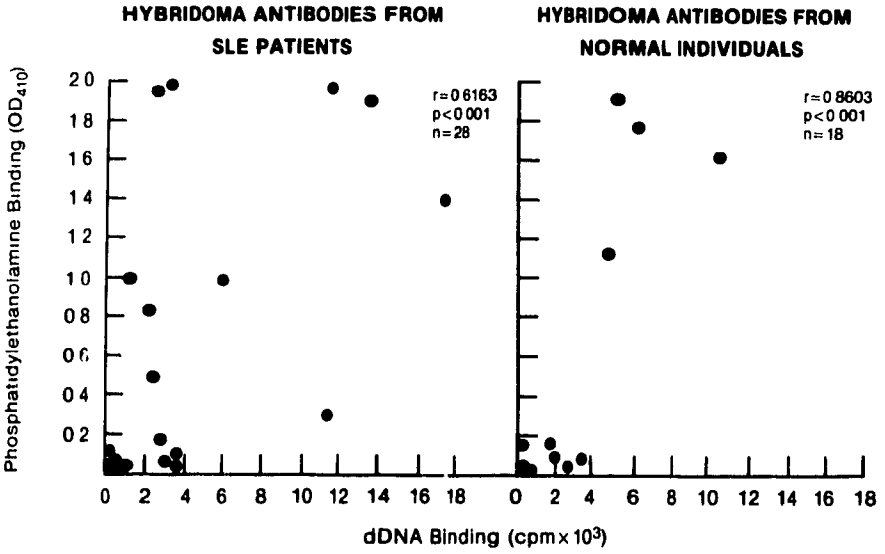

Table 4. Correlation of Anti-Platelet Reactivity with Anti-Phospholipid Reactivities of Hybridoma Antibodies Derived from SLE Patients and Normal Individuals

Correlation of Anti-Platelet Activity with:	Correlation Coefficient (r)*	
	Antibodies from SLE	Antibodies from Normals
Anti-PS	n=27, r=0.45, p<0.05	n=9, r=0.93, p<0.001
Anti-PI	n=25, r=0.47, p<0.02	n=8, r=0.62, p<0.05
Anti-PG	n=27, r=0.58, p<0.001	n=7, r=0.87, p<0.001
Anti-CL	n=29, r=0.71, p<0.001	n=15, r=0.80, p<0.001
Anti-PE	n=28, r=0.78, p<0.001	n=15, r=0.90, p<0.001
Anti-PC	n=27, r=0.81, p<0.001	n=10, r=0.94, p<0.001

* Pearson's correlation coefficients (r) were determined from the numerical data obtained in repeated assays (part of which is shown in Table 2), and not based on the assignment of positive or negative assay results. The n represents the number of individual antibodies in the particular category indicated.

Identification of Platelet Protein Binding Specificities of Human Hybridoma Antibodies by SDS PAGE and Western Blotting

In order to characterize the epitopes responsible for the binding of normal and SLE-derived hybridoma antibodies to platelets, antibody binding to platelet proteins separated by SDS PAGE was analyzed by the Western blotting technique. Figure 3 shows an immunoblot of whole platelet proteins with some representative platelet-binding and non-platelet-binding SLE and normal-derived hybridoma antibodies. The two groups of hybridoma antibodies showed very different patterns of reactivity. Strips incubated with SLE or normal-derived nonplatelet-binding hybridoma antibodies (B109, B110, B111, B3700, and B3123) did not show any reactivity with platelet proteins. In some of the negative control strips (B3700, B3123, 9702, GM 4672, and casein blocked control), faint bands with mobilities of μ (M.W. 70,000) or τ (M.W. 50,000) heavy chains were caused by reactivity of platelet-bound immunoglobulin with the peroxidase-conjugated anti-immunoglobulin antibodies. Platelet-binding hybridoma antibodies from both SLE and normal donors bound to a protein of M.W. 45,000, which had the same mobility as actin (e.g. B122, 9200, 9500, 9706 and 1206), or to several protein bands with relative mobilities between 65,000 and 90,000 M.W. (B105, B122, B135, 9200, 9500, 9706 and 1407). However, the 4 normal-derived polyspecific platelet-binding hybridoma antibodies (4003, B105, B122 and B135) also bound to casein blocked nitrocellulose membrane, producing very high background binding, and did not show unique reactivity with any platelet component. In contrast to the normal-derived antibodies,

Figure 3. Immunoblot of Platelet Proteins with Hybridoma Antibodies Derived from SLE and Normal Individuals

Normal-derived platelet-binding antibodies showed high nonspecific background binding to casein blocked nitrocellulose membrane, with some binding to several common protein bands. SLE-derived platelet-binding antibodies showed reactivity to various different platelet proteins, as well as binding to some proteins in common with the normal-derived antibodies. An SLE-derived platelet-binding antibody (103-4) with similar polyspecific reactivities to the normal-derived polyspecific antibodies in solid phase immunoassays showed neither nonspecific binding nor specific reactivity by Western blotting.

From left to right, Lane 1: M.W. standards; Lane 2: whole platelet proteins; Lane 3: casein blocked control strip incubated with peroxidase-conjugated anti-human IgM; Lanes 4-6: normal-derived, nonplatelet-binding hybridoma IgM antibodies B109, B110 and B111; Lanes 7-10: normal-derived, platelet-binding hybridoma IgM antibodies 4003, B105, B122 and B135; Lanes 11-21: SLE-derived platelet-binding hybridoma IgM antibodies 9200, 9500, 9502, 9604, 9700, 9703, 9706, 1206, 1305, 1407 and 103-4; Lanes 22-23: SLE-derived nonplatelet-binding hybridoma IgM antibodies B3700 and B3123; Lane 24: SLE-derived nonplatelet-binding hybridoma IgG antibody 9702; Lane 25: GM 4672 IgG control; Lane 26: casein blocked control strip incubated with peroxidase anti-human IgG.

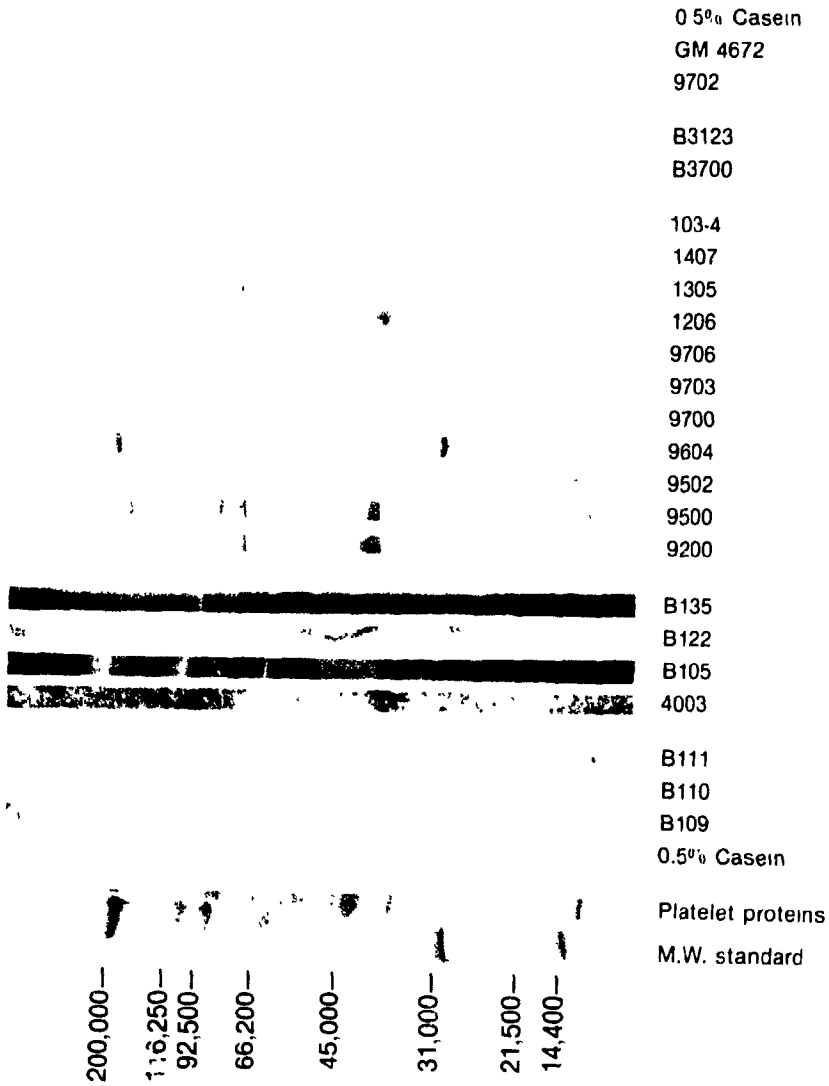
0.5% Casein
GM 4672
9702
B3123
B3700
103-4
1407
1305
1206
9706
9703
9700
9604
9502
9500
9200
B135
B122
B105
4003
B111
B110
B109
0.5% Casein
Platelet proteins
M.W. standard
200,000
116,250
92,500
66,200
45,000
31,000
21,500
14,400

Figure 3

several SLE-derived platelet-binding antibodies reacted with unique protein bands. One very clear example was antibody 9604, which bound to platelet proteins of approximately 200,000 and 32,000 M.W., found in isolated platelet membranes (data not shown) as well as in whole platelet lysates (Figure 3). One SLE-derived polyspecific antibody, 103-4, which showed an identical pattern of reactivity to the 4 normal-derived polyspecific antibodies in solid phase immunoassays on 8 different antigens (Table 2) showed low background binding (in contrast to the normal-derived antibodies) and did not bind to any platelet proteins on the blot. These results demonstrate that SLE-derived polyspecific hybridoma platelet-binding antibodies showed more specific reactivity than similar normal-derived antibodies on Western blots of platelet proteins.

Cytotoxicity of Human Hybridoma Autoantibodies

Cytotoxicity studies, using a chromium-51 release assay, were used to compare the functional effects of hybridoma antibodies derived from SLE patients and normal individuals. Platelets were used as the specific target cells and lymphocytes as control target cells. Figure 4 shows the cytotoxicity of hybridoma culture supernatants from SLE and normal-derived clones. In these experiments, hybridoma cells were grown in hypoxanthine-thymidine (HT) medium to avoid the high background lysis caused by aminopterin in the supernatants. All SLE and normal-derived hybridoma supernatants used in this assay contained between 2.0 and 10.0 µg/ml of IgM hybridoma antibody. Five of 9 SLE-derived hybridoma supernatants (9604, 9703, 9703-5, 9705 and 1109) caused increased ^{51}Cr release (19.3-32.4%) from platelets,

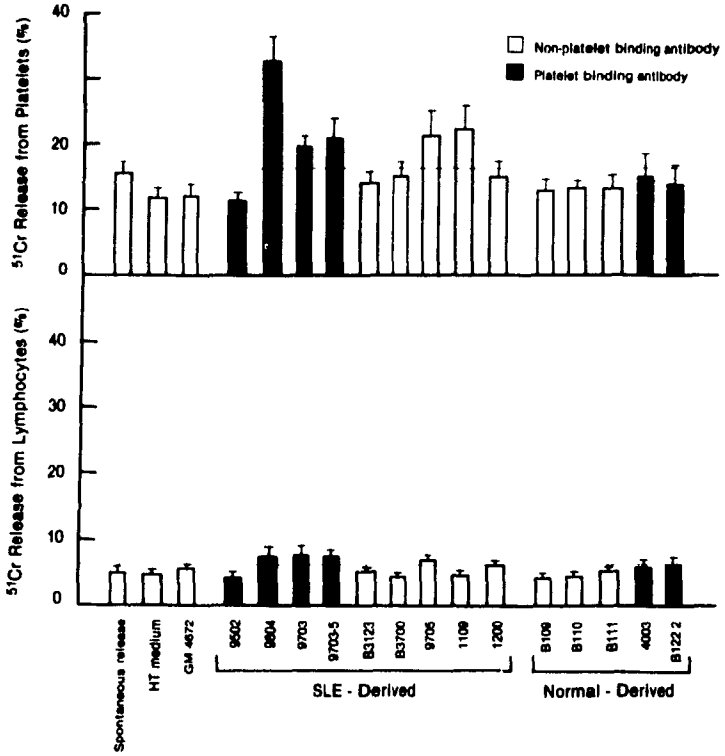

Figure 4. Cytotoxic Effects of SLE-Derived and Normal-Derived Hybridoma Culture Supernatants on Platelets (Top Panel) and Lymphocytes (Bottom Panel)

All hybridomas used in this study were IgM-secreting clones. Five of the nine SLE-derived antibodies caused increased ^{51}Cr release from platelets but had little or no effect on lymphocytes, while none of the normal-derived antibodies were cytotoxic to either of these cells. Representative results of repeated assays in which supernatants were tested in quadruplicate are shown. The columns show the mean percentage of chromium-51 released from the target cells (platelets or lymphocytes). Error bars show the SD of quadruplicate samples within one individual assay (i.e. internal variation). The negative cut-off values were calculated from the mean plus 2 SD of three negative controls (spontaneous release, HT medium and GM 4672 supernatant) run in each assay and are represented by dotted lines.

while 4 of these were only slightly cytotoxic (6.2-7.6%) to lymphocytes. In contrast, all of the normal-derived hybridoma supernatants tested, including those which contained platelet-binding antibodies by ELISA, were not cytotoxic to either platelets or lymphocytes. To confirm that the cytotoxicity of the SLE-derived antibodies was due to the antibodies rather than to a nonspecific effect of the supernatants, affinity-purified hybridoma antibodies were used in further experiments shown in Table 5. In these studies, the values of ^{51}Cr release for the negative controls (mean 7.8%) were much lower than in the supernatant studies (mean 12.8%). All 8 affinity-purified SLE-derived platelet-binding hybridoma antibodies, including antibodies 9703 and 9604 which were positive in supernatant form (Figure 4), showed cytotoxicity (14.7-21.0% release) to platelets. Five (1104, 1109, 1311, 9702 and 9603) of the 8 non-platelet-binding SLE-derived antibodies were also cytotoxic to platelets, and 4 of these 5 antibodies had lupus anticoagulant activity. However, one lupus anticoagulant antibody, 9705, which was cytotoxic as a culture supernatant, lost this activity in its affinity-purified form. Using a Fisher's exact test, no statistically significant association was found between platelet cytotoxicity and either lupus anticoagulant or anti-CL activity (Table 2). In contrast to the positive cytotoxic results of the SLE-derived antibodies, none of the affinity-purified normal-derived platelet-binding antibodies, including antibodies 4003, B122-2, and B105 which were strongly reactive to platelets in a solid phase immunoassay, had cytotoxic effects on platelets. These results demonstrate that the SLE-derived

Legend to Table 5

[a] The values shown are the means of quadruplicate samples, plus or minus 1 SD. All samples were assayed at 5 µg/ml, as determined by RIA for IgM or IgG.

[b] Hybridoma 9703-5 is a subclone of hybridoma 9703.

[c] The cut-off value was the mean + 2 SD (7.81 + 0.66, or 8.47) of the 4 negative controls shown (S.R., PBS-A, GM 4672 and HIgM).

[d] S.R. indicates spontaneous ^{51}Cr release from platelets in the absence of complement.

[e] HIgM was pooled normal human IgM.

[f] MCA 167, a commercial monoclonal anti-platelet antibody (see Methods), served as positive control.

Table 5. Cytotoxic Effects of SLE-Derived and Normal-Derived Affinity-Purified Hybridoma Antibodies on Platelets

Non-Platelet Binding	^{51}Cr-Release (%)[a]	Platelet Binding	^{51}Cr-Release (%)[a]
SLE-Derived Antibodies:			
B3123	8.33 ± 0.02	9200	14.69 ± 0.02
B3700	6.96 ± 0.91	9500	18.83 ± 1.16
9705	8.21 ± 0.01	9502	18.59 ± 2.05
1104	13.90 ± 0.09	9604	19.57 ± 1.70
1109	11.18 ± 0.07	9700	21.02 ± 0.08
1311	9.84 ± 1.12	9703	19.40 ± 0.05
9702	24.66 ± 1.02	9703-5[b]	19.65 ± 1.14
9603	8.54 ± 1.05	9706	18.17 ± 3.81
Mean	11.45	Mean	18.74
Normal-Derived Antibodies:			
B109	7.58 ± 0.04	B122-2	7.88 ± 0.07
B110	7.87 ± 0.03	B105	8.21 ± 0.05
B111	8.08 ± 0.03	4003	8.16 ± 0.03
Mean	7.84	Mean	8.11
Negative and Positive Controls[c]:			
S.R.[d]	8.06 ± 0.03	HIgM[e]	7.36 ± 0.04
PBS-A buffer	7.91 ± 0.03	MCA 167[f]	19.44 ± 0.04
GM4672 IgG	7.52 ± 0.04		

antibodies had potent cytolytic effects on platelets, while the normal-derived antibodies were unable to cause significant platelet lysis.

DISCUSSION

The present study was designed to investigate whether platelet-binding antibodies derived from SLE patients differ from those derived from normal individuals. We addressed this question using human hybridoma antibodies produced from peripheral blood lymphocytes of SLE patients and normal individuals which were analyzed for anti-platelet, anti-dDNA, and lupus anticoagulant activities and were defined as either monospecific, polyspecific, or nonreactive antibodies. One of the major technical difficulties in comparing SLE-derived and normal-derived antibodies is the difference in the numbers of clones and reactive antibodies produced by fusions of peripheral blood lymphocytes from these donors. In the present study, 24 SLE-derived antibodies with known activities were found from 11 of 13 donors, while 9 normal-derived hybridoma antibodies with known activities were derived from only 2 donors. In many fusions using normal-derived lymphocytes, few or no clones secreting antibodies with activities were obtained (19 of 23 fusions using SLE lymphocytes produced hybridoma clones, while only 7 of 19 fusions using normal lymphocytes produced hybridoma clones and 4 of these produced only 1 clone per fusion). Thus, selection of the hybridoma antibodies analyzed in the present study was based both upon the availability of hybridoma supernatants and an attempt at representation of several

different fusion donors. As most normal-derived antibodies did not show any reactivity to the antigens tested, there were far more negative antibodies in this group than in the SLE-derived group. The above selection process could certainly create a bias which is difficult to avoid unless all hybridoma antibodies obtained were to be analyzed.

As the majority of platelet-binding hybridoma antibodies were polyspecific, regardless of their origin from SLE patient or normal individual, we evaluated whether these polyspecific platelet-binding antibodies bound to the same or different epitopes by solid phase immunoassays on dDNA and phospholipids, and by Western blotting analysis on whole platelet proteins. The results of the solid phase immunoassays (RIA and ELISA) demonstrated that the polyspecific platelet-binding antibodies from normal donors bound to all antigens tested, while most polyspecific platelet-binding antibodies from SLE patients, with the exception of antibody 103-4, showed diverse reactivities to dDNA and various phospholipids. This finding was further supported by a statistical analysis of the binding reactivities of SLE-derived and normal-derived antibodies. Strong positive correlations of dDNA binding activity with anti-platelet and anti-phospholipid reactivities were found for the hybridoma antibodies derived from normal individuals, while no correlations of these reactions were found for the SLE-derived antibodies, with the exception of a correlation of anti-DNA with anti-PE reactivity. These data imply that antibodies from normal donors show either positive or negative reactions to all autoantigens tested in solid phase

immunoassay systems, with positive reactivity apparently due to a polyspecific type of interaction. The binding did not appear to be nonspecific, as these antibodies did not bind to bovine serum albumin, gelatin or Tween 20 coated tubes or plates. In contrast, most antibodies from SLE patients showed more restricted specificity to the antigens tested. These results were confirmed by Western blotting analysis, in which polyspecific antibodies from normal individuals did not bind to unique protein bands, but often showed high nonspecific backgrounds with no distinct bands (e.g. 4003) or reactivity to a series of common bands (e.g. B105, B122 and B135), also detected by some of the SLE-derived antibodies. In contrast, SLE-derived antibodies exhibited several different patterns of reactivity and some antibodies (e.g. 9604) recognized unique platelet proteins. In an attempt to identify the protein(s) bound by antibody 9604, various antibodies and lectins with defined specificities were used to probe the blots. No corresponding reactivity was found with monoclonal antibodies against GPIb, GPIIb and GPIIIa; monoclonal antibodies against cytoskeletal proteins; polyclonal antibody against human factor VIII, a high molecular weight glycoprotein present in platelet granules; and concanavalin A and wheat germ agglutinin, which bind to different platelet membrane glycoproteins (data not shown).

Another important difference between the SLE and normal-derived antibodies was revealed in their functional cytotoxic activity. All SLE-derived platelet-binding antibodies were cytotoxic to platelets in vitro. In addition, several SLE-derived antibodies which did not

bind to platelets by ELISA or Western blotting analysis, but which had lupus anticoagulant activity, were also cytotoxic to platelets. Platelet cytotoxicity did not show a statistically significant association with lupus anticoagulant activity among the 16 affinity-purified antibodies analyzed, but this may have been due to the small number of lupus anticoagulant negative and platelet cytotoxic negative antibodies analyzed. In contrast to the SLE-derived antibodies, none of the affinity-purified normal-derived hybridoma antibodies, including those with strong polyspecific and platelet-binding properties, were cytotoxic to platelets. These results demonstrate that while the SLE-derived antibodies had potent cytolytic effects specific for platelets, normal-derived antibodies were unable to cause significant platelet lysis. Moreover, there was a vast discrepancy between the reactivity of normal-derived "autoantibodies" in solid phase assay systems and in the in vitro cytolytic assay and APTT assay, suggesting that the latter functional assays may be more realistic measures of potential activities in vivo.

Polyspecificity to several different antigens is characteristic of both SLE and normal-derived hybridoma autoantibodies[5,6,9-14,16-21]. Monoclonal hybridoma lupus anti-DNA autoantibodies have been reported to bind to cytoskeletal proteins[14,21], platelets[13,18,23], Sm antigen[17], various polynucleotides and phospholipids[12,19,20], and polypeptides on cell membranes[16]. Several laboratories, including our own, have also demonstrated that human hybridomas derived from normal donors can produce anti-DNA antibodies, many of which show polyspecificity[9-11,21,23]. In addition, antibodies with

autoreactivity have been reported in sera of normal mice and humans, suggesting that individuals without apparent autoimmune disease have B cells which can be activated to secrete autoantibodies[1-8]. The apparent similarities of some polyspecific antibodies derived from normal individuals and SLE patients suggest that immunoglobulin genes encoding autoantibodies exist in normal individuals[4,9,11,21,22,29]. This, however, requires an explanation of why autoimmune individuals produce highly specific and pathogenic autoantibodies, while normal individuals do not.

Antibodies defined as "polyspecific" by their ability to react with more than one antigen in solid phase immunoassays and lupus anticoagulant assays were produced from both patients with SLE and normal individuals. Although it is premature to draw conclusions, because of the relatively small number of antibodies analyzed from normal individuals, our results show that most polyspecific autoantibodies produced by SLE patients exhibited more restricted antigen specifi- city than antibodies derived from normal individuals. Moreover, SLE-derived autoantibodies exhibited functional activity in in vitro assays for lupus anticoagulant activity and platelet cytotoxicity, while antibodies from normal donors had little or no effect in these in vitro systems. Anti-phospholipid and lupus anticoagulant antibo- dies have been shown to be associated with thrombosis, thrombocyto- penia, and multiple spontaneous abortions in SLE patients[30-33]. However, it is not clear whether these hemostatic abnormalities are the result of pathogenic mechanisms involving lupus anticoagulant, anti-phospholipid, or anti-

platelet antibodies or whether all of these antibodies are involved. In addition, the epitopes responsible for anti-platelet antibody reactivity in patients with SLE remain unknown[18,23]. The binding and functional characteristics of the SLE-derived polyspecific autoantibodies suggest that these antibodies may play some role in the hemostatic disturbances found in SLE, while polyspecific antibodies from normal individuals may have little significance in vivo. Although the epitopes involved in the platelet reactivity of SLE-derived hybridoma antibodies remain to be defined, our results are consistent with the hypothesis that SLE patients produce autoantibodies with greater specificity for target autoantigens (e.g. platelets) than normal individuals.

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REFERENCES

1. Avrameas S, Guilbert B, Dighiero G. Natural antibodies against tubulin, actin, myoglobulin, thyroglobulin, fetuin, albumin, and

transferrin are present in normal human sera, and monoclonal immunoglobulins from multiple myeloma and Waldenstrom's macroglobulinemia may express similar antibody specificities. Ann Immunol (Inst Pasteur) 1981; 132C:231-6

2. Guilbert B, Dighiero G, Avrameas S. Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation and characterization. J Immunol 1982; 128:2779-87

3. Dighiero G, Guilbert B, Avrameas S. Naturally occurring antibodies against nine common antigens in humans. II. High incidence of monoclonal Ig exhibiting antibody activity against actin and tubulin and sharing antibody specificities with natural antibodies. J Immunol 1982; 128:2788-92

4. Madaio MD, Schattner A, Schattner M, Schwartz RS. Lupus serum and normal human serum contain anti-DNA antibodies with the same idiotype marker. J Immunol 1986; 137:2535-40

5. Dighiero G, Lymberi P, Holmberg D, Lundquist I, Coutinho A, Avrameas S. High frequency of natural autoantibodies in normal newborn mice. J Immunol 1985; 134:765-71

6. Holmberg D, Freitas AA, Portnoi D, Jacquemart F, Avrameas S, Coutinho A. Antibody repertoires of normal BALB/c mice: B lymphocyte populations defined by state of activation. Immunol Rev 1986; 93:147-69

7. Holmberg D, Forsgren S, Ivars F, Coutinho A. Reactions among IgM antibodies derived from normal, neonatal mice. Eur J Immunol 1984; 14:435-41

8. Vakil M, Dearney JF. Functional characterization of monoclonal

auto-anti-idiotype antibodies isolated from the early B cell repertoire of BALB/c mice. Eur J Immunol 1986; 16:1151-8

9. Cairns E, Block J, Bell DA. Anti-DNA autoantibody-producing hybridomas of normal human lymphoid cell origin. J Clin Invest 1984; 74:880-7

10. Cairns E, Komar R, Bell DA. Cytoskeletal binding of monoclonal anti-DNA antibodies derived from tonsillar lymphoid cells of a normal subject. Arthritis Rheum 1986; 29:1351-8

11. Logtenberg T, Kroon A, Gmelig-Meyling FHJ, Ballieux RE. Analysis of the human tonsil B cell repertoire by somatic hybridization: occurrence of both "monospecific" and "multispecific" (auto)antibody-secreting cells. Eur J Immunol 1987; 17:855-9

12. Lafer EM, Rauch J, Andrzejewski C Jr, Mudd D, Furie BC, Furie B, Schwartz RS, Stollar BD. Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J Exp Med 1981; 153:897-909

13. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC, Furie B, Stollar BD, Schwartz RS. Production of autoantibodies by human-human hybridomas. J Clin Invest 1982; 70:205-8

14. André-Schwartz J, Datta SK, Shoenfeld Y, Isenberg DA, Stollar BD, Schwartz RS. Binding of cytoskeletal proteins by monoclonal anti-DNA lupus autoantibodies. Clin Immunol Immunopathol 1984; 31:261-71

15. Massicotte H, Rauch J, Shoenfeld Y, 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 1984 3:215-21

16. Jacob L, Tron F, Bach J-F, Louvard D. A monoclonal anti-DNA antibody also binds to cell-surface protein(s). Proc Natl Acad Sci USA 1984; **81**:3843-5

17. Pisetsky DS, Hoch SO, Klatt CL, O'Donnell MA, Keene JD. Specificity and idiotypic analysis of a monoclonal anti-Sm antibody with anti-DNA activity. J Immunol 1985; **135**:4080-5

18. Asano T, Furie BC, Furie B. Platelet binding properties of monoclonal lupus autoantibodies produced by human hybridomas. Blood 1985; **66**:1254-60

19. Rauch J, Tannenbaum M, Tannenbaum H, Ramelson H, Cullis PR, Tilcock CPS, Hope MJ, Janoff AS. Human hybridoma lupus anticoagulants distinguish between lamellar and hexagonal phase lipid system. J Biol Chem 1986; **261**:9672-7

20. Rauch J, Tannenbaum H, Senécal J-L, Janoff AS, Cullis PR, Frojmovic MM. Polyfunctional properties of hybridoma lupus anticoagulant antibodies. J Rheumatol 1987; **14(Suppl.13)**:132-7

21. Senécal J-L, Rauch J. Hybridoma lupus autoantibodies can bind major cytoskeletal filaments in the absence of DNA-binding activity. Arthritis Rheum 1988; **31**:864-75

22. Datta SK, Naparstek Y, Schwartz RS. In vitro production of an anti-DNA idiotype by lymphocytes of normal subjects and patients with systemic lupus erythematosus. Clin Immunol Immunopathol 1986; **38**: 302-18

23. Rauch J, Meng Q-h, Tannenbaum H. Lupus anticoagulant and anti-platelet properties of human hybridoma autoantibodies. J Immunol 1987; **139**:2598-604

24. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Shaller JG, Talal N, Winchester RJ. The 1982 revised criteria for the diagnosis of SLE. Arthritis Rheum 1982; **25**: 1271-77

25. Rauch J, Massicotte H, Tannenbaum H. Specific and shared idiotypes found on hybridoma anti-DNA autoantibodies derived from rheumatoid arthritis and systemic lupus erythematosus patients. J Immunol 1985; **135**: 2385-92

26. Rauch J, Massicotte H, Tannenbaum H. Hybridoma anti-DNA autoantibodies from patients with rheumatoid arthritis and systemic lupus erythematosus demonstrate similar nucleic binding characteristics. J Immunol 1985; **134**:180-6

27. Pipkin Fb. Correlation and regression. In: Pipkin FB, ed. Medical Statistics Made Easy. Churchill-livingston, New York, 1984; 94-96.

28. Swinscow TDV. Exact probability test. In: Swinscow TDV, ed. Statistics at Square One. British Medical Association, Dawson & Goodall Ltd., The Mendip Press, Bath, 1981; 54-7

29. Schwartz RS, Stollar BD. Origins of anti-DNA antibodies. J Clin Invest 1985; **75**:321-7

30. Naparstek Y, André-Schwartz J, Manser T, Wysocki LJ, Breitman L, Stollar BD, Gefter M, Schwartz RS. A single germline V_H gene segment of normal A/J mice encodes autoantibodies characteristic of systemic lupus erythematosus. J Exp Med 1986; **164**:614-26

31. Schleider MA, Nachman RL, Jaffee EA, Coleman M. A clinical study of the lupus anticoagulant. Blood 1976; **48**:499-509

32. Mueh JR, Herbst KD, Rapaport SI. Thrombosis in patients with

the lupus anticoagulant. Ann Intern Med 1980; **92**:156-9

33. Firkin BG, Howard MA, Radford N. Possible relationship between lupus inhibitor and recurrent abortion in young women. Lancet 1980; 2:366

34. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GRV. Anticardiolipin antibodies: Detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet 1983; **2**:1211-4

CHAPTER V

A SYSTEMIC LUPUS ERYTHEMATOSUS-DERIVED HUMAN HYBRIDOMA AUTOANTIBODY REACTIVE WITH ANTIGENS EXPRESSED ON ADP-ACTIVATED PLATELETS

ABSTRACT

Hematological complications seen in systemic lupus erythematosus (SLE) patients may be caused by the binding of specific autoantibodies to platelets, but the epitopes on platelets responsible for antibody binding and the mechanisms by which autoantibodies induce hemostatic abnormalities in SLE patients remain unknown. We have previously demonstrated that both specific and polyspecific platelet-binding antibodies are present in SLE patients. In the present study, we have characterized an SLE-derived hybridoma antibody, 9604, which did not bind to fixed intact platelets in enzyme-linked immunoassays (ELISA), but reacted with lysed, or ADP-activated platelets in ELISA and with live or fixed washed platelets in a radioimmunoassay. Antibody 9604 had lupus anticoagulant activity in a dilute activated partial thromboplastin time assay and was strongly cytotoxic to platelets in an *in vitro* ^{51}Cr release assay, but did not react with phospholipids or fibrinogen by direct binding ELISA. By Western blotting analysis, 9604 reacted mainly with polypeptides of approximately 200,000 and 32,000 molecular weight (M.W.) in platelets. In blots of endothelial cell proteins, 9604 reacted with a band of approximately 200,000 M.W., but no 32,000 M.W. reactive band was observed. Based on these findings, we postulate that antibody 9604 may bind to a protein or proteins exposed on the platelet surface during platelet activation. Further characterization of these proteins may provide insight into the mechanisms responsible for the production and pathogenesis of anti-platelet autoantibodies in patients with SLE.

INTRODUCTION

The sera of patients with systemic lupus erythematosus (SLE) frequently contain multiple autoantibodies, including antibodies to platelets[1-2]. These patients often display hematological abnormalities, including thrombosis and thrombocytopenia[2-6]. It has been reported that the activation or destruction of platelets in immune thrombocytopenia may be caused by the binding of specific autoantibodies to platelets, the binding of immune complexes to platelets, or an increase in IgG bound to platelets via Fc receptors[7-10]. The mechanisms which result in abnormal hemostasis in SLE are unknown, but may involve any or all of the above three binding interactions. A better understanding of the platelet antigens recognized by autoantibodies present in patients with SLE might help to clarify the mechanisms of thrombocytopenia and thrombosis in these patients and in patients with other autoimmune diseases.

Platelets are important cells involved in the initiation and propagation of hemostasis and thrombosis. These anucleate cells circulate in the blood in a resting (inactive) form. Once they are activated by various factors, platelets undergo a series of morphological and functional changes which include the formation of pseudopods and development into spherocytes, adhesion to an exposed subendothelial or injured endothelial surface, release of granule contents, and aggregation and participation in the formation of a hemostatic or thrombotic plug[11]. The molecular changes on the platelet membrane which accompany platelet activation have been partially characterized and may involve binding of exogenous proteins

to the surface or exposure of endogenous antigens on the surface. When platelets are stimulated by adenosine diphosphate (ADP), collagen, thrombin or epinephrine, various proteins including thrombospondin (glycoprotein G)[12], Factor V[13], Factor VIII-related antigen (von Willebrand factor)[14], and protein S[15] are released from platelet granules and become associated with the membrane. Factor XIa[16], Factor XIIIa[17], plasminogen[18], fibronectin[19] and fibrinogen[20] bind specifically to activated platelet surfaces. The fibrinogen receptor, composed of glycoproteins IIb and IIIa, is expressed only on platelets activated with agents including ADP, epinephrine and thrombin[20-22]. Receptors for high molecular weight kininogen on stimulated human platelets have also been reported[23], and studies comparing surface structures on resting and activated platelets have demonstrated that actin and a 149,000 M.W. α-granule protein are newly expressed on the surface after platelet secretion in response to thrombin[24].

Hybridoma antibodies have been described which identify structures present on activated but not on resting platelets. Monoclonal anti-platelet antibodies, KC4[25,26] and S12[27,28], were produced from mice immunized with thrombin-activated washed platelets and recognized antigens expressed on thrombin- or ADP-activated platelets but not present on resting platelets. These antibodies were reactive with a platelet membrane glycoprotein of 140,000 M.W. Another murine monoclonal anti-platelet antibody bound to a 53,000 M.W. platelet granule protein found on the surface of activated platelets[29]. Similar reactivity was also demonstrated by a human

monoclonal antibody derived from a patient with immune thrombocytopenia[30]. However, it is not known whether autoantibodies derived from SLE patients can distinguish between resting and activated platelets, although we and others have demonstrated that some SLE-derived hybridoma antibodies bind to live and fixed platelets[31-33]. In the present study, we report the characteristics of one such SLE-derived hybridoma autoantibody called 9604, which bound to glutaraldehyde fixed ADP-activated platelets, but not to fixed resting platelets. The ability of this antibody to distinguish between activated and resting platelets may provide insight into the mechanisms by which autoantibodies interfere with platelet function in patients with SLE.

MATERIALS AND METHODS

Production and Purification of Human Hybridoma Autoantibodies.

Human hybridoma autoantibodies were produced from fusions of GM 4672 lymphoblastoid cells with peripheral blood lymphocytes isolated from patients with SLE (antibodies 9604, 9500, 9703, and B3123) or normal individuals (antibodies B110 and B111), as previously described[34]. The SLE patients fulfilled the American Rheumatism Association revised criteria for the classification of SLE[35]. The purification of hybridoma antibodies was performed by affinity chromatography using a rabbit anti-human IgM antibody conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) column[36]. Bound immunoglobulin was eluted from the column using 0.1 M glycine-HCl buffer, pH 2.3, and dialyzed against phosphate buffered saline,

pH 7.3 (PBS).

Isolation and Activation of Platelets

Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood (9 volume blood : 1 volume citrate) from a healthy donor at 180 x g for 20 minutes. The PRP was diluted with an equal volume of Tyrode's buffer without Mg++ and Ca++ containing 4 mM EDTA (Tyrode's-EDTA) and centrifuged at 1600 x g for 15 minutes to pellet the platelets. The isolated platelets were then washed 3 times and resuspended in the same buffer.

Activation of platelets was done in PRP. ADP was added to the PRP at a final concentration of 1.0 µg/ml (2.34 µM), which was maximum ADP concentration without inducing irreversible aggregation under the present experimental conditions and was the concentration reported previously being able to activate platelets, and gently swirled for 2 minutes at room temperature. The activated platelets were fixed immediately after activation by adding glutaraldehyde to the PRP or platelet suspension at a final concentration of 2.5% and then the suspension was swirled very gently on a rotator for 30 minutes at room temperature. The coating and ELISA procedure were the same as described for the platelet-binding ELISA above.

Platelet-Binding ELISA

The platelet-binding ELISA was performed following the method of Asano et al.[32] as previously described[33]. Fixed resting platelets were fixed in PRP, as described above, while fixed washed platelets were isolated as in the platelet radioimmunoassay (RIA) procedure, using a Percoll density gradient, as previously described[33]. In the

platelet ELISA, antibody 9500, which bound to platelets both in ELISA and RIA, was used as a positive control and antibodies B110 and B111, which did not bind to platelets in either ELISA or RIA, were used as negative controls. All negative control antibodies gave similar results and their use in a given assay was dependent only upon availability of these antibodies.

Platelet-Binding Radioimmunoassay

The platelet radioimmunoassay was performed as previously described[33].

Pretreatment of Intact Platelets with Enzymes

Washed intact platelets were resuspended in Hank's buffer and incubated with DNAase (Cooper Biomedical, Malvern, PA) at 1mg/ml + 6.25 mM $MgSO_4$ + 5 mM $CaCl_2$, RNAase (Cooper Biomedical) at 1mg/ml, phospholipase A_2 (Boehringer Mannheim, Dorval, Quebec) at 0.5 mg/ml + 5 mM $CaCl_2$, phospholipase C (Cooper Biomedical) at 0.5 mg/ml + 40 mM $CaCl_2$, S. aureus strain V_8 protease (Cooper Biomedical) at 0.5 mg/ml + 5 mM $CaCl_2$, or trypsin (Cooper Biomedical) at 0.5 mg/ml for 45 minutes at 37°C. The platelets were then washed twice with assay buffer and subjected to the platelet-binding assay described above.

Platelet Lysate Binding ELISA

Pellets of 10^8 isolated platelets were stored at -20°C, freeze-thawed 3 times, and then resuspended in 1.0 ml 10 mM TBS buffer, pH 7.4. The suspension was sonicated in a bath sonicator (Laboratory Supplies Company Inc., Hicksville, New York) for 5 minutes to lyse the cells completely. ELISA plates (Immulon-2, Dynatech, Chantilly, Virginia) were coated with 100 µl of the cell lysate per well and the

plates were incubated uncovered for 16 hours at 37°C. The rest of the ELISA procedure was as described for the platelet-binding ELISA.

Preparation of Platelet and Endothelial Cell Proteins

SDS solubilization of whole cells: Pooled normal platelets or umbilical vein endothelial cells, which were prepared as described in Chapter VI, were solubilized in SDS sample buffer (2% SDS, 10% glycerol in 15 mM Tris-HCl buffer, pH 6.7) with or without 2-mercaptoethanol (5%) at a concentration of 2 x 10^9 cells/ml for platelets and 2 x 10^7 cells/ml for endothelial cells, which was the optimal condition for electrophoretical resolution.

Preparation of platelet membrane proteins: Isolated normal platelet pellets were frozen at -70°C and thawed and refrozen 3 times at room temperature and sonicated for 5 minutes. The cell lysate was centrifuged at 100,000 x g for 1 hour. The membrane pellet was washed twice with PBS, pH 7.3 and then solubilized in SDS sample buffer with 2-mercaptoethanol at a concentration equivalent to 2 x 10^9 cells/ml.

Enzyme treatment of platelet preparation: Lysed platelets (3 x 10^9/ml) were incubated with various enzymes at a final concentration of 0.25 mg/ml for 1 hour at 37°C and then solubilized with an equal volume of SDS sample buffer. Enzymes used in these studies included DNAase (Cooper Biomedical, Malvern, PA), RNAase (Cooper Biomedical), phospholipase A_2 (Boehringer Mannheim, Dorval, Quebec), phospholipase C (Cooper Biochemical), protease (Cooper Biomedical), proteinase K (Cooper Biomedical) and trypsin (GIBCO, Grand Island, N.Y.).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Western Blotting

Solubilized platelet or endothelial cell proteins were electrophoretically separated in an 8-12% linear gradient SDS polyacrylamide gel or a 7.5% mini-SDS polyacrylamide gel and electrophoretically transferred onto a 0.45 µm nitrocellulose membrane (Bio Rad, Richmond, CA) at 60 volts for 16 hours at 4°C. Protein transfer was shown to be complete by Coomassie blue staining of the gel following transfer. The membrane was blocked with 0.5% casein in 10 mM TBS, pH 7.4 at 37°C for 1 hour and then cut into vertical strips. The strips were incubated with hybridoma antibodies for 3 hours at 37°C, washed 6 times and further incubated with peroxidase- labeled goat anti-human IgM (µ specific) antibody (Tago, Burlingame, CA) diluted 1:1000 in 0.5% casein in PBS, pH 7.4 for 1 hour at 37°C. The protein bands reactive with hybridoma antibodies were visualized with 0.05% diaminobenzidine in 50 mM Tris buffer, pH 7.4. Molecular weights were calculated from linear plots of molecular weights against relative mobilities of the high and low molecular weight standards run on each gel.

Electroelution of Platelet Proteins from SDS Polyacrylamide Gel

Platelet proteins were separated by SDS PAGE. Gel bands with mobilities of M.W. 200,000 and 32,000 (i.e. those bands with which antibody 9604 was reactive) were cut and sliced into 0.5x0.5 cm^2 pieces. The gel slices were placed into glass tubes of an electroelutor (Bio Rad, Richmond, CA) and the proteins were electroeluted from the gel at 10 milliamps per tube for 3 hours at room tempera- ture.

The electroeluted material was collected and dialyzed against PBS, pH 7.3 for 24 hours at 25°C with 2 changes and then concentrated using Centricon 30 concentrating units (Amicon, Danvers, Massachusetts).

Glycoprotein Staining of SDS Gel

Glycoproteins were stained with fuchsin-sulphite reagent following the procedure of Zacharius et al[37].

Isoelectric Focusing (IEF)

Monoclonal myeloma IgM, Bor, was kindly provided by Dr. Marianna Newkirk. Polyclonal human IgM was purchased from Cappel (West Chester, PA). The IEF was performed using a modification of the method of Gibson et al.[38], as previously described[39]. Briefly, affinity-purified hybridoma IgM was incubated with 10 mM dithiothreitol for 60 minutes at 25°C and then with 20 mM iodoacetamide for another 10 minutes at 25°C. The samples were dialyzed and concentrated in the presence of 1% glycerol in Centricon 30 concentrating units and electrophoresed in a urea-formate gel at 120 volts, 50 mAmps and 10 watts for 17 hours at 10°C. The gel bands containing the light chains were cut out of the gel, placed on the anode side of an IEF gel containing 2 M urea and pH 3.5-10.0 ampholines (LKB, Brevete, Sweden), and IEF was performed at a maximum current of 250 mAmps, constant power of 8 watts, and maximum voltage of 1200 volts at 4°C until 20 minutes after the voltage reached 1200 volts. The gel was fixed with 12.5% trichloroacetic acid and 4% sulfosalacylic acid and stained with Coomasie blue.

RESULTS

Binding and Functional Activities of Antibody 9604

The binding and functional characteristics of antibody 9604 are summarized in Table 1. Using solid phase immunoassays, antibody 9604 was shown to bind to dDNA in a RIA, but did not bind to any of the phospholipids tested nor to fibrinogen by ELISA. Antibody 9604 also had lupus anticoagulant antibody activity in a dilute APTT assay and was strongly cytotoxic to platelets in vitro[40].

Reactivity of Hybridoma Antibody 9604 with Intact Resting and Activated Platelets

Table 2 compares the direct binding reactivities of hybridoma antibody 9604 with fixed resting and fixed washed platelets in both the ELISA and RIA. In the ELISA, antibody 9604 did not bind to glutaraldehyde fixed resting platelets, but bound to fixed washed platelets and to platelet lysate. In the RIA, antibody 9604 bound to fixed resting platelets, but binding was significantly higher to fixed or unfixed washed platelets. In both assays, positive control antibody 9500 and negative control antibody E110 showed similar reactivities, regardless of whether platelets were resting, washed, or fixed.

In further experiments, antibody 9604 was tested for binding to normal resting platelets and platelets activated with ADP. As shown in Table 3, both hybridoma culture supernatant from 9604 cells and affinity-purified 9604 IgM bound to platelets activated with 1 µg/ml ADP (2.34 µM) and fixed with glutaraldehyde in plasma, but did not bind to fixed resting platelets, while positive and negative control

Table 1. General Characteristics of Antibody 9604

Activities Tested	Reaction
Anti-dDNA	+
Anti-Cardiolipin	-
Anti-Phosphatidic Acid	-
Anti-Phosphatidylcholine	-
Anti-Phosphatidylethanolamine	-
Anti-Phosphatidylglycerol	-
Anti-Phosphatidylinositol	-
Anti-Phosphatidylserine	-
Anti-Fibrinogen	-
Lupus Anticoagulant	+
Platelet Cytotoxicity	+

Table 2. Binding of Hybridoma Antibody 9604 to Live and Fixed Platelets in the ELISA and Radioimmunoassay

Platelet Preparation	Platelet Binding Reactivities of Hybridoma Antibody		
	9604	9500[a]	B110[a]
ELISA[b]			
Fixed Resting Platelets	0.08 ± 0.02	0.58 ± 0.01	0.07 ± 0.02
Fixed Washed Platelets	0.17 ± 0.01**	0.64 ± 0.08	0.02 ± 0.02
Platelet Lysate	0.65 ± 0.42	0.62 ± 0.12	0.01 ± 0.02
Radioimmunoassay[c]			
Fixed Resting Platelets	13,756	15,007	1,697
Fixed Washed Platelets	20,286	17,166	2,818
Unfixed Washed Platelets	21,169	14,257	1,150

[a] 9500 is a platelet-binding hybridoma IgM antibody, run as a positive control; and B110 is a nonplatelet-binding hybridoma IgM antibody, run as a negative control, respectively.

[b] Values represent means of OD_{410} values in the platelet-binding ELISA plus or minus 2 SE, calculated from duplicate wells run in 2 separate assays. A binding value of $OD_{410} \geq 0.08$ was considered to be positive.

[c] Values represent the mean cpm bound to platelets plus or minus 2 SE, calculated from duplicate tubes run in 2 separate assays. A binding value > 3000 cpm was considered to be positive.

Table 3. Comparison of the Binding of Hybridoma Antibody 9604 to Resting and ADP-Activated Platelets

Hybridoma Antibody	Resting Platelets	ADP-Activated Platelets
Supernatants		
9604	0.01 ± 0.03[a]	0.29 ± 0.18
9500[b]	0.58 ± 0.25	0.42 ± 0.18
B110[b]	0	0
IgM (5 μg/ml)[c]		
9604	0.05 ± 0.02	0.32 ± 0.14
B110	0.01 ± 0.02	0

[a] Values represent the mean OD_{410} readings plus or minus 2 SE, calculated from assays done in duplicate and repeated 7 times for 9604 supernatant and 4 times for 9604 IgM.

[b] 9500 is a platelet-binding hybridoma IgM antibody, run as a positive control; and B110 is a nonplatelet-binding hybridoma IgM antibody, run as a negative control.

[c] Hybridoma IgM was purified by affinity-chromatography (see Methods) and tested at an IgM concentration of 5 μg/ml.

antibodies, 9500 and B110, showed consistent results on fixed resting and activated platelets. As shown in Figure 1, the binding of affinity-purified 9604 IgM to fixed ADP-activated platelets increased in a dose-dependent manner.

Binding of Antibody 9604 to Platelet Proteins by Western Blotting Analysis

The reactivity of antibody 9604 with platelet proteins was analyzed by Western blotting and endothelial cell proteins were used as a control to determine whether the reactivity of 9604 was restricted to platelets. As shown in Figure 2A, when whole platelet proteins were solubilized and run under reducing conditions in SDS PAGE, the antibody reacted mainly with protein bands of approximate molecular weights (M.W.) 200,000 (doublet) and 32,000, as well as with multiple bands of molecular weights between 100,000 and 45,000 (Lane 3). A strip blotted with platelet-binding antibody, 9500 (Lane 4), showed binding predominantly to a band of 100,000 M.W., a pattern which was different from that of antibody 9604. A strip blotted with control nonplatelet-binding antibody, B111 (Lane 5), did not show any reactive protein bands. On blots of whole endothelial cell proteins, antibody 9604 bound to a single protein of approximately 200,000 M.W., but the 32,000 M.W. protein was completely absent from these blots (Lane 7). Antibody 9500 bound strongly to a group of endothelial cell protein bands with mobilities close to actin (45,000 M.W.) (Lane 8), while control antibody B111 showed no reactivity with endothelial cell proteins (Lane 9). Interestingly, when the platelet proteins were solubilized and run under non-reducing conditions in

SDS PAGE (Figure 2B), the 200,000 and 32,000 M.W. bands reactive with 9604 disappeared from the blot, and 9604 reactive platelet protein bands appeared mainly at 50,000, 92,000 and higher molecular weights (≥200,000) (Lane 5). Negative control IgM antibodies B3123 and B110 (Lanes 3 and 4), and 9703 (Lane 6), a platelet-binding antibody with phospholipid but not protein reactivity, showed no bands on Western blots. Antibody 9500 showed several major reactive bands between 30,000 and 90,000 M.W. (Lane 7).

Figure 2C shows the binding of antibody 9604 to isolated platelet membrane proteins under reducing conditions. The 9604 reactive 200,000 and 32,000 M.W. bands remained the most reactive when platelet membrane proteins were used in place of whole platelet proteins (Lane 5). The negative control antibodies (Lanes 3 and 4) showed no reactivity except for a band of approximately 80,000 M.W., which is likely to be the mu chain of surface bound IgM recognized by the peroxidase anti-human IgM antibody. Antibody 9500 bound to a major band of approximately 45,000 M.W., but also exhibited reactivity with multiple other bands in the platelet membrane preparation (Lane 7).

In order to isolate and characterize two of the three platelet proteins reactive with 9604, platelet proteins were electrophoretically separated in a SDS gradient gel and proteins with mobilities of approximately 200,000 and 32,000 M.W were electroeluted from the gel and rerun on a SDS-minigel (Figure 3A). The isolated 32,000 and 200,000 M.W. bands are shown in Lanes 2 and 3, respectively. A mixture of these two fractions revealed the same two

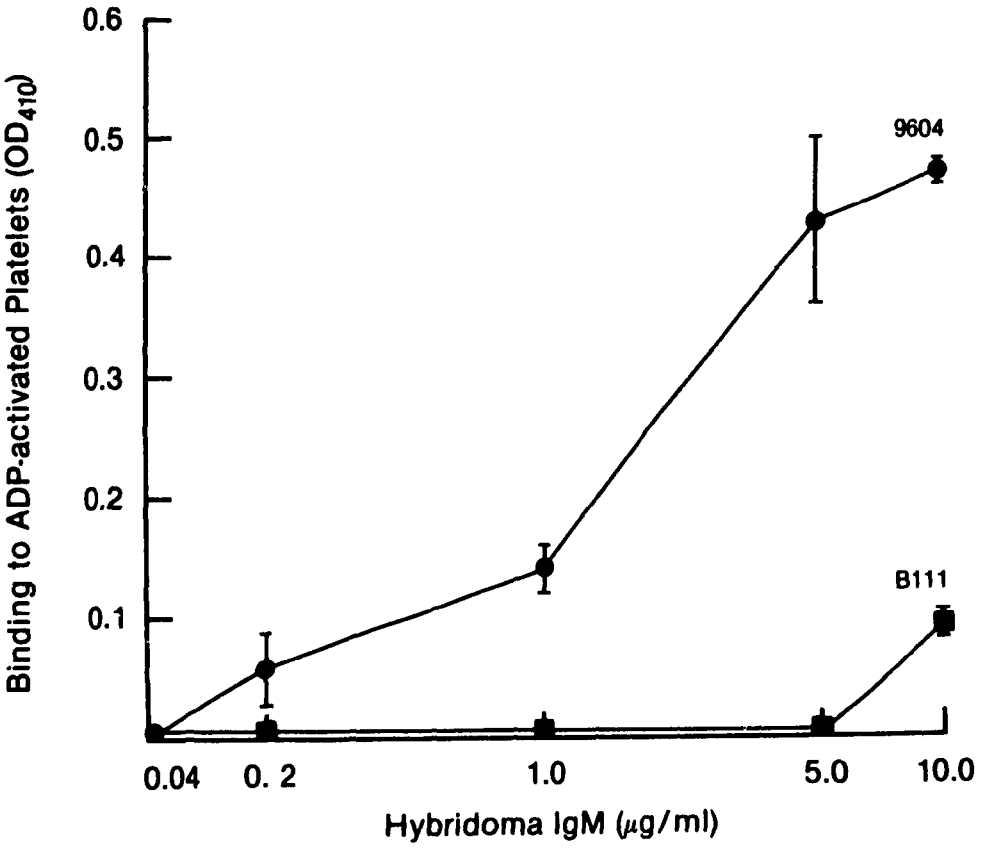


Figure 1. Dose-Dependent Binding of 9604 IgM to ADP-Activated Platelets

Platelets were activated with 1.0 μg/ml (2.34 μM) ADP, fixed with glutaraldehyde and incubated with 9604 IgM or the negative control B111 IgM, affinity-purified from hybridoma culture supernatants.

bands seen in the individual fractions (Lane 4). By Western blotting, both of the isolated proteins retained reactivity to antibody 9604 (Figure 3B, Lanes 4 and 5).

Glycoprotein Staining of Platelet Proteins Reactive with Antibody 9604

Glycoprotein staining with fuchsin-sulfite of SDS polyacrylamide gels showed that the 200,000 M.W. platelet protein band contained carbohydrate, while the 32,000 M.W. protein band did not (data not shown).

Binding of Antibody 9604 to Enzyme-Treated Platelets

The enzyme sensitivity of the 9604 reactive platelet proteins was examined by treating lysed platelets with protease, proteinase K, trypsin, DNAase, RNAase, phospholipase A_2 or phospholipase C and then using these enzyme treated platelets in Western blotting analysis. All bands reactive with antibody 9604 were completely abolished by treatment of platelets with trypsin, protease, and proteinase K (Figure 4A, Lanes 4 to 6), while only a few high molecular weight bands (100,000 and > 200,000 M.W.) were affected by the treatment with DNAase, RNAase, phospholipase A_2, and phospholipase C (Figure 4A) (Lanes 7 to 10). DNAase, RNAase, phospholipase A_2, and phospholipase C did not affect the reactivity of 9604 with the 200,000 M.W. and 32,000 M.W. bands. Reactivity which appeared at approximately 40,000 M.W. after protease digestion was due to the binding of 9604 to components in the enzyme preparation as can be seen in the blot of protease with 9604 (Lanes 5 and 12). These results are consistent with results obtained in a platelet binding

Figure 2. Western Blotting of Hybridoma Antibodies on Platelet and Endothelial Cell Proteins

In panel A, cells were solubilized in SDS sample buffer with 2-mercaptoethanol and run under reducing conditions. Lane 1: M.W. standards; Lane 2: whole platelet proteins stained with amido black; Lanes 3-5: whole platelet proteins blotted with hybridoma antibodies 9604, 9500, and B111, respectively; Lane 6: whole endothelial cell proteins stained with amido black; Lanes 7-9: whole endothelial cell proteins blotted with hybridoma antibodies 9604, 9500, and B111 respectively.

In panel B, platelets were solubilized in SDS sample buffer without 2-mercaptoethanol and run under nonreducing conditions. Lane 1: M.W. standards; Lane 2: whole platelet proteins stained with amido black; Lanes 3-7: whole platelet proteins blotted with hybridoma antibodies B3123, B110, 9604, 9703, and 9500, respectively.

In panel C, isolated platelet membranes were solubilized in SDS sample buffer with 2-ME and run under reducing conditions. Lane 1: M.W. standards; Lane 2: platelet membrane proteins stained with amido black; Lanes 3-7: platelet membrane proteins blotted with hybridoma antibodies B3123, B110, 9604, 9703, and 9500, respectively.

Figure 2A

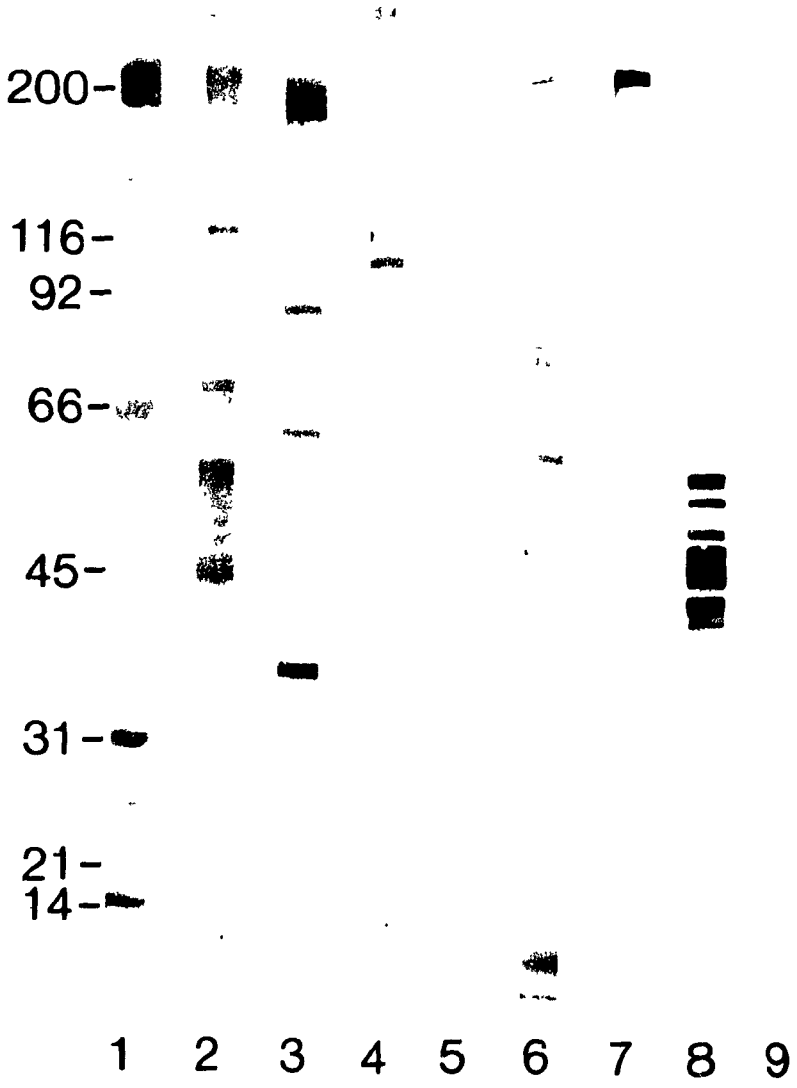


Figure 2B

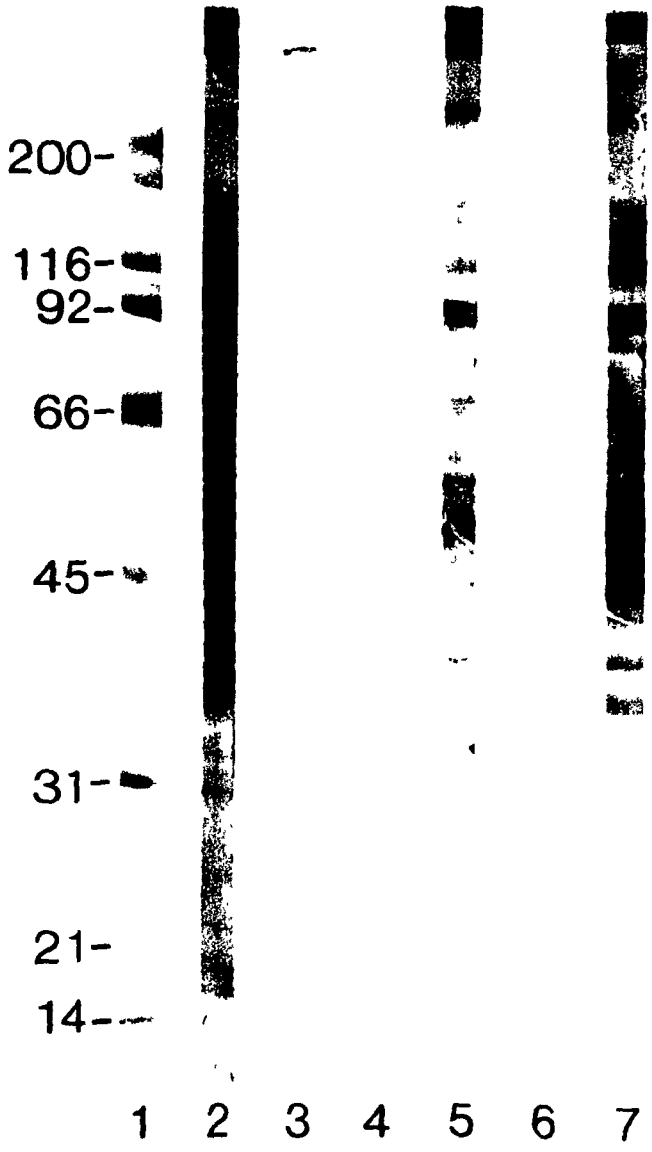
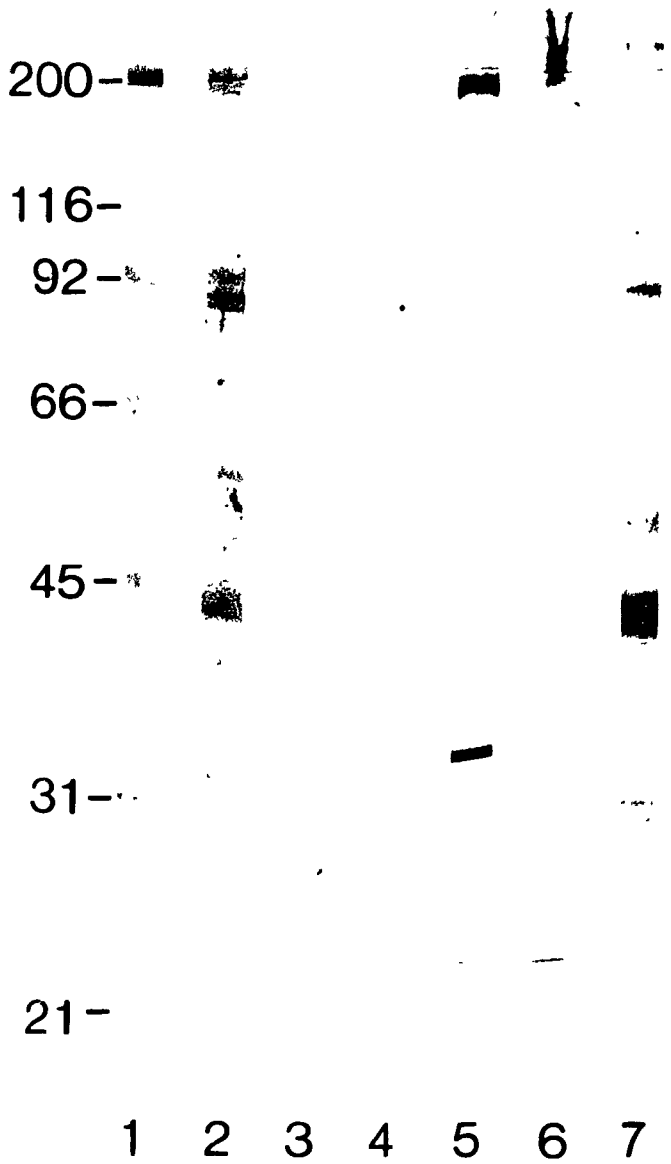


Figure 2C



RIA, in which intact platelets were first incubated with the same enzymes and then tested for binding of antibody 9604. The results, shown in Figure 4B, demonstrated that the binding of antibody 9604 to platelets was significantly decreased by treatment of platelets with trypsin (25%) and slightly decreased by treatment with protease and DNAase (10-12%), but was not affected by RNAase, phospholipase A_2, or phospholipase C.

Monoclonality of Antibody 9604

Since antibody 9604 bound to several platelet proteins, it was necessary to identify whether these reactivities were due to more than one antibody present in the preparation or due to the polyspecificity of a single antibody. The monoclonality of antibody 9604 was demonstrated by isoelectric focusing gel electrophoresis. As shown in Figure 5, the light chain of 9604 IgM showed a single major band which focused at a pI of approximately 5.8 (Lane 3). This figure also shows the isoelectric pattern of light chains of a monoclonal myeloma IgM protein (Bor) (Lanes 1 and 2) and of polyclonal human serum IgM (Lane 4) for comparison.

Figure 3. SDS PAGE and Western Blotting of 9604 Reactive Platelet Proteins Isolated by Electroelution

In panel A, SDS polyacrylamide gels were run under reducing conditions and stained with Coomassie blue. Lane 1: whole platelet proteins; Lane 2: isolated 32,000 M.W. platelet protein; Lane 3: isolated 200,000 M.W. platelet protein; Lane 4: mixture of isolated 200,000 and 32,000 M.W. platelet proteins. Lane 5: M.W. standards.

In panel B, SDS PAGE were run under reducing conditions and proteins in the polyacrylamide gels were electrophoretically transferred onto nitrocellulose membrane and analyzed by Western blotting. Lane 1: M.W. standards; Lane 2: whole platelet proteins stained with amido black; Lanes 3-5: 9604 was incubated with blots of: whole platelet proteins (Lane 3), isolated 200,000 M.W. platelet protein (Lane 4), and isolated 32,000 platelet protein (Lane 5).

Figure 3A

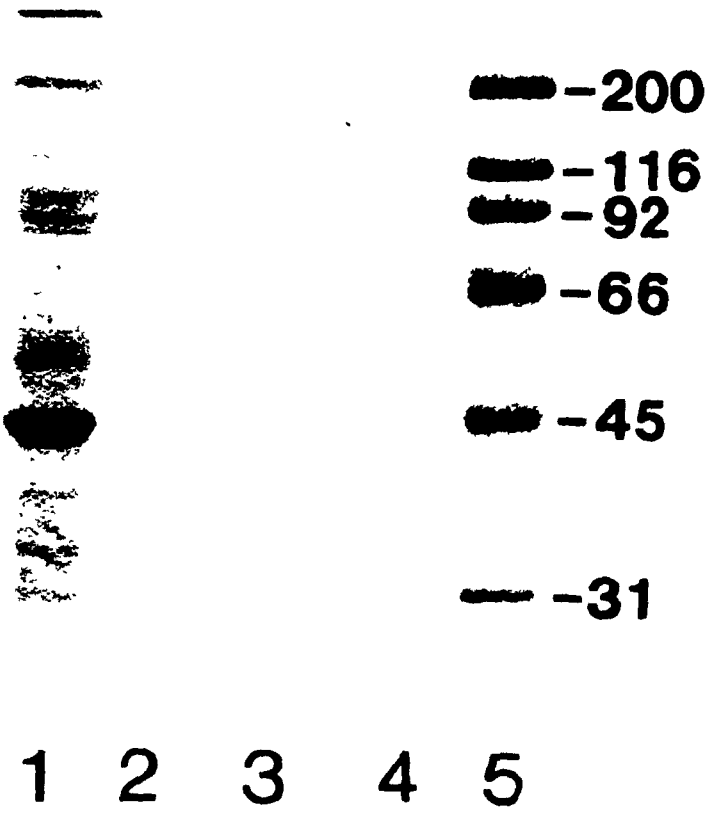


Figure 3B

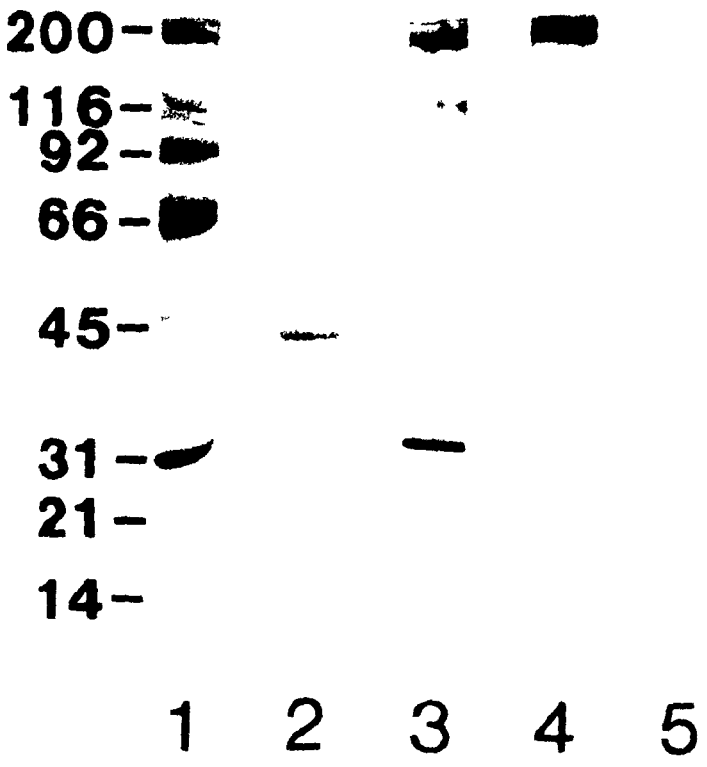


Figure 4. Western Blotting Analysis and RIA of the Binding of Antibody 9604 to Enzyme Treated Platelets

In panel A, SDS gel was run under reducing conditions. Lane 1: M.W.standards; Lane 2: whole platelet proteins stained with amido black; Lanes 3-10 show platelet lysates treated with buffer or enzyme and then blotted with 9604 supernatant: nontreated whole platelet proteins (Lane 3), platelet proteins treated with trypsin (Lane 4), platelet proteins treated with protease (Lane 5), platelet proteins treated with proteinase K (Lane 6), platelet proteins treated with DNAase (Lane 7), platelet proteins treated with RNAase (Lane 8), platelet proteins treated with phospholipase A_2 (Lane 9), platelet proteins treated with phospholipase C (Lane 10). Lanes 11-17 show the enzymes alone blotted with 9604 supernatant: trypsin (Lane 11), protease (Lane 12), proteinase K (Lane 13), DNAase (Lane 14), RNAase (Lane 15), phospholipase A_2 (Lane 16), phospholipase C (Lane 17).

In panel B, intact live platelets were treated with various enzymes and then tested for 9604 binding by RIA. The values represent percent binding and are means of duplicate samples.

Figure 4A

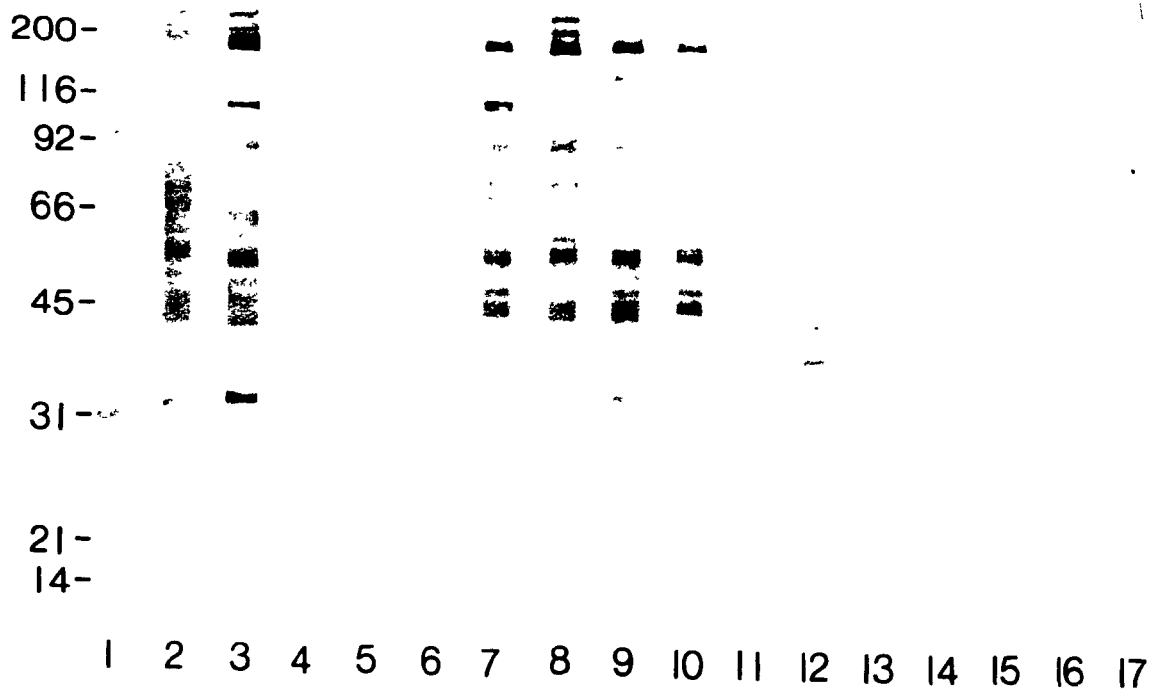
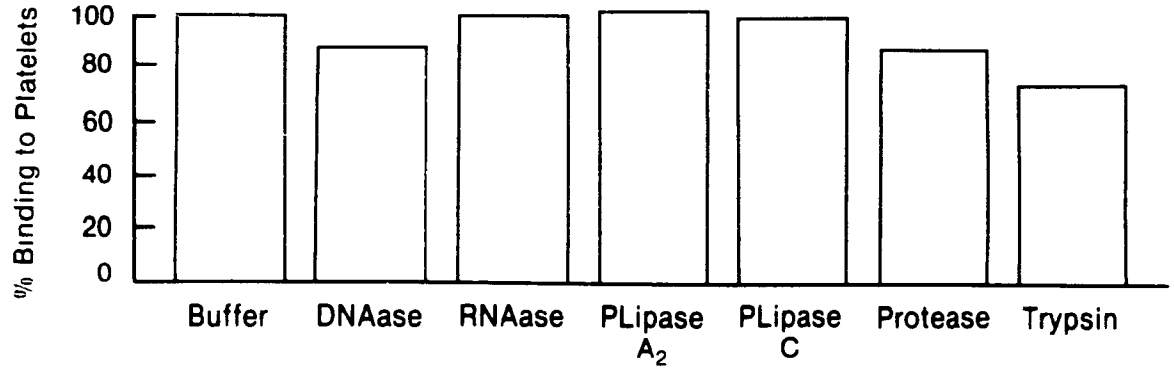


Figure 4B


% Binding to Platelets
100
80
60
40
20
0
Buffer
DNAase
RNAase
PLipase A2
PLipase C
Protease
Trypsin

DISCUSSION

Hybridoma autoantibody 9604 was derived from the fusion of GM 4672 human lymphoblastoid cells with peripheral blood lymphocytes from an SLE patient. In a previous study which characterized the platelet-binding properties of similarly derived SLE hybridoma autoantibodies, most antibodies showed similar reactivity in a RIA using live platelets and in an ELISA using glutaraldehyde fixed normal platelets[33]. Hybridoma antibody 9604, however, was different in that it did not bind to fixed intact platelets in ELISA, but bound to live platelets in the RIA. This antibody also reacted with lysed platelets in ELISA, bound to unique platelet proteins in Western blotting analysis, and was strongly cytotoxic to platelets using an in vitro ^{51}Cr release assay[40]. It is noteworthy that 9604 reacted with live platelets in the radioimmunoassay and cytotoxicity assays, but not with fixed unactivated platelets in the ELISA, suggesting that there may have been some activation and expression of the 9604 reactive protein during the washing steps in these in vitro assays. This was confirmed by experiments showing that antibody 9604 reacted with fixed washed platelets in the ELISA and that washing of the platelets enhanced the binding of 9604 to fixed or unfixed platelets in the RIA. It is not clear whether the binding of 9604 to fixed resting platelets in the RIA but not in the ELISA is due to differences in assay conditions or assay sensitivity.

It has been noted by several investigators that the activation of platelets is accompanied by the expression of new antigens on the cell surface, rearrangement of membrane components, or binding of

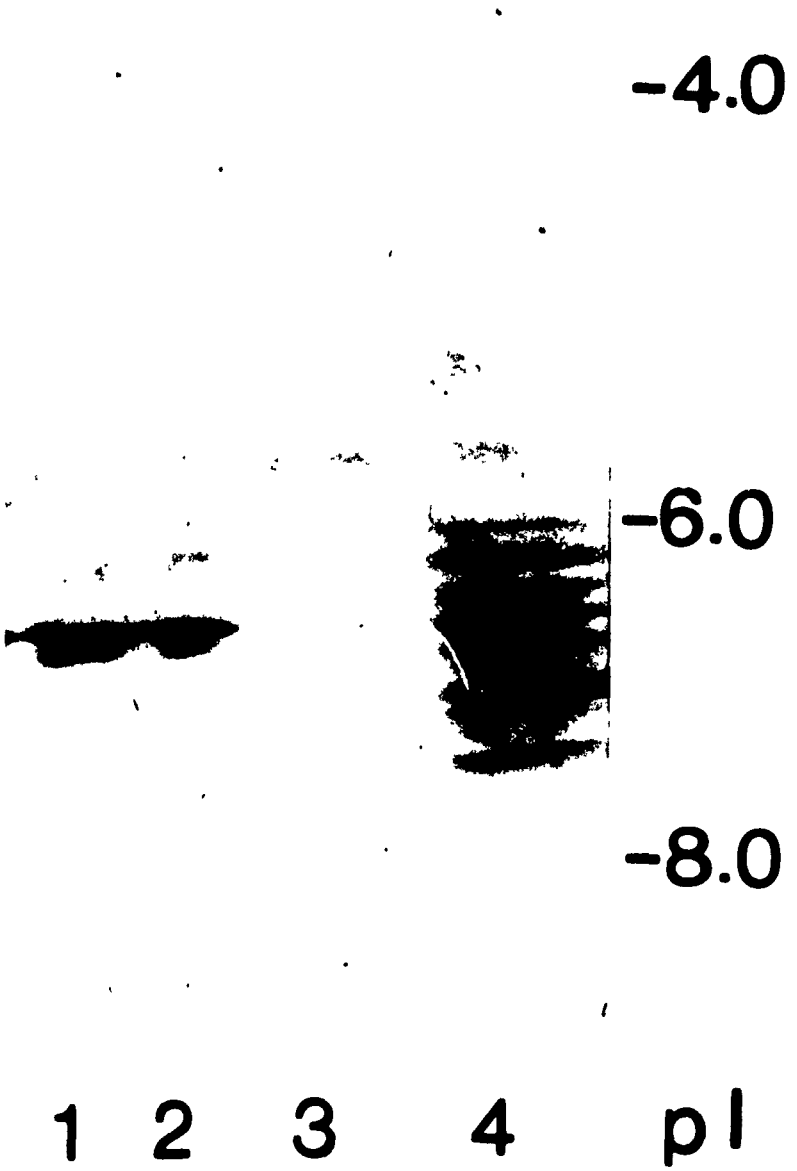


Figure 5. Isoelectric Focusing of the Light Chain of 9604 IgM

Lanes 1 and 2: light chain of myeloma IgM protein (Bor); Lane 3: light chain of 9604 IgM; Lane 4: light chains of polyclonal human IgM.

exogenous proteins to the surface[12-24]. A mouse monoclonal antibody, KC4, identified a 140,000 M.W. integral membrane protein that was newly expressed on the surface of thrombin-activated platelets and associated with platelet secretion[25-28]. Another mouse monoclonal antibody, 2.28, was reported to react with a secreted lysosome-like granule protein of 53,000 M.W. on the surface of activated platelets[29]. A similar phenomenon was noted for a human IgM monoclonal autoantibody, 5E5, derived from a patient with immune-mediated thrombocytopenic purpura, which recognized a neoantigen on glycoprotein IIIa which was expressed on platelets activated by thrombin or stored for more than 3 days[30]. The epitope responsible for antibody 5E5 binding was a protein with an apparent molecular weight of 95,000. To our knowledge, there has not been any report of similar reactivities of autoantibodies derived from SLE patients.

In the present study, we have characterized hybridoma antibody 9604 and the platelet components to which this antibody binds. Our results demonstrate that this SLE-derived antibody 9604 binds to antigen(s) exposed on platelets activated by ADP in the presence of plasma. The finding that antibody 9604 does not bind to fibrinogen excludes the possibility that this antibody reacts with plasma fibrinogen bound to surface receptors which are exposed only on activated platelets.

We have attempted to identify the proteins responsible for the binding of 9604 to platelets using Western blotting analysis. The results of these studies demonstrated that antibody 9604 bound mainly to two platelet protein bands of M.W. 200,000 and 32,000 in SDS gels

run under reducing conditions. These proteins were also demonstrable in isolated membrane preparations of lysed platelets. The 200,000 M.W., but not the 32,000 M.W. band, was shown to be a glycoprotein by fuchsin-sulphite staining. The 200,000 M.W. band was also seen on blots incubated with other hybridoma antibodies and blots of endothelial cell proteins incubated with 9604. This is a wide band composed of several platelet proteins with mobilities of approximately 200,000 M.W. in SDS gels. Platelet proteins of approximately 200,000 M.W. include the Fc receptor (210,000 M.W.)[40], filamin (250,000 M.W.), talin (235,000 M.W.) and myosin (200,000 M.W.)[41]. Experiments using commercially available specific antibodies to these high molecular weight proteins excluded the possibility that 9604 binds to filamin and Western blotting analysis using Triton X100 extracts of platelets[41] demonstrated that 9604 does not bind to talin (data not shown). The 32,000 M.W. protein band was seen only on blots of platelet proteins incubated with antibody 9604. This polypeptide appeared to be labile and often disappeared when the gradient of the gel was varied (data not shown). Moreover, when the gel was run under non-reducing conditions, both 9604 reactive bands of 32,000 and 200,000 M.W. disappeared and antibody 9604 showed binding to higher molecular weight protein(s). Taken together, these data suggest that the 9604 bound protein(s) are located in the platelet membrane and may be composed of polypeptide subunits which are linked by disulfide bonds.

In an attempt to identify the protein(s) bound by antibody 9604, various antibodies and lectins with defined specificities were used

to probe the blots. No corresponding reactivity was found with concanavalin A and wheat germ agglutinin, which bind to different platelet membrane glycoproteins; monoclonal antibodies against GPIb, GPIIb and GPIIIa; monoclonal antibodies against cytoskeletal proteins; or polyclonal antibody against human factor VIII-related antigen, a high molecular weight glycoprotein present in platelet granules. Enzyme digestion of lysed platelets showed that the 9604 reactive epitopes were completely degraded by protease, proteinase K or trypsin, suggesting that the determinants responsible for 9604 binding are protein in nature. Trypsin digestion of live platelets also decreased the binding of 9604 by 25%, suggesting that trypsin had limited access to the antigen in intact platelets. treatment of lysed platelets with DNAase, RNAase, and phospholipases A_2 and C showed a loss of reactivity of 9604 with minor platelet protein bands in Western blotting, suggesting that some 9604 reactive platelet components may contain phosphodiester or phospholipid moieties. However, treatment with these enzymes did not affect the 200,000 or 32,000 M.W. components.

Platelet-binding antibodies[1,2,32,33] and abnormalities of platelet function[3-6,42] have been demonstrated in patients with SLE. Karpatkin et al.[1] reported that anti-platelet antibodies could be detected in 78% of patients with SLE but that only 14% of these patients were thrombocytopenic. Efforts have been made to characterize the relationship between anti-platelet antibodies and platelet function and to identify epitopes on platelets responsible for autoantibody binding. Kaplan et al.[2] studied the reactivities of

sera from SLE patients with platelet membrane proteins derived from normal and autologous platelets using an immunoblotting technique. Two target antigens of 108,000 and 66,000 M.W. were found for some SLE sera. In another study, it was reported that all (4/4) sera from SLE patients caused aggregation and immunofluorescent staining of normal platelets and that the active fractions in these sera contained antibodies to C1q, C1s and beta$_2$-microglobulin[43,44]. Weissbarth et al.[45] demonstrated that anti-platelet antibodies induce serotonin release from platelets from SLE and rheumatoid arthritis patients. Furthermore, they found that binding of IgG-containing immune complexes to platelet Fc receptors was associated with platelet phagocytosis, which induced release of granule contents and the aggregation of circulating platelets. These data, however, give little indication of the fine specificities of the platelet-binding autoantibodies found in SLE patients.

To date, there have been relatively few studies on the reactivities of human hybridoma anti-platelet antibodies derived from patients with SLE. Shoenfeld et al.[31] demonstrated that some SLE-derived hybridoma antibodies showed platelet-binding activity. Asano et al.[32] reported that SLE-derived hybridoma autoantibodies to single-stranded DNA could crossreact with platelets and that the platelet epitope(s) did not appear to involve DNA, protein or sialic acid. We have previously shown that several SLE and normal-derived hybridoma antibodies, initially selected for anti-DNA and lupus anticoagulant activity, reacted with platelets. Platelet reactivity of these hybridoma antibodies was highly correlated with binding to

DNA, cardiolipin, and phosphatidylethanolamine and was sometimes affected by pretreatment of the platelets with trypsin[33]. However, it still remains unclear as to how anti-platelet antibodies cause abnormalities in platelet function or disturbances in hemostasis in SLE. Although we have not examined the effects of 9604 on platelet function, we have previously demonstrated that 9604 is highly cytotoxic to platelets in in vitro chromium-51 release assays[40]. This suggests one possible mechanism by which autoantibodies, such as 9604, could affect hemostasis in vivo. In addition, the reactivity of 9604 with ADP-activated platelets, but not with resting platelets, suggests that the reactivity of 9604 with platelets in vivo would be dependent upon factors or other autoantibodies capable of initiating platelet activation. Further characterization of the molecular components on the platelet membrane to which this antibody binds may provide insight into the mechanisms by which platelet-binding autoantibodies interfere with normal platelet function.

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REFERENCES

1. Karpatkin S, Strick N, Karpatkin MB, Siskind GW: Cumulative experience in the detection of antiplatelet antibody in 234 patients with idiopathic thrombocytopenic purpura, systemic lupus erythematosus and other clinical disorders. Am J Med 52:776, 1972

2. Kaplan C, Champeix P, Blanchard D, Muller JY, Cartron JP: Platelet antibodies in systemic lupus erythematosus. Br J Haematol 67:89, 1987

3. Rick ME, Hoyer LW: Hemostatic abnormalities in systemic lupus erythematosus. J Clin Rheum Dis 1:583, 1975

4. Lee SL, Miotti AB: Disorders of hemostatic function in patients with systemic lupus erythematosus. Semin Arthritis Rheum 4:241, 1975

5. Budman DR, Rosse WF: Hematologic aspects of systemic lupus erythematosus. Ann Intern Med 86:220, 1977

6. Gladman DD, Urowitz MB, Tozman EC, Glynn MFX: Hemostatic abnormalities in systemic lupus erythematosus. Quart J Med 207:424, 1983

7. Dixon RH, Rosse WF: Platelet antibody in autoimmune thrombocytopenia. Br J Haematol 31:129, 1975

8. McMillan R: Immune thrombocytopenias. Clin Haematol 12:69, 1983

9. Mueller-Eckhardt C, Kayser W, Mersch-Baumert K, Mueller-Eckhardt G, Breidenbach M, Kugel HG, Graubner M: The clinical significance of platelet associated IgG: a study on 298 patients with various disorders. Br J Haematol 46:123, 1980

10. Hegde UM, Williams K, Devereux S, Bowes A, Powell D, Film D,

Fischer D: Platelet associated IgG and immune thrombocytopenia in lymphoproliferative and autoimmune disorders. Clin Lab Haematol 5:9, 1983

11. Frojmovic MM and Milton JG: Physical, chemical and functional changes following platelet activation in normal and "giant" platelets. Blood Cells 9:359-82, 1983.

12. Phillips DR, Jennings LD, Prasanna HR: Ca^{2+}-mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. J Biol Chem 255: 11629, 1980

13. Vicic WJ, Lages B, Weiss HJ: Release of human platelet factor V activity is induced by both collagen and ADP and is inhibited by aspirin. Blood 56:448, 1980

14. Koutts J, Walsh PN, Plow E F, Fenton JW II, Bouma BN, Zimmerman TS: Active release of human platelet factor VIII-related antigen by adenosine diphosphate, collagen, and thrombin. J Clin Invest 62:1255, 1978

15. Harris KW, Esmon CT: Protein S is required for bovine platelets to support activated protein C binding and activity. J Biol Chem 260:2007, 1985

16. Sinha D, Seaman FS, Koshy A, Knight LC, Walsh PN: Blood coagulation factor XIa binds specifically to a site on activated human platelets distinct from that for Factor XI. J Clin Invest 73:1550, 1984

17. Greenberg CS, Shuman MA: Specific binding of blood coagulation Factor XIIIa to thrombin-stimulated platelets. J Biol Chem 259:14721, 1984

18. Miles LA, Plow EF: Binding and activation of plasminogen on the platelet surface. J Biol Chem 260:4303, 1985

19. Plow EF, Ginsberg MH: Specific and saturable binding of plasma fibronectin to thrombin stimulated platelets. J Biol Chem 256:9477, 1981

20. Bennett JS, Vilaire G: Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393, 1979

21. Marguerie GA, Plow EF, Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254:5357, 1979

22. Kornecki E, Tusxzynski GP, Niewiarowski S: Identification of human platelet membrane fibrinogen receptors by immunochemical techniques. Haematologia 17:387, 1984

23. Greengard JS, Griffin JH: Receptors for high molecular weight kininogen on stimulated washed human platelets. Biochemistry 23:6863, 1984

24. George JN, Lyons RM, and Morgan RK: Membrane change associated with platelet activation. Exposure of actin on the platelet surface after thrombin-induced secretion. J Clin Invest 66:1, 1980

25. Hsu-Lin SC, Berman CL, Furie BC, August D, Furie B: A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. J Biol Chem 259:9121, 1984

26. Berman CL, Yeo EL, Wencel-Drake JD, Furie BC, Ginsberg MH, Furie B: A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and

subcellular localization of platelet activation-dependent granule-external membrane protein. J Clin Invest 78:130-7, 1986

27. McEver RP, Martin MN: A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. J Biol Chem 259:9799, 1984

28. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF: A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. J Cell Biol 101:880, 1985

29. Nieuwenhuis HK, van Oosterhout JJG, Rozemuller E, van Iwaarden F, Sixma JJ: Studies with a monoclonal antibody against activated platelets: Evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. Blood 70:838, 1987

30. Nugent DJ, Kunicki TJ, Berglund C, Bernstein D: A human monoclonal autoantibody recognizes a neoantigen on glycoprotein IIIA expressed on stored and activated platelets. Blood 70:16, 1987

31. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC, Furie B, Stollar BD, Schwartz RS: Production of autoantibodies by human-human hybridomas. J Clin Invest 70:205, 1982

32. Asano T, Furie BC, Furie B: Platelet binding properties of monoclonal lupus autoantibodies produced by human hybridomas. Blood 66:1254, 1985

33. Rauch J, Meng Q-h, Tannenbaum H: Lupus anticoagulant and antiplatelet properties of human hybridoma autoantibodies. J Immunol 139:2598, 1987

34. Massicotte H, Rauch J, Shoenfeld Y, 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, 1984

35. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Shaller JG, Talal N, Winchester RJ: The 1982 revised criteria for the diagnosis of SLE. Arthritis Rheum 25:1271, 1982

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

37. Zacharius RM, Zeu TE, Morrison JH: Glycoprotein staining following electrophoresis acrylamide gels. Anal Biochem 30:148, 1969.

38. Gibson DM, Taylor BA, Cherry M: Evidence for close linkage of a mouse light chain marker with the Ly-2.3 locus. J Immunol 121:1585, 1978

39. Rauch J, Tannenbaum H, Straaton K, Wild J: Human-human hybridoma autoantibodies with both anti-DNA and rheumatoid factor activities. J Clin Invest 77:106, 1985

40. Meng Q-h, Rauch J: Differences between human hybridoma platelet-binding antibodies derived from systemic lupus erythematosus patients and normal individuals. Autoimmunity 1989 (In press)

41. O'Halloran T, Beckerle MC, Burridge K: Identification of talin as a major cytoplasmic protein implicated in platelet activation. Nature 317:449, 1985

42. Regan MG, Lackner H, Karpatkin S: Platelet function and

coagulation profiles in lupus erythematosus. Studies in 50 patients. Ann Intern Med 81:462, 1974

43. Gerhard K. M. Endresen, Øystein FØrre: Studies on the binding of proteins to the human platelet surface: Relation to platelet activation. Throm Haemo 53:360, 1985

44. Falus A, Merétey K, Bagdy D, Dioszegi M, Bohm U, Csak E, Bozsoky S: Beta2-microglobulin-specific autoantibodies cause platelet aggregation and interfere with ADP-induced aggregation. Clin Exp Immunol 47:103, 1982

45. Weissbarth E, Baruth B, Mielke H, Liman W, Deicher H: Platelets as target cells in rheumatoid arthritis and systemic lupus erythematosus: A platelet specific immunoglobulin inducing the release reaction. Rheum Int 2:67, 1972

46. Stricker RB, Reyes PT, Corash L, Shuman MA: Evidence that a 210,000 molecular weight glycoprotein (GP210) serves as a platelet Fc receptor. J Clin Invest 79:1589, 1987

CHAPTER VI

EFFECTS OF LUPUS AUTOANTIBODIES ON ENDOTHELIAL CELLS AND ON THE INTERACTIONS BETWEEN ENDOTHELIAL CELLS AND PLATELETS

INTRODUCTION

As described in Chapter 1, the interactions between platelets and endothelial cell (EC)s involve many factors and are very complex. In general, any trauma to or modification of the endothelium will permit platelet adherence. Once adhesion has occurred, the cascade of coagulation is initiated and will result in hemostasis or thrombosis. Immune mechanisms may also be involved in endothelial injury. In order to investigate whether lupus autoantibodies play a role in the interactions between platelets and ECs, the present study was designed to examine the binding and cytotoxicity of human hybridoma autoantibodies to ECs and their effects on the adhesion of platelets to endothelial monolayers in vitro.

MATERIALS AND METHODS

Culturing of Human Umbilical Vein Endothelial Cells

Human umbilical vein EC monolayers were prepared from umbilical veins following the method of Jaffe et al. (213). Human umbilical cords were placed in normal saline and used within 6 hours of delivery. Both ends of the cord were cut and the vein was cannulated with butterfly needles (#6) and clamped. The vein was washed with Hank's buffer until the wash no longer contained any blood and looked clear. Ten to twenty milliliters (depending on the size of the cord)

of 0.1% collagenase in Hanks' buffer was injected into the vein and incubated for 30 minutes at 37°C in 5% CO_2. The cell suspension in the vein was then withdrawn using a syringe and centrifuged at 250 x g for 10 minutes. The cell pellet was washed twice with Hanks' buffer, resuspended in 10 ml of RPMI 1640 medium containing 15% fetal calf serum, 50 U/ml heparin (Leo Laboratories, Pickering, Ontario) and 10 µg/ml endothelial cell growth supplement (ECGS) (Collaborative Research, Inc., Bedford, MA) (EC medium) and placed into 75 cm^2 flasks (Costar, Cambridge, MA), which were precoated with 0.2% gelatin for one hour at room temperature. The cells were incubated at 37°C in 5% CO_2 and fed twice a week. When the cells grew to confluence, they were trypsinized and passaged at a 1:2 dilution and kept for 3 passages only.

Preparation of Endothelial Cell Monolayers in Microtiter Wells

Confluent EC monolayers in 75 cm^2 flasks were washed twice with Tyrodes' buffer without calcium and magnesium, but containing 4 mM EDTA (Tyrodes-EDTA) and incubated with 5 ml of 0.02% trypsin-EDTA (GIBCO, Grand Island, NY) at 37°C until all cells were completely detached from the flask (approximately 10 to 15 minutes). The cells were then washed 3 times with Tyrodes-EDTA, resuspended in EC medium at a concentration of 2×10^5 cells/ml, and plated in Falcon flat-bottom 96-well microtiter plates (Becton Dickinson & Company, Oxnard, CA) precoated with 100 µl per well of a 2.5 µg/ml solution of fibronectin in distilled water. The plates were incubated at 37°C in 5% CO_2 for 24 to 48 hours or until the cells formed confluent monolayers. The cells were identified as ECs by their "cobblestone"

appearance (polymorphism) (213) and by their positivity with anti-Factor VIII antibody (see Table 3 of Chapter X).

Direct Binding ELISA on Endothelial Cells

The EC monolayers in microtiter wells were washed twice with Hanks' buffer and fixed by adding 100 µl of 2.5% glutaraldehyde in Hanks' buffer to each well and incubating for 30 minutes at room temperature. The plates were then washed 4 times with 10 mM Tris-buffered saline, pH 7.4 (TBS) and blocked with 0.5% gelatin in TBS at 37°C for 1 hour. The wells were washed 5 times with TBS and 75 µl of hybridoma supernatant or affinity-purified hybridoma antibody at 5 µg/ml were added to each well in duplicate and incubated for 90 minutes at room temperature. The plates were washed 5 times with TBS, and then incubated with alkaline phosphatase-conjugated anti-human polyvalent immunoglobulin (Sigma Chemical Co., St. Louis, Mo) at room temperature for another 90 minutes. After 5 washes with TBS, the colour was developed with p-nitrophenyl phosphate disodium (1 mg./ml) and read at OD_{410} using a Dynatech MR600 ELISA reader.

Effects of Hybridoma Antibodies on the Adhesion of Platelets to Endothelial Cell Monolayers

Platelets (3×10^8) isolated from normal citrated blood were radiolabeled by incubating with 100 µl ^{51}Cr sodium chromate (1 mCi/ml) (Frosst, Kirkland, Quebec) for 1 hour at 37°C in 5% CO_2.

Endothelial monolayers in microtiter wells were washed 3 times with Hanks' buffer containing 0.5% bovine serum albumin (Hanks-BSA). One hundred microliters of hybridoma supernatant were added to each well in duplicate and incubated for 1 hour at 37°C in 5% CO_2. The

plates were then washed 3 times with Hanks-BSA and 50 µl of ^{51}Cr-labeled platelets (0.3 - 0.6 x 10^8) were added to each well and incubated for 1 hour at 37°C. The wells were washed 6 times with Hanks -BSA and the cells remaining in the well were solubilized with 200 µl of 0.1% Triton X-100 and 100 µl from each well were counted in a Beckman gamma-counter to determine platelet adherence (as detected by bound ^{51}Cr) to the endothelial monolayers.

Cytotoxicity of Hybridoma Antibodies to Endothelial Cells

Two different procedures were used to prepare radiolabeled ECs for the cytotoxic ^{51}Cr-release assay. EC monolayers in microtiter wells were radiolabeled with sodium chromate ^{51}Cr (1 mCi/ml) (Frosst, Kirkland, Quebec) diluted in 10 ml EC medium (100 µl/well) for 1 hour in 5% CO_2 at 37°C, or trypsinized ECs were radiolabled with 100 µl sodium chromate ^{51}Cr (1 mCi/ml), washed to remove free radioactivity, resuspended in EC medium and plated in V-bottom microtiter wells at 3 x 10^5 cells/well. The ^{51}Cr-release assay was performed using the same procedure as that described for the platelet cytotoxicity assay (Chapter IV). A commercial anti-EC antibody, MCA 117 (Serotec, Blackthorn, England), was used as positive control.

Analysis of Hybridoma Antibody Binding to Endothelial Cell Proteins by Western Blotting

SDS PAGE was run under reducing conditions and the Western blotting procedure was the same as that described for Western blotting analysis on platelet proteins in Chapter V (Materials and Methods).

RESULTS

Direct Binding of Hybridoma Antibodies to Human Umbilical Vein Endothelial Cells

A total of 26 SLE-derived and 14 normal-derived human hybridoma antibodies were tested for their direct binding reactivity to glutaraldehyde fixed human umbilical vein ECs by ELISA. Eight of the 26 SLE-derived and 4 of the 14 normal-derived antibodies showed positive reactions to ECs (Figure 1A). A comparison of the platelet-binding activity of these same antibodies (Figure 1B) showed that there were fewer antibodies which bound to ECs in the SLE group, while all of the normal-derived platelet-binding antibodies also reacted with ECs. Moreover, the binding levels of most EC-reactive antibodies were lower on ECs than on platelets.

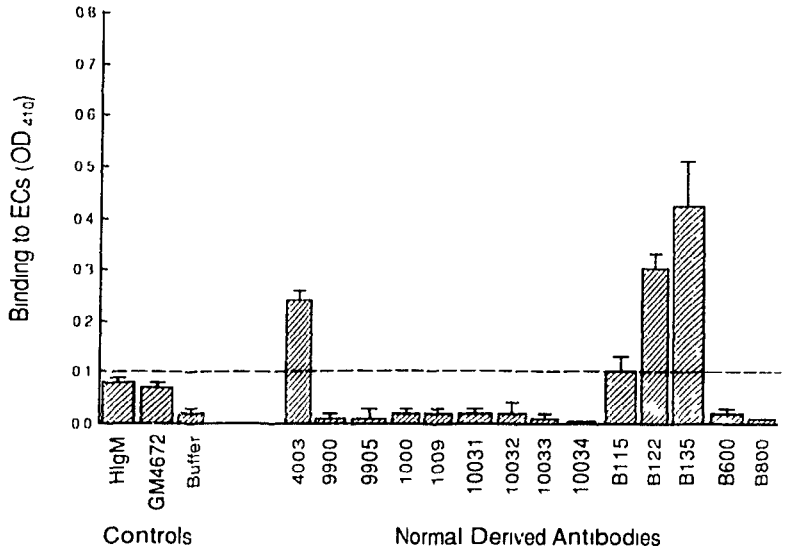
Effects of Hybridoma Antibodies on the Adhesion of Platelets to Endothelial Cells

In order to establish the optimal conditions for this study, a preliminary study of the spontaneous adhesion of platelets to EC monolayers was performed using different numbers of ^{51}Cr-labeled platelets (0.4 - 12.5 x 10^6/well) (Figure 2). A concentration of between 1.5 - 3.0 x 10^6/well (0.3 - 0.6 x 10^8/ml) labeled platelets was selected for further study because this concentration fell within the linear portion of the curve and gave sufficiently high cpm values to be detected. Representative results of the effects of hybridoma antibodies on the adhesion of platelets to EC monolayers are shown in Table 1. Using a baseline value calculated from the negative controls of EC medium and GM 4672 cell supernatant, neither SLE-derived nor

Figure 1. Binding Reactivity of Hybridoma Antibodies to Fixed Human Umbilical Vein Endothelial Cells and Platelets

A reaction in this assay was considered positive if the OD_{410} was greater than 0.10 for ECs and greater than 0.08 for platelets, which are the means + 2 SE of three control samples tested in duplicate in 4 repeated assays. Antibody binding levels to ECs (Figure 1A) and platelets (Figure 1B) are shown as the mean plus or minus 2 SE of samples tested in duplicate and reproduced in 2 separate assays.

Figure 1A


Binding to ECs (OD 410)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HIgM
GM4672
Buffer
4003
9900
9905
1000
1009
10031
10032
10033
10034
B115
B122
B135
B600
B800
Controls
Normal Derived Antibodies

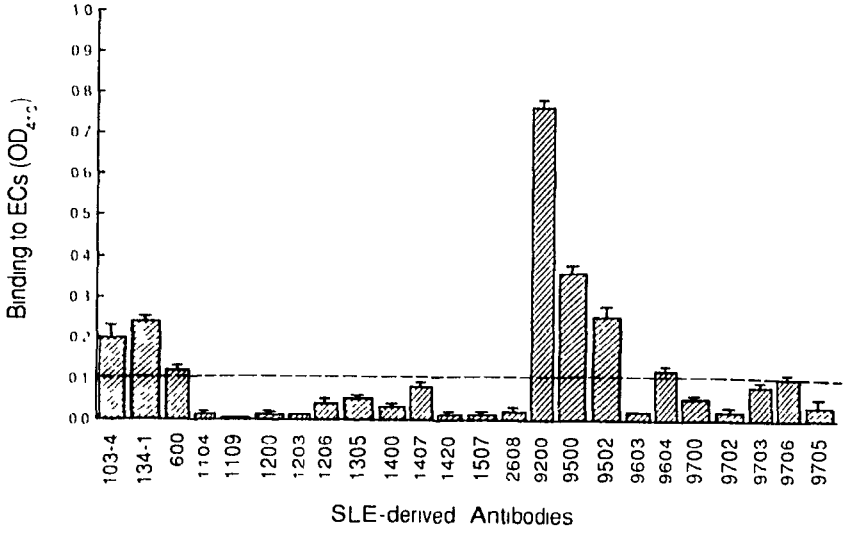
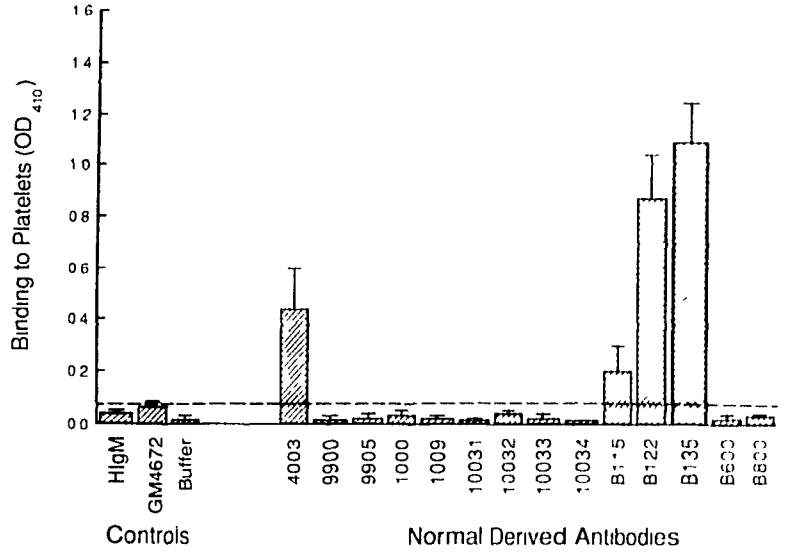
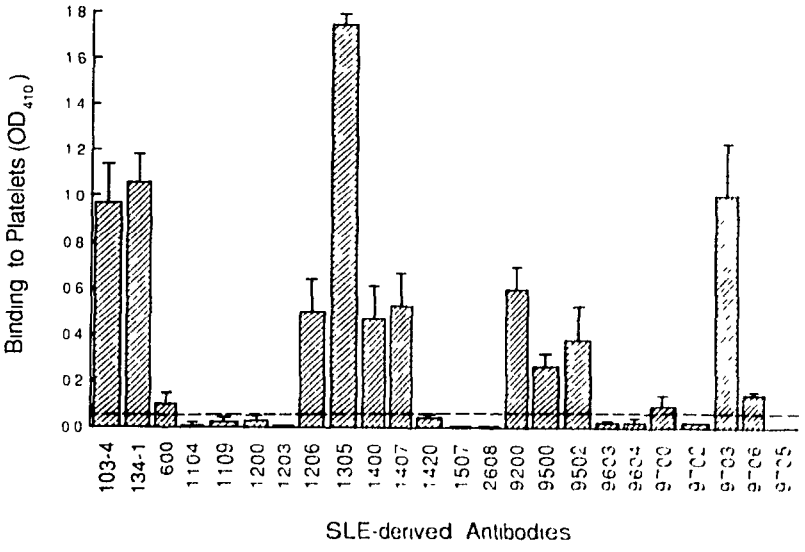

Binding to ECs (OD 410)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
103-4
134-1
600
1104
1109
1200
1203
1206
1305
1400
1407
1420
1507
2608
9200
9500
9502
9603
9604
9700
9702
9703
9706
9705
SLE-derived Antibodies

Figure 1B


Binding to Platelets (OD_{410})
16
14
12
10
08
06
04
02
00
HIgM
GM4672
Buffer
4003
9900
9905
1000
1009
10031
10032
10033
10034
B122
B135
Controls
Normal Derived Antibodies


Binding to Platelets (OD_{410})
18
16
14
12
10
08
06
04
02
00
103-4
134-1
600
1104
1109
1200
1203
1206
1305
1400
1420
2608
9200
9500
9502
SLE-derived Antibodies

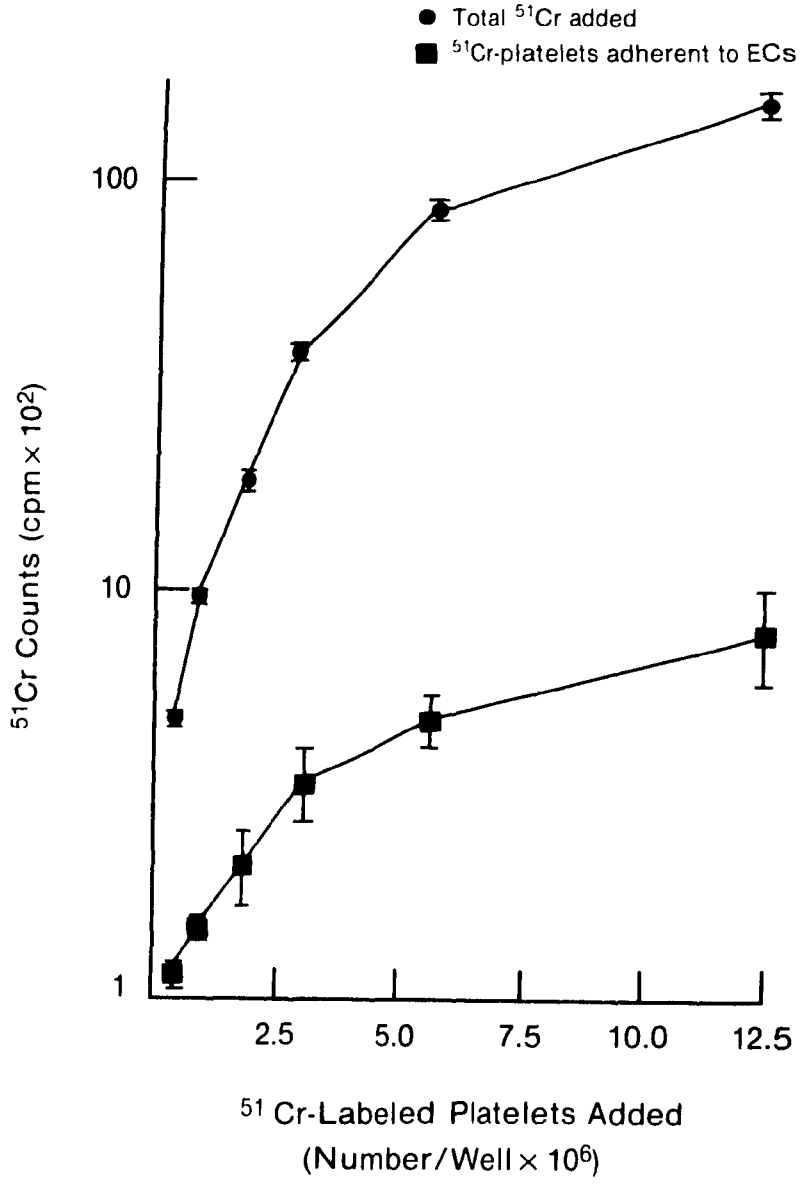


Figure 2. Spontaneous Adhesion of ^{51}Cr-Labeled Platelets to Endothelial Cell Monolayers

Each point on the curve represents the mean of quadruplicate samples plus or minus one SD.

Table 1. Effects of Hybridoma Antibodies on the Adhesion of Platelets to Endothelial Cell Monolayers[a]

Hybridoma Antibody	Adherence of ^{51}Cr-Labelled Platelets to EC (% of Total Label Added)[b]
Normal-Derived	
B109	8.00 ± 0.66[c]
B110	8.54 ± 0.50
B111	8.24 ± 0.99
B105	8.00 ± 1.11
B122	8.74 ± 0.80
4003	7.57 ± 1.12
SLE-Derived	
1104	6.47 ± 0.80
1109	8.54 ± 0.61
1200	8.56 ± 0.98
1420	9.02 ± 0.74
9200	6.35 ± 0.70
9502	7.17 ± 1.17
9603	6.94 ± 0.62
9604	8.35 ± 1.40
9700	7.18 ± 0.77
9703	7.12 ± 0.24
9703-5	7.71 ± 0.95
9705	6.63 ± 0.35
B3123	7.35 ± 0.73
Control Samples	
Medium	8.53 ± 0.36
GM 4672	8.80 ± 0.83
Total label added	5315 ± 251 cpm

[a] The results in this table are from 1 representative experiment of a series of 5 repeated assays on hybridoma supernatants.

[b] The cut-off value in this assay is 9.04%, which was calculated from mean plus 2 SD of the 3 control samples (8.66 + 0.38%).

[c] Each value represents the mean plus or minus one SD of 4 or 8 replicate samples in the same assay.

normal-derived hybridoma antibodies showed significant effects (increase or inhibition) on the adhesion of platelets to EC monolayers.

Cytotoxicity of Hybridoma Antibodies to Endothelial Cells

Chromium-51 release assays on ECs were employed to examine the possible role of autoantibodies in endothelial injury. The assays were performed using 2 different procedures: (1) intact EC monolayers were labelled with sodium chromate ^{51}Cr diluted 1 to 100 in EC medium or (2) trypsinized ECs were labeled with sodium chromate ^{51}Cr and then plated in microtiter wells for the cytotoxicity assay. Most experiments were performed using the second method since the internal variation between replicate wells was lower using this procedure. Representative results using this method are shown in Table 2. There was no significant effect of either SLE or normal-derived hybridoma supernatants on ^{51}Cr-release by ECs. Although the commercial anti-EC antibody, MCA 117, always caused some increase in ^{51}Cr-release over the mean + 2 SD of the negative controls, increases in ^{51}Cr-release from the EC monolayers caused by the purified hybridoma antibodies (400), 1109, 1311 and 9702) were not reproducible in repeated assays.

Legend to Table 2

[a] Results shown for hybridoma supernatants are from 1 representative experiment of 8 repeated assays. Results shown for hybridoma antibodies were obtained using affinity-purified hybridoma IgM or IgG at a final concentration of 2 µg/ml and are from 1 representative experiment of 3 repeated assays.

[b] ^{51}Cr release is shown as a percentage of the total ^{51}Cr labeled in platelets.

[c] Spontaneous release.

[d] HIgM and HIgG are normal pooled human IgM and IgG, respectively.

[e] MCA 117, a commercial mouse monoclonal antibody to ECs (Serotec, Blackthorn, England) and normal mouse gamma globulins were used as positive and negative controls, respectively.

Table 2. Cytotoxicity of Hybridoma Supernatants and Affinity-Purified Hybridoma Antibodies to Endothelial Cells

Culture Supernatants[a]	^{51}Cr-Release from ECs (%)[b]	Purified Antibodies[a]	^{51}Cr-Release from ECs (%)
Normal-Derived:			
B109	15.26 ± 0.88	B109 IgM	12.12 ± 1.54
B110	16.84 ± 0.97	B110 IgM	13.61 ± 1.64
B111	15.12 ± 1.02	B111 IgM	12.18 ± 0.79
B105	15.91 ± 1.28	10032 IgM	12.44 ± 2.11
B122-2	18.18 ± 1.29	B122-2 IgM	15.78 ± 3.53
4003	17.65 ± 0.90	4003 IgM	24.68 ± 1.69
SLE-Derived:			
1109	18.12 ± 2.00	1109 IgM	23.64 ± 4.30
1200	16.18 ± 0.86	1104 IgM	13.35 ± 2.94
9200	15.18 ± 1.49	1311 IgM	25.26 ± 2.45
9502	18.42 ± 0.64	9200 IgM	14.29 ± 1.56
9603	16.53 ± 0.63	9500 IgM	14.18 ± 2.57
9604	17.33 ± 1.17	9502 IgM	18.98 ± 2.48
9700	15.68 ± 0.58	9603 IgM	19.27 ± 3.30
9703	19.37 ± 0.85	9604 IgM	21.49 ± 1.60
9703-5	15.97 ± 0.56	9700 IgM	21.92 ± 2.40
9705	16.28 ± 2.05	9702 IgG	27.73 ± 1.49
		9703 IgM	22.91 ± 1.33
		9703-5 IgM	21.83 ± 1.55
		9705 IgM	11.70 ± 1.38
		9706 IgM	13.54 ± 1.65
		B3123 IgM	13.01 ± 1.49
Control Samples:			
S.R.[c]	16.73 ± 1.05	S.R.	12.60 ± 1.49
Medium	18.97 ± 0.63	Medium	12.43 ± 1.73
GM 4672	17.16 ± 1.00	GM 4672 IgG	18.97 ± 0.69
Human IgM[d]	18.75 ± 1.84	Human IgM	11.37 ± 0.27
MCA 117[e]	21.29 ± 0.53	Human IgG[d]	19.94 ± 1.26
Mouse IgG[e]	19.18 ± 1.78	MCA 117	24.27 ± 2.09
Total Release	4303 ± 104 cpm	Total Release	1952 ± 228 cpm
Mean ± 2 SD	17.90 ± 2.25	Mean ± 2 SD	15.06 ± 8.10

Western Blotting Analysis of Hybridoma Antibodies on Endothelial Cell Proteins

Western blotting analysis of hybridoma antibodies on EC proteins, shown in Figure 3, was performed using the same panel of antibodies as used in the platelet studies (Figure 3 of Chapter VI). The protein binding patterns of these antibodies on EC proteins differed from those observed on platelet proteins. Strips blotted with negative controls (0.5% casein and GM 4672 cell supernatant) and non-EC binding antibodies (normal-derived antibodies B109, B110, and B111, and SLE-derived antibodies 9700, 9703, 1206, 1305, 1407 and 9702) did not show any reactive protein bands. Strips blotted with normal-derived EC-binding antibodies showed high background binding without clearly defined bands (4003 and B135) or reactivity with common bands (B105 and B122) at approximately 80,000 M.W., which may be due to reactivity with EC-bound IgM immunoglobulin. Among the SLE-derived EC-binding antibodies, antibody 9500 with lupus anticoagulant activity showed strong and specific binding to a group of EC proteins with mobilities similar to actin (45,000 M.W.). Antibody 9604 reacted mainly with a 200,000 M.W. protein which was also observed in blots of this antibody with platelet proteins. This particular 9604 supernatant also showed reactivity with many other EC proteins, which were not seen in several other blots of EC proteins with 9604 antibody (refer to Figures 3 and 4 of Chapter X). Of the other antibodies which reacted with ECs in ELISA, antibodies 9200, 9502 and 9706 showed weak binding to 2 or 3 bands between 30,000 and 40,000 M.W., while 103-4 did not bind to any protein bands.

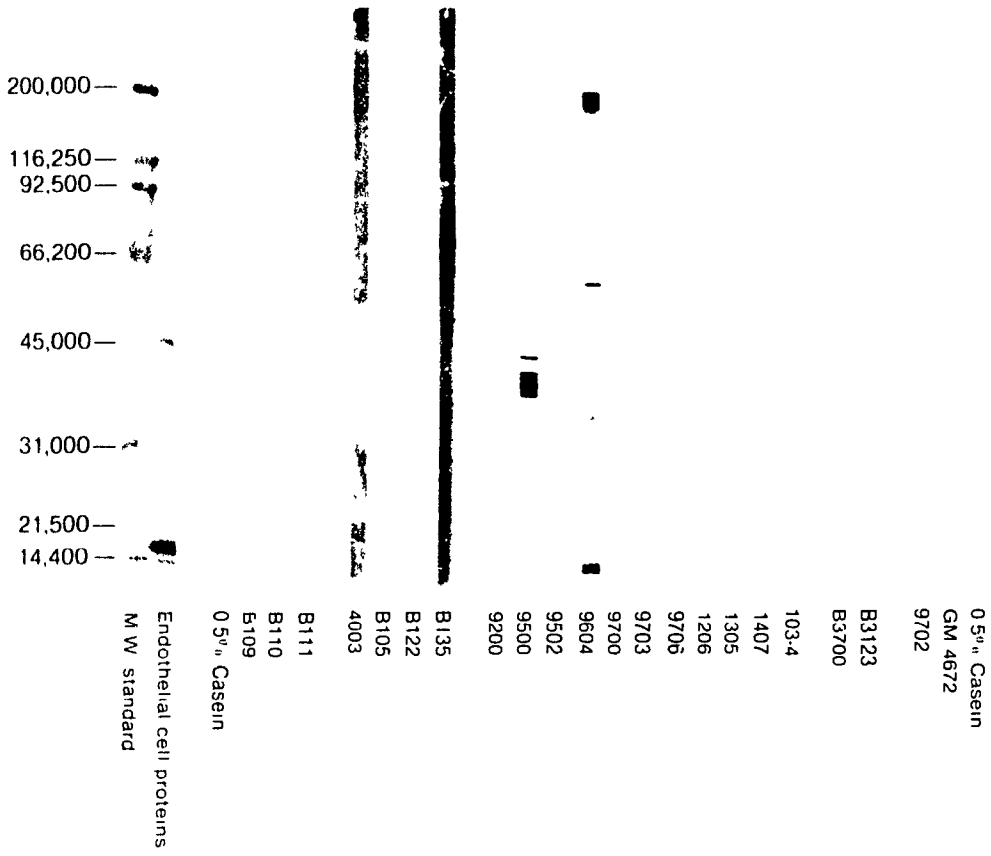


Figure 3. Western Blotting of Hybridoma Antibodies on Endothelial Cell Proteins

Human umbilical vein ECs were solubilized in SDS sample buffer and SDS gels were run under reducing conditions. From left to right, Lanes 1 and 2 were M.W. standards and whole EC proteins stained with amido black; Lanes 3 to 26 were whole EC proteins blotted with hybridoma antibodies or control samples: Lane 3, casein blocked control strip incubated with peroxidase anti-human IgM; Lanes 4-10: normal-derived hybridoma IgM antibodies B109, B110, B111, 4003, B105, B122 and B135; Lanes 11-23: SLE-derived hybridoma IgM antibodies 9200, 9500, 9502, 9604, 9700, 9703, 9706, 1206, 1305, 1407, 103-4, B3700 and B3123; Lane 24: SLE-derived hybridoma IgG antibody 9702; Lane 25: GM 4672 IgG control; Lane 26: casein blocked control strip incubated with peroxidase anti-human IgG.

DISCUSSION

In the present study, a panel of human hybridoma autoantibodies which had been studied for reactivity with platelets was examined for reactivity with ECs. Fewer antibodies were found to react with ECs than with platelets by direct binding ELISA. In experiments examining the effects of these antibodies on the interactions between platelets and ECs and the cytotoxicity of these antibodies to ECs, there were no consistent positive reactions for any of the antibodies tested. The lack of consistent positive reactions may have been due to methodological problems, such as the detachment of the EC monolayers in the presence of some antibodies and during the many buffer washes, and the high level of spontaneous ^{51}Cr release by EC in the cytotoxicity assay. Alternatively, it may reflect a true lack of functional effects of these antibodies on ECs.

Immune participation in endothelial injury has been reported in blood vessel diseases in SLE patients (346). There is evidence that EC antigens expressed on the surface of injured cells may promote immune complex binding to cultured human ECs (122,347) or Fc-dependent monocyte binding to rabbit aorta ECs (348), although it is still not clear whether the intact endothelium is a target of autoantibodies, immune cells or immune complexes (349,350). The demonstration of Fc receptors (351) and C1q receptors (352) on human endothelial cells provides a possible mechanism in which antibodies or antibody-antigen complexes can bind to the endothelium and cause EC damage. Since autoantibodies and immune complexes persist in the circulation of SLE patients, it will be important to determine

whether these autoantibodies and immune complexes can bind to normal intact endothelium and thus change the nonthrombogenic surface to induce platelet adhesion. Alternatively, an altered endothelium could induce the production of autoantibodies which mediate immune injury.

This raises the question of whether EC-binding autoantibodies are the cause or the result of EC injury in SLE patients. In a serum study using human umbilical vein ECs, Cines et al. (60) demonstrated that sera from SLE patients deposited significant amounts of IgG onto ECs and also fixed appreciable amounts of C3 to the EC surface. Furthermore, the binding of IgG was accompanied by a disruption of the endothelial monolayer as well as morphological changes in the ECs, secretion of PGI_2 by ECs, and diffuse platelet adhesion to EC monolayers (60). Morphological changes (a rounded appearance, loss of contact with neighboring cells, and detachment of EC monolayers from plastic surfaces) were also noted in our experiments when live EC monolayers were incubated with some, but not all, EC-binding hybridoma lupus antibodies (data not shown). The negative results in our studies of the effects of hybridoma antibodies on EC cytotoxicity and the interaction between EC and platelets may reflect the detachment of ECs from microtiter wells. In the cytotoxicity experiments, EC detachment may have influenced the results if EC-binding antibodies were not able to bind to detached cells. Similarly, this could have resulted in an apparent lack of effect on platelet adherence to ECs in cases where the EC monolayers became detached.

Mechanisms other than a direct effect of antibodies on ECs have

been proposed. Antibodies with lupus anticoagulant activity have been shown to inhibit PGI_2 synthesis in vascular tissues, possibly by interfering with the liberation of arachidonic acid from membrane phospholipids (150,221,268). Carreras et al. (150) found that an lupus anticoagulant-containing IgG fraction, isolated from a patient with a history of arterial thrombosis and multiple intrauterine deaths, reduced the release of PGI_2 from rat aorta rings or pregnant human myometrium and the production of 6-keto-prostaglandin $F_{1\alpha}$ by bovine endothelial cells. The inhibitory effect was abolished in the presence of arachidonic acid. In a subsequent study, PGI_2 production by vascular tissues was inhibited by plasmas of 8 of 14 patients with LA, 6 of whom had thrombosis (268). Similar findings were obtained by some but not all groups of investigators (278-281). Others argue that the thrombosis associated with anti-phospholipid antibodies cannot be explained by effects on EC and platelet prostanoid synthesis (282) and that the significantly increased EC procoagulant activity induced by sera from patients with SLE and LA may account for the increased incidence of thrombosis in these patients (283). These discrepant results may be due to the heterogeneous properties of LA antibodies or to the effects of other autoantibodies in the plasma of these patients. More direct evidence is still required to explain the association of LA and anti-PL with thrombosis and thrombocytopenia. In our laboratory, we plan to undertake similar studies using hybridoma LA and anti-PL antibodies to identify whether monoclonal antibodies of these specificities are able to inhibit or enhance PGI_2 production.

In summary, our studies using hybridoma LA, anti-PL and anti-DNA autoantibodies demonstrated that fewer of these antibodies bound to ECs than to platelets. Furthermore, neither EC-binding nor non-EC-binding antibodies had significant cytotoxic effects on ECs in vitro, nor was there any reproducible effect of these antibodies on the adherence of platelets to EC monolayers. These results support the possibility that mechanisms other than direct immune injury to ECs may be involved in causing thrombosis in SLE patients.

CHAPTER VII

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The present thesis describes our studies on the characterization of reactivities of SLE and normal-derived human hybridoma lupus anticoagulant, anti-phospholipid and anti-dDNA autoantibodies with platelets and endothelial cells. This work was based on the hypothesis that autoantibodies might play a role in hemostatic disturbances which are frequently seen in SLE patients (249-251). Although it has been known for some time that hybridoma antibodies with autoreactivities can be derived from both SLE and normal individuals, few studies have examined the differences between the epitopes recognized by SLE and normal-derived antibodies and in the functional effects of these antibodies on cells. It was also not clear what, if any, relationship exists between the multiple reactivities (anti-DNA, anti-PL, LA, anti-platelet and anti-EC), or polyspecificity, of these autoantibodies and whether specific epitopes on platelets and ECs are recognized by these polyspecific antibodies.

The four main approaches employed in our studies were: 1) the production of human hybridoma autoantibodies from fusions of GM 4672 human lymphoblastoid cells with peripheral blood lymphocytes from SLE patients and normal individuals; 2) an examination of the binding reactivities of these antibodies by solid phase immunoassays and Western blotting analysis, and of their functional activities by APTT assay and chromium-51 release cytotoxicity assays; 3) a statistical analysis of the relationship between different individual

reactivities (e.g. lupus anticoagulant and anti-platelet reactivities), examined by analyzing SLE and normal-derived hybridoma antibodies both together and as separate groups; and 4) an investigation of the epitopes recognized by an interesting SLE-derived platelet-binding autoantibody which reacted with ADP-activated but not with resting platelets.

In the first part of our studies (**Chapter III**), a panel of hybridoma autoantibodies derived from normal individuals and SLE patients was tested and analyzed for LA, anti-PL, anti-dDNA, and anti-platelet reactivities. Since the direct binding reactivities were tested at an early stage of hybridoma production and only limited amounts of supernatant were available from most hybridomas, we were unable to evaluate and adjust the Ig concentrations prior to screening on the different antigens. However, most direct binding and cytotoxicity assays using affinity-purified hybridoma Ig at equivalent concentrations showed consistent results with the supernatant studies. The data suggested that anti-platelet antibodies may recognize different epitopes on the platelet membrane. Anti-platelet reactivity of these antibodies was correlated with DNA and PL-binding activities, but not with LA activity. Thus, many anti-PL antibodies bound directly to platelets in vitro, while most LA antibodies did not.

In the above-mentioned studies, SLE and normal-derived hybridoma antibodies were analyzed as part of the same group. There was a positive correlation seen between anti-dDNA and anti-platelet activities (Figure 2 of Chapter III). However, when hybridoma

antibodies were grouped according to their origin, from either SLE patient or normal donor, the correlation analyses revealed different findings. Among the normal-derived antibodies, there was a strong positive correlation seen between anti-platelet and anti-dDNA activities, but among SLE-derived antibodies, there was no correlation between these two reactivities (Figure 1 of Chapter IV). These results suggested that normal and SLE-derived antibodies may differ and led to the second part of our studies (**Chapter IV**), in which the binding and functional activities of SLE and normal-derived hybridoma antibodies were compared. The data demonstrated that hybridoma platelet-binding autoantibodies derived from SLE patients exhibited greater antigen specificity and functional activity than similar antibodies derived from normal individuals, suggesting that SLE and normal-derived polyspecific platelet-binding hybridoma antibodies may bind to different epitopes on platelets or bind to platelets via different mechanisms.

An interesting SLE-derived hybridoma antibody, 9604, was noted in these latter studies. In contrast to most other hybridoma antibodies, which displayed consistent reactions in direct binding assays on live platelets (by RIA) and on glutaraldehyde-fixed platelets (by ELISA), antibody 9604 did not bind to fixed intact platelets in ELISA, but did react with live platelets in RIA. It was also strongly cytotoxic to platelets <u>in vitro</u>. We postulated that antibody 9604 might react with an antigen(s) which is normally not expressed on resting platelets. Further experiments (**Chapter V**) to identify the 9604 reactive epitope(s) demonstrated that antibody 9604

did not bind to fixed intact resting platelets, but reacted with freeze-thawed lysed platelets and with fixed ADP-activated platelets in ELISA. Western blotting analysis identified specific 9604 reactive epitopes on reducible polypeptides of approximately 200,000 and 32,000 M.W. The finding that antibody 9604 binds to proteins present on activated but not on resting platelets suggests that additional factors may be required to explain the pathogenetic role of this kind of lupus autoantibody. These additional factors could include other autoantibodies or serum factors which induce platelet activation and thus permit the binding of 9604-like antibodies to activated platelets, and possibly, immune damage by these antibodies.

The objective of the fourth part of our studies was to examine the effects of SLE autoantibodies on ECs and on the interactions between ECs and platelets (Chapter VI). Using the same panel of hybridoma antibodies as was studied for platelet reactivity, fewer antibodies were found to bind to ECs than to platelets by ELISA and Western blotting analysis. Experiments examining the effects of these antibodies on the adherence of platelets to ECs and the cytotoxicity of these antibodies to ECs were repeated numerous times using different procedures. However, no consistent positive results have been obtained to date. This may reflect a true lack of functional effects of these hybridoma antibodies on ECs, or alternatively, may be due to the differences between ECs present in arteries, veins and capillaries, and to certain methodological problems which arise in working with cultured ECs.

Several general conclusions may be drawn from all of the data

obtained in the above studies: 1) The direct binding and cytotoxicity of SLE-derived hybridoma autoantibodies to platelets suggest that these antibodies may play a direct role *in vivo* in causing some disturbances of hemostasis in SLE patients. 2) Western blotting studies and statistical analysis of antibody binding reactivities suggest that SLE-derived platelet-binding antibodies may react with different epitopes on platelets or bind to platelets via different mechanisms than normal-derived antibodies. Furthermore, SLE-derived antibodies exhibited more specific binding and greater functional activity than normal-derived platelet-binding antibodies. 3) The characterization of an SLE-derived platelet-binding antibody, 9604, demonstrated that some SLE-derived autoantibodies are able to distinguish between resting and activated platelets. Taken together, these data suggest that several kinds of autoantibodies may be involved in causing hemostatic disorders in SLE patients. Although our studies provide some insight into the involvement of autoantibodies in hemostatic abnormalities seen in SLE patients, much work remains to be done in determining the precise epitopes responsible for the binding of these antibodies to platelets.

There is no doubt that platelet-binding antibodies can be detected in both sera (55-59) and hybridoma autoantibodies (76) derived from SLE patients. Efforts have been made to identify the epitopes on platelets responsible for autoantibody binding (58,59). Platelet target antigens of approximately 108,000 and 66,000 M.W. (58) and 120,000 and 80,000 M.W. (59) were found for some SLE sera. In our studies (Chapter V and VI), we have demonstrated that SLE-

derived autoantibodies can bind to certain epitopes on platelets and ECs. One antibody, 9604, reacted with a 32,000 M.W. polypeptide on platelets and a 200,000 M.W. protein present on both platelets and ECs, while another antibody, 9500, was reactive with a group of proteins in ECs with relative mobilities similar to actin (approximately 45,000 M.W.). Antibody 9604 was able to distinguish between resting and activated platelets, suggesting that the reactive epitopes may be present on activated platelets, but not on resting platelets, and may be related to platelet activation antigens, some of which are analogous to VLA family of proteins (222,227). Further identification and characterization of the platelet and EC proteins reactive with these hybridoma autoantibodies may provide a better understanding of the biological functions and significance of these proteins. Furthermore, the in vivo effects of these autoantibodies on these proteins may help to explain the mechanisms which result in hemostatic abnormalities in SLE patients. Production of mouse monoclonal antibodies which mimic the binding and functional properties of these human hybridoma autoantibodies could provide useful antibody probes and would help to overcome the technical difficulties inherent in producing large quantities of highly purified human hybridoma antibodies.

Platelet activation results not only in changes in surface protein antigens (173-185), but also in a rearrangement of platelet membrane PLs. In particular, PE is exposed on the activated but not on the resting platelet surface, as detected by trinitrobenzene-sulfonate (TNBS) labeling (125). It will be important to examine

whether antibodies reactive with PE (located mainly on the inside of the platelet membrane in resting platelets) or with other PLs, and which do not bind to intact resting platelets, are able to bind to activated platelets.

As discussed above, 9604-like antibodies or other antibodies which bind to activated but not to resting platelets may require the action of additional antibodies or factors on platelets to permit their binding and subsequent effects. Further exploration of the individual and cooperative role of these antibodies in immune pathogenesis in SLE may reveal a kind of autoantibody cascade mechanism.

Although some authors have proposed that LA may inhibit the production and/or release of PGI_2, and thus promote thrombosis, most of these observations have been made in vitro using antibodies derived from plasma or serum (150,268,278-280). Furthermore, a controversy exists as to whether LA antibodies affect PGI_2 production (282,283). The discrepancy between the results from different laboratories may result from the use of ECs from different tissue sources (vascular tissues compared with cultured human umbilical vein ECs) or to the presence of autoantibodies other than LA in SLE sera. These issues can be directly addressed by examining the influence of monoclonal hybridoma autoantibodies with defined reactivities on PGI_2 production by ECs and on TXA_2 production by platelets.

The use of human hybridoma autoantibodies from SLE patients and patients with other autoimmune hemostatic abnormalities should provide a valuable approach to elucidate the mechanisms by which autoantibodies result in various disturbances of hemostasis.

CHAPTER VIII

STATEMENT OF ORIGINALITY

* The platelet and endothelial cell-binding properties of a panel of human hybridoma lupus anticoagulant, anti-phospholipid and anti-DNA autoantibodies were assessed and the relationship between these different reactivities was analyzed.

* Chromium-51 release assays were developed to test the cytotoxic effects of hybridoma antibodies on platelets and endothelial cells.

* The direct binding reactivities and functional characteristics (cytotoxicity to platelets and endothelial cells, and lupus anticoagulant activity) of SLE-derived hybridoma antibodies were compared with normal-derived antibodies.

* A technique using the parental human lymphoblastoid GM 4672 cells as a feeder layer was developed and successfully employed in cloning the hybridomas.

* A procedure for isoelectric focusing of human hybridoma IgM was developed to identify the monoclonality of the hybridoma antibodies.

* An SLE-derived human hybridoma autoantibody, 9604, was shown

to react with reducible polypeptides which were present on ADP-activated platelets, but not on the cell surface of resting platelets. These 9604-reactive platelet polypeptides have been isolated and partially characterized.

CHAPTER IX

REFERENCES

1. Osler W: On the visceral manifestations of the erythemal group of skin diseases (third paper). Am J Med Sci 127:1-23, 1904.
2. Rothfield NF: Systemic lupus erythematosus: Clinical and laboratory aspects. In Arthritis and Allied Conditions, ninth edition. Ed. by McCarty DJ, Lea & Febiger, Philadelphia, P.691-714, 1979.
3. Hargraves MM, Richmond H, and Morton R: Presentation of two bone marrow elements: The "tart" cell and the "L.E." cell. Pro Staff Meet Mayo Clin 23:25-8, 1948.
4. Holman HR, and Kunkel HG: Affinity between the lupus erythematosus serum factor and cell nuclei and nucleoprotein. Science 126:162-3, 1957.
5. Okudaira K, Tanimoto K, Nakamura T, and Horiuchi Y: Spontaneously enhanced in vitro immunoglobulin synthesis by B cells in systemic lupus erythematosus. Clin Immunol Immunopathol 16:267-78, 1980.
6. Kumagai S, Sredni B, House S, Steinberg AD, and Green I: Defective regulation of B lymphocyte colony formation in patients with systemic lupus erythematosus. J Immunol 128:258-62, 1982.
7. Morimoto C, Abe T, Hara M, and Homma M: In vitro TNP specific antibody formation by peripheral lymphocytes from patients with systemic lupus erythematosus. Scand J Immunol 6:575-9, 1977.

8. Blaese RM, Grayson J, and Steinberg AD: Elevated immunoglobulin secreting cells in the blood of patients with active systemic lupus erythematosus: Correlation of laboratory and clinical assessment of disease activity. Am J Med 69:345-50, 1980.

9. Tan PL, Pang GT, Cullinane G, and Wilson ID: Immunoglobulin secreting cells in SLE correlation with disease activity. J Rheumatol 7:807-13, 1980.

10. Tan EM, Schur PH, Carr RI, and Kunkel HG: Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest 45:1732-40, 1966.

11. Koffler D, Schur PH, and Kunkel HG: Immunological studies concerning the nephritis of systemic lupus erythematosus. J Exp Med 126:607-24, 1967.

12. Koffler D, Agnello V, and Kunkel HG: Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. Am J Pathol 74:109-22, 1974.

13. Winfield JB, Faiferman I, and Koffler D: Avidity of anti-DNA antibodies in serum and IgG glomerular eluates from patients with systemic lupus erythematosus. Association of high affinity anti-native DNA with glomerulonephritis. J Clin Invest 59:90-6, 1977.

14. Hashimoto H, Utagawa Y, Yamagata J, Okada T, Shiokawa Y, Suzuki S, and Tomiyama T: The relationship of renal histopathological lesions to immunoglobulin classes and complement fixation of anti-native ENA antibodies in systemic lupus erythematosus. Scand J Rheumatol 12:209-14, 1983.

15. Brentjens JR, Sepulueda M, Baliah T, Bentzel C, Erlanger BF, Elwood C, Montes M, Hsu KC, and Andres GA: Interstitial immune complex nephritis in patients with systemic lupus erythematosus. Kidney Int 7:342-50, 1975.

16. Abe T, and Homma M: Immunological reactivity in patients with systemic lupus erythematosus (humoral antibody and cellular immune response). Acta Rheum Scand 17:35-46, 1971.

17. Winchester RJ, Winfield JB, Siegel F, Wernet P, Bentwich Z, and Kunkel HG: Analysis of lymphocytes from patients with rheumatoid arthritis and systemic lupus erythematosus. Occurrence of interfering cold-reactive antilymphocyte antibodies. J Clin Invest 54:1082-92, 1974.

18. Winfield JB, Winchester RJ, and Kunkel HG: Various types of antilymphocyte autoantibodies in patients with systemic lupus erythematosus (SLE) and their relationship with lymphopenia. Arthritis Rheum 18:284-5, 1975 (abstract).

19. De Horatius RJ, and Messner RP: Lymphocytotoxic antibodies in family members of patients with systemic lupus erythematosus. J Clin Invest 55:1254-8, 1975.

20. Raveche ES, and Steinberg AD: Lymphocyte and lymphocyte functions in systemic lupus erythematosus. Semin Hematol 16:344-70, 1979.

21. Suciu-Foca N, Buda JA, Thiem T, and Reemtsmu K: Impaired responsiveness of lymphocytes in patients with systemic lupus erythematosus. Clin Exp Immunol 18:295-301, 1974.

22. Glinski W, Gershwin ME, Budman DR, and Steinberg AD: Study of

lymphocyte subpopulations in normal humans and patients with SLE by fractionation of peripheral blood lymphocyte on a discontinuous Ficoll-gradient. Clin Exp Immunol 26:228-38, 1976.

23. Bell DA: Cell mediated immunity in systemic lupus erythematosus: Observations on in vitro cell-mediated immune response in relationship to number of potentially reactive T cells, disease activity and treatment. Clin Immunol Immunopathol 11:301-7, 1978.

24. Streldauskas JA, Callery RT, Borel Y, and Schlossman SF: Direct evidence of loss of human suppressor cells during active autoimmune disease. Proc Natl Acad Sci USA 75:5150-4, 1978.

25. Clough JD, Frank SA, and Calabrese LH: Deficiency of T cell mediated regulation of anti-DNA production in systemic lupus erythematosus. Arthritis Rheum 23:24-9, 1980.

26. Sakane T, Kotani H, Takada S, Murakawa Y, and Ueda Y: A defect in the suppressor circuits among OKT4+ cell population in patients with systemic lupus erythematosus occurs independently of a defect in the OKT8+ suppressor T cell function. J Immunol 131:753-61, 1983.

27. Hoffman T: Natural Killer function in systemic lupus erythematosus. Arthritis Rheum 23:30-5, 1980.

28. Carpentier B, Carnand C, and Bach JF: Selector depression of the xenogeneic cell-mediated lympholysis in systemic lupus erythematosus. J Clin Invest 64:351-60, 1979.

29. Fessel WJ: Epidemiology of systemic lupus erythematosus. In Rheumatic Disease Clinics of North America, Ed. by Klippel JH,

W.B. Saunders Company, Philadelphia, P.15-23, 1988.

30. Kunkel HG, Holman HR, and Deicher HRG: Multiple "autoantibodies" to cell constituents in systemic lupus erythematosus. CIBA Foundation Symposium on Cellular Aspects of Immunity 8:429-37, 1960.

31. Ceppellini R, Polli C, and Celada F: A DNA-reacting serum factor in serum of a patient with lupus erythematosus diffusus. Proc Soc Exp Biol Med 96:572-82, 1957.

32. Miescher P, and Strassle R: New serological methods for the detection of the L.E. factor. Vox Sang 2:283-7, 1957.

33. Dameshek W: Systemic lupus erythematosus: a complex autoimmune disorder? Ann Intern Med 48:707-30, 1958.

34. Arana R, and Seligmann M: Antibodies to native and denatured deoxyribonucleic acid in systemic lupus erythematosus. J Clin Invest 46:1867-82, 1967.

35. Friou GJ: Identification of the nuclear component of the interaction of lupus erythematosus globulin and nuclei. J Immunol 80:476-81, 1958.

36. Tan EM: An immunologic precipitin system between soluble nucleoprotein and serum antibody in systemic lupus erythematosus. J Clin Invest 46:735-45, 1967.

37. Stollar BD: Varying specificity of systemic lupus erythematosus sera for histonic factors and a periodate-sensitive antigen associated with histone. J Immunol 103:804-8, 1969.

38. Fritzler MJ, and Tan EM: Antibodies to histones in drug induced and idiopathic lupus erythematosus. J Clin Invest 62:560-7,

1978.

39. Rekvig OP, and Hannestad K: Human autoantibodies that react with both cell nuclei and plasma membranes display specificity for the octamer of histones H2A, H2B, H3 and H4 in high salt. J Exp Med 152:1720-33, 1980.

40. Tan EM, and Kunkel HG: Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. J Immunol 96:464-71, 1966.

41. Mattioli M, and Reichlin M: Characterization of a soluble nuclear ribonucleoprotein antigen reactive with SLE sera. J Immunol 107:1281-90, 1971.

42. Northway JD, and Tan EM: Differentiation of antinuclear antibodies giving speckled staining patterns in immunofluorescence. Clin Immun Immunopathol 1:140-54, 1972.

43. Kurata N, and Tan EM: Identification of antibodies to nuclear acidic antigens by counterimmunoelectrophoresis. Arthritis Rheum 19:574-80, 1976.

44. Lerner MR, and Steitz JA: Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc Natl Acad Sci USA 76:5495-9, 1979.

45. Reichlin M: Clinical and immunological significance of antibodies to Ro and La in systemic lupus erythematosus. Arthritis Rheum 25:761-71, 1982.

46. Elkon KB, and Jankowski PW: fine specificities of autoantibodies directed against the Ro, La, Sm, RNP and Jo-1 proteins defined by two-dimensional gel electrophoresis and immunoblotting. J

Immunol 134:3819-24, 1985.

47. Akizuki M, Powers R Jr, and Holman HR: A soluble acidic protein of the cell nucleus which reacts with serum from patients with systemic lupus erythematosus. J Clin Invest 59:264-72, 1977.

48. Schur PH, and Monroe M: Antibodies to ribonucleic acid in systemic lupus erythematosus. Proc Natl Acad Sci USA 63:1108-12, 1969.

49. Sturgill BC, and Carpenter RR: Antibody to ribosomes in systemic lupus erythematosus. Arthritis Rheum 8:213-8, 1965.

50. Asherson GL: Antibodies against nuclear and cytoplasmic constituents in systemic lupus erythematosus and other diseases. Br J Exp Path 40:209-15, 1959.

51. Deicher HRG, Holman HR and Kunkel HG: Anticytoplasmic factors in the sera of patients with systemic lupus erythematosus and certain other diseases. Arthritis Rheum 3:1-15, 1960.

52. Alcover A, Molano J, Renart J, Gil-Aguado A, and Nieto A: Antibodies to vimentin intermediate filaments in sera from patients with systemic lupus erythematosus. Arthritis Rheum 27:922-8, 1984.

53. Strelkauskas AJ, Callery RT, Borel Y, and Schlossman SF: Functional characteristics of human T-lymphocyte subjects identified by sera of patients with systemic lupus erythematosus. Clin Immunol Immunopathol 14:47-55, 1979.

54. Bluestein HG, Williams GW, and Steinberg AD: Cerebrospinal fluid antibodies to neuronal cells: association with neuropsychiatric manifestations of systemic lupus erythematosus. Am J Med

70:240-6, 1981.

55. Karpatkin S, Strick N, Karpatkin MB, Siskind GW: Cumulative experience in the detection of antiplatelet antibody in 234 patients with idiopathic thrombocytopenic purpura, systemic lupus erythematosus and other clinical disorders. Am J Med 52:776-85, 1972.

56. Weissbarth E, Baruth B, Mielke H, Liman W, and Deicher H: Platelets as target cells in rheumatoid arthritis and systemic lupus erythematosus: A platelet specific immunoglobulin inducing the release reaction. Rheumatol Int 2:67-71, 1972.

57. Gerhard KM, Endresen GKM, and FØrreØ: Studies on the binding of proteins to the human platelet surface: Relation to platelet activation. Thromb Haemost 53:360-5, 1985.

58. Kaplan C, Champeix P, Blanchard D, Muller JY, and Cartron JP: Platelet antibodies in systemic lupus erythematosus. Br J Haematol 67:89-93, 1987.

59. Howe SE, and Lynch DM: Platelet antibody binding in systemic lupus erythematosus. J Rheum 14:482-6, 1987.

60. Cines DB, Lyes AP, Reeber M, Bina M, and DeHoratius RJ: Presence of complement-fixing anti-endothelial cell antibodies in systemic lupus erythematosus. J Clin Invest 73:611-25, 1984.

61. Rosenbaum J, Pottinger BE, Woo P, Black CM, Loizou S, Byron MA, and Pearson JD: Measurement and characterisation of circulating anti-endothelial cell IgG in connective tissue diseases. Clin Exp Immunol 72:450-56, 1988.

62. Vismara A, Meroni PL, Tincani A, Harris EN, Barcellini W,

Brucato A, Khamashta M, Hughes GRV, Xanussi C, and Balestrieri G: Relationship between anti-cardiolipin and anti-endothelial cell antibodies in systemic lupus erythematosus. Clin Exp Immunol 74:247-53, 1988.

63. Castro O, Farber LR, and Clyne LP: Circulating anticoagulants against factors IX and XI in systemic lupus erythematosus. Ann Intern Med 77:543-8, 1972.

64. Robboy SJ, Lewis EJ, and Schur PH: Circulating anticoagulants to factor VIII. Immunochemical studies and clinical response to factor VIII concentrates. Am J Med 49:742-52, 1970.

65. Hazeltine M, Rauch J, Danoff D, Esdaile JM, and Tannenbaum H: Antiphospholipid antibodies in systemic lupus erythematosus: Evidence of an association with positive Coombs' and hypocomplementemia. J Rheumatol 15:80-6, 1988.

66. Conley CL, and Hartmann RC: A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. J Clin Invest 31:621-2, 1952.

67. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, and Hughes GRV: Anticardiolipin antibodies: Detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet 2:1211-4, 1983.

68. Estes D, and Christian CL: The natural history of systemic lupus erythematosus by prospective analysis. Medicine (Baltimore) 50:85-95, 1971.

69. Kanai Y, Kawaminami Y, Miwa M, Matsushima T, and Sugimura T: Naturally occurring antibodies to poly (ADP-ribose) in patients

with systemic lupus erythematosus. Nature 265:175-7, 1977.

70. Lafer EM, Rauch J, Andrzejewski C Jr, Mudd D, Furie B, Furie B, Schwartz RS, and Stollar BD: Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J Exp Med 153:897-909, 1981.

71. Shoenfeld Y, Rauch J, Massicotte H, Datta SK, André-Schwartz J, Stollar D, and Schwartz RS: Polyspecificity of monoclonal lupus autoantibodies produced by human-human hybridomas. N Eng J Med 308:414-20, 1983.

72. Hoch S, and Schwaber J: Specificity analysis of human anti-DNA antibodies. J Immunol 136:882-7, 1986.

73. Rauch J, Tannenbaum H, Senécal J-L, Janoff AS, Cullis PR, and Frojmovic MM: Polyfunctional properties of hybridoma lupus anticoagulant antibodies. J Rheumatol 14 (Suppl.13):132-7, 1987.

74. André-Schwartz J, Datta SK, Shoenfeld Y, Isenberg DA, Stollar BD, and Schwartz RS: Binding of cytoskeletal proteins by monoclonal anti-DNA lupus autoantibodies. Clin Immunol Immunopathol 31:261-71, 1984.

75. Senécal J-L, and Rauch J: Hybridoma lupus autoantibodies can bind major cytoskeletal filaments in the absence of DNA-binding activity. Arthritis Rheum 31:864-75, 1988.

76. Asano T, Furie BC, and Furie B: Platelet binding properties of monoclonal lupus autoantibodies produced by human hybridomas. Blood 66:1254-60, 1985.

77. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC,

Furie B, Stollar BD, and Schwartz RS: Production of autoantibodies by human-human hybridomas. J Clin Invest 70:205-8, 1982.

78. Rauch J, Meng Q-h, and Tannenbaum H: Lupus anticoagulant and antiplatelet properties of human hybridoma autoantibodies. J Immunol 139:2598-604, 1987.

79. Pisetsky DS, Hoch SO, Klatt CL, O'Donnell MA, and Keene JD: Specificity and idiotypic analysis of a monoclonal anti-Sm antibody with anti-DNA activity. J Immunol 135:4080-5, 1985.

80. Jacob L, Tron F, Bach J-F, and Louvard D: A monoclonal anti-DNA antibody also binds to cell-surface protein(s). Proc Natl Acad Sci USA 81:3843-5, 1984.

81. Gilliam AC, Lang D, and Lo Spalluto JJ: Antibodies to ds-DNA: purification and characterization of binding specificities. J Immunol 125:874-85, 1980.

82. Andrzejewski C Jr, Stollar BD, Lalor TM, and Schwartz RS: Hybridoma autoantibodies to DNA. J Immunol 124:1499-502, 1980.

83. Braun RP, Wordsworth ML, and Lee JS: The immunogenic properties of poly(dI), poly(rI), poly(dC) and poly(rI)-poly(dC)-analysis by monoclonal antibodies. Mol Immunol 23:685-91, 1986.

84. Friou GJ, Finch SC, and Detre KD: Interaction of nuclei and globulin from lupus erythematosus serum demonstrated with fluorescent antibody. J Immunol 80:324-9, 1957.

85. Hughes GRV, Cohen SA and Christian CL: Anti-DNA activity in systemic lupus erythematosus: a diagnostic and therapeutic guide. Ann Rheum Dis 30:259-64, 1971.

86. Voss EW Jr, and Casperson GF: Anti-DNA antibodies in SLE:

Historical perspective. In Anti-DNA antibodies in SLE. Ed. by Voss EW Jr. CRC Press, Boca Raton, Florida, P.1-15, 1988.

87. Bourdage JS, and Voss EW: Comparative immunological analyses of anti-deoxyribonucleotide and anti-single-stranded DNA antibodies. In Anti-DNA Antibodies in SLE. Ed. by Voss EW Jr, CRC Press, Boca Raton, Florida, P.17-67, 1988.

88. Rauch J, Tannenbaum H, Stollar BD, and Schwartz RS: Monoclonal anti-cardiolipin antibodies bind to DNA. Eur J Immunol 14:529-34, 1984.

89. Koffler D: Immunopathogenesis of systemic lupus erythematosus. Ann Rev Med 25:149-64, 1974.

90. Clough JD, and Valenzuela R: Relationship of renal histopathology in SLE nephritis to immunoglobulin class of anti-DNA. Am J Med 68:80-5, 1980.

91. Miniter MF, Stollar BD, and Agnello V: Reassessment to the clinical significance of native DNA antibodies in SLE. Arthritis Rheum 22:959-68, 1979.

92. Hahn BH: Characteristics of pathogenic subpopulations of antibodies to DNA. Arthritis Rheum 25:747-52, 1982.

93. Yoshida H, Yoshida M, Izui S, and Lambert PH: Distinct clonotypes of anti-DNA antibodies in mice with lupus nephritis. J Clin Invest 76:685-94, 1985.

94. Ebling F, and Hahn BH: Restricted subpopulations of DNA antibodies in kidneys of mice with systemic lupus. Comparison of antibodies in serum and renal eluates. Arthritis Rheum 23:392-402, 1980.

95. Faaber P, Rijke TPM, van de Putte LBA, Capel PJA, and Berden JHM: Cross-reactivity of human and murine anti-DNA antibodies with heparan sulfate. The major glycosaminoglycan in glomerular basement membranes. J Clin Invest 77:1824-30, 1985.

96. Madaio MP, Carlson J, and Cataldo J: Murine monoclonal anti-DNA antibodies bind directly to glomerular antigens and form immune deposits. J Immunol 138:2882-9, 1987.

97. Lake RA, Morgan A, Henderson B, and Staines NA: A key role for fibronectin in the sequential binding of native dsDNA and monoclonal anti-DNA antibodies to components of the extracellular matrix: its possible significance in glomerulonephritis. Immunology 54:389-95, 1985.

98. Winfield JB, Faiferman I, and Koffler D: Avidity of anti-DNA antibodies and serum and IgG glomerular eluates from patients with SLE. Association of high avidity anti-native DNA antibodies with glomerulo nephritis. J Clin Invest 59:90-6, 1977.

99. Mueller-Eckhardt C, Kayser W, Mersch-Baumert K, Mueller-Eckhardt G, Breidenbach M, Kugel HG, and Graubner M: The clinical significance of platelet associated IgG: a study on 298 patients with various disorders. Br J Haematol 46:123-31, 1980.

100. Hegde UM, Williams K, Devereux S, Bowes A, Powell D, Film D, and Fischer D: Platelet associated IgG and immune thrombocytopenia in lymphoproliferative and autoimmune disorders. Clin Lab Haematol 5:9-15, 1983.

101. Chong BH, Berndt MC, Koutts J, and Castaldi PA: Quinidine-

induced thrombocytopenia and leukopenia: Demonstration and characterization of distinct antiplatelet and antileukocyte antibodies. Blood 62:1218-23, 1983.

102. Worlledge SM, Carstairs KC, Dacie JV: Autoimmune hemolytic anemia associated with α-methyldopa therapy. Lancet 2:135-9, 1966.

103. Kelton JG, Meltzer D, Moore J, Giles AR, Wilson WE, Bart R, Hirsch J, Neame PB, Powers PJ, Walter I, Bianchi F, Carter CJ: Drug-induced thrombocytopenia is associated with increased binding of IgG to platelets both in vivo and in vitro. Blood 58:524-9, 1981.

104. Stricker RB, and Shuman MA: Quinidine purpura: Evidence that Glycoprotein V is a target platelet antigen. Blood 67: 1377-1381, 1986.

105. Savona S, Nardi MA, Lennette ET, Karpakin S: Thrombocytopenic purpura in narcotics addicts. Ann Intern Med 102:737-4, 1985.

106. Mason D, and McMillan R: Platelet antigens in chromic idiopathic thrombocytopenic purpura in sexually active homosexual men. N Engl J Med 311:635-9, 1984.

107. Stricker RB, Corash DIAL, and Shuman MA: Target platelet antigen in homosexual men with immune thrombocytopenia. N Engl J Med 313:1375-80, 1985.

108. Dixon RH, and Rosse WF: Platelet antibody in autoimmune thrombocytopenia. Br J Haematol 31:129-33, 1975.

109. McMillan R: Immune thrombocytopenias. Clin Haematol 12:69-88, 1983.

110. van Leeuwen EF, van der Van J THM, Engelfriet CP, and von dem Borne AEGKr: Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 59:23-6, 1982.

111. Beardsley DS, Spiegel JE, Jacobs MM, Handin RI, and Lux IV SE: Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J Clin Invest 74:1701-7, 1984.

112. Woods VL Jr, Kurata Y, Montgomery RR, Tani P, Mason D, Oh EH, and McMillan R: Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood 64:156-60, 1984.

113. Mason D, and McMillan R: Platelet antigens in chronic idiopathic thromcytopenic purpura. Br J Haematol 56:529-34, 1985.

114. Winiarski J, and Ekelund E: Antibody binding to platelet antigens in acute and chronic idiopathic thrombocytopenic purpura: a platelet membrane ELISA for the detection of antiplatelet antibodies in serum. Clin Exp Immunol 63:459-65, 1986.

115. McMillan R, Tani P, Millard F, Berchtold P, Renshaw L, and Woods VL Jr: Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. Blood 70:1040-5, 1987.

116. Tomiyama Y, Kurata Y, Mizutani H, Kanakura Y, Tsubakio T, Yonezawa T, and Tarui S: Platelet glycoprotein IIb as a target antigen in two patients with chronic idiopathic thrombocytopenic purpura. Br J Haematol 66:535-8, 1987.

117. van Vliet HHDM, Kappers-Klunne MC, van der Hel JWB, and Abels J: Antibodies against glycosphingolipids in sera of patients with

idiopathic thrombocytopenic purpura. Br J Haematol 67:103-8, 1987.

118. Nugent DJ, Kunicki TJ, Berglund C, and Bernstein ID: A human monoclonal autoantibody recognizes a neoantigen on glycoprotein IIIa expressed on stored and activated platelets. Blood 70:16-22, 1987.

119. Hsu-Lin SC, Shoenfeld Y, Furie B, and Schwartz RS: Human hybridoma-produced monoclonal antibodies that bind to platelets. Blood 60 (Suppl. 1):187a, 1982.

120. Stemerman MB: Vascular intimal components: precursors of thrombosis. Prog Hemost Thromb 2:1-47, 1974.

121. Spaet TH, and Ts'ao CH: Vascular endothelium and thrombogenesis. In Thrombosis. Ed. by Sherry S, Brinkhous KM, Genton E, and Stegle JM, National Academy Sciences, Washington, P.416, 1969.

122. Ryan VS, Schultz DR, and Ryan JW: Fc and C3b receptors on pulmonary endothelial cells: induction by injury. Science 214: 557-8, 1981.

123. Marcus AJ: The role of lipids in platelet function: With particular reference to the arachidonic acid pathway. J Lipid Res 19:793-826, 1978.

124. Majerus AJ, Ullman HL, and Safier LB: Lipid composition of subcellular particles of human blood platelets. J Lipid Res 10:108-14, 1969.

125. Schick PK, Lirica LB, and Chacko GK: Location of phosphatidyl-ethanolamine and phosphatidylserine in the human platelet plasma membrane. J Clin Invest 1221-6, 1976.

126. Chap HJ, Zwaal RFA, and van Deemen LLM: Action of highly purified phospholipases on blood platelets. Evidence for an asymmetric distribution of phospholipids in the surface membrane. Biochem Biophys Acta 467:146-64, 1977.

127. Cullis PR, Hope MJ, de Kruijff B, Verkleij AJ, and Tilcock CPS: Structural properties and functional roles of phospholipids in biological membranes. In Phospholipids and Cellular Regulation. Ed. by Kuo JF, CRC Press, Boca Raton, Florida, P.1-59, 1985.

128. Alving CR: Antibodies to liposomes, phospholipids and phosphate esters. Chem Phys Lipids 40:303-14, 1986.

129. Landsteiner K, and Simms S: Production of heterogenetic antibodies with mixtures of the binding part of the antigen and protein. J Exp Med 38:127-138, 1923.

130. Eagle H: Studies in the serology of syphilis. VI. The induction of the antibodies to tissue lipids (a positive Wassermann reaction) in normal rabbits. J Exp Med 55:667-81, 1932.

131. Fowler E, and Allen RH. Studies on the production of Wassermann regin. J Immunol 88:591-4, 1962.

132. Inoue K, and Nojima S: Immunochemical studies of phospholipids. I. Production of antibody to cardiolipin. Biochem Biophys Acta 144:409-14, 1967.

133. Aho K, Rostedt I, and Saris NE: Reactivity of various anti-cardiolipin antibodies with intact mitochondria. Clin Exp Immunol 14.573-9, 1973.

134. DeSiervo A: Anti-cardiolipin and anti-phosphatidylglycerol antibodies prepared against bacterial phospholipids. Infect

Immunol 9:835-8, 1974.

135. Buchheim W, Drendshahn D, and Lullman-Rauch R: Freeze-fracture studies of cytoplasmic inclusions occurring in experimental lipidosis as induced by amphiphilic cationic drugs. Biochim Biophys Acta 575:71-80, 1979.

136. van Venetie R, and Verkleij AJ: Possible role of non-bilayer lipids in the structure of mitochondria. A freeze-fracture electron microscopy study. Biochim Biophys Acta 692:397-405, 1982.

137. Gruner SM: Intrinsic curvature hypothesis for biomembrane lipid composition: a role for nonbilayer lipids. Proc Natl Acad Sci (U.S.A.) 82:3665-9, 1985.

138. Cullis PR, Hope MJ, and Tilcock CP: Lipid polymorphism and the role of lipids in membranes. Chem Phys Lipids 40:127-44, 1986.

139. Rauch J, Tannenbaum M, Tannenbaum H, Ramelson H, Cullis PR, Tilcock CPS, Hope MJ, and Janoff AS: Human hybridoma lupus anticoagulants distinguish between lamellar and hexagonal phase lipid system. J Biol Chem 261:9672-7, 1986.

140. Janoff AS and Rauch J: The structural specificity of anti-phospholipid antibodies in autoimmune disease. Chem Phys Lipids 40:315-332, 1986.

141. Banerji B, Lyon JA, and Alving CR: Membrane lipid composition modulates the binding specificity of a monoclonal antibody against liposomes. Biochim Biophys Acta 689:319-26, 1982.

142. Alving CR: Natural antibodies against phospholipids and liposomes in humans. Biochem Soc Trans 12:342-4, 1984.

143. Pangborn MC: Isolation and purification a serologically active

phospholipid from beef heart. J Biol Chem 143:247-56, 1942.

144. Moore JE, and Mohr CF: The incidence and etiologic background of chronic biologic false-positive reactions in serologic tests for syphilis. Ann Int Med 37:1156-61, 1952.

145. Yanemasa Y: Electron microscopic observation of lipid antigens. With special reference to the Wassermann antigen. Japan J Microbiol 18:193-202, 1974.

146. Frick PG: Acquired circulating anticoagulants in systemic "collagen disease"; autoimmune thromboplastin deficiency. Blood 10:691-706, 1955.

147. Laurell AB, and Nilson IM: Hypergammaglobulinemia, circulating anticoagulant, and biologic false positive Wassermann reaction. J Lab Clin Med 49:694-707, 1957.

148. Hamsten A, Norberg R, Biorkbolm M, De Faire U, and Holm G: Antibodies to cardiolipin in young survivors of myocardial infarction: an association with recurrent cardiovascular events. Lancet 1:113-6, 1986.

149. Firkin BG, Howard MA, and Radford N: Possible relationship between lupus inhibitor and recurrent abortion in young women. Lancet 2:366, 1980.

150. Carreras LO, Defreyn G, Mechin SJ, Vermylen J, Deman R, Spitz B. and Van-Assche A: Arterial thrombosis, interauterine death and "lupus" anticoagulant: detection of immunoglobulin interfering with prostacyclin formation. Lancet 1:244-6, 1981.

151. Reece EA, Romero R, Clyne LP, Kriz NS, and Hobbins JC: Lupus-like anticoagulant in pregnancy. Lancet 1:344-5, 1984.

152. Prentice RL, Gatenby PA, Loblay RH, Shearman RP, Kronenberg H, and Basten A: Lupus anticoagulant in pregnancy. Lancet 2:464, 1984.

153. Gleicher N, and Friberg J: IgM gammopathy and the lupus anticoagulant syndrome in habitual abortion. J Am Med Assoc 253:3278-81, 1985.

154. Lockshin MD, Druzin ML, Goei S, Qamar T, Magid MS, Jovanovic L, and Ferenc M: A predictor of fetal distress or death in pregnant patients with systemic lupus erythematosus. N Engl J Med 313:152-6, 1985.

155. Medal LS, and Lisker R: Circulating anticoagulants in disseminated lupus erythematosus. Haematol 5:284-93, 1959.

156. Margolius A, Jackson DP, and Ratnoff OD: Circulating anticoagulants: a study of 40 cases and a review of the literature. Medicine (Baltimore) 40:145-202, 1961.

157. Bowie EJW, Thompson JH Jr, and Pascuzzi CA: Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. J Lab Clin Med 62:416-30, 1963.

158. Lechner K: Acquired inhibitors in nonhemophilic patients. Haemostasis 3:65-93, 1974.

159. Schleider MA, Nachman RL, and Jaffe EA: A clinical study of the lupus anticoagulant. Blood 48:499-509, 1976.

160. Mueh JR, Herbst KD, and Rapaport SI: Thrombosis in patients with the lupus anticoagulants. Ann Int Med 92:156-9, 1980.

161. Boey M, Colaco CB, Gharavi AE, Elkon KB, Loizou S, and Hughes GRV: Thrombosis in systemic lupus erythematosus: striking

association with the presence of circulating lupus anticoagulant. Br Med J 287:1021-3, 1983.

162. Harris EN, Loizou S, Englert H, Derue G, Chan JK, Gharavi AE, and Hughes GR: Anticardiolipin antibodies and lupus anticoagulant. Lancet 2:1099, 1984.

163. Harris EN, Gharavi AE, Tincani A, Chan JK, Englert H, Mantelli P, Allego F, Ballestrieri F, and Hughes GR: Affinity purified anti-cardiolipin and anti-DNA antibodies. J Lab Clin Immunol 17:155-62, 1985.

164. Glueck HI, Kant KS, Weiss MA, Pollak VE, Miller MA, and Coots M: Thrombosis in systemic lupus erythematosus. Relation to the presence of circulating anticoagulants. Arch Intern Med 145:1389-95, 1985.

165. Triplett DA: The clinical Importance of lupus anticoagulants. Thromb Haemost 7:1-14, 1985.

166. Colaco CB, and Male DK: Anti-phospholipid antibodies in syphilis and a thrombotic subset of SLE: distinct profiles of epitope. Clin Exp Immunol 59:449-56, 1985.

167. Harris EN, Chan JK, Asherson RA, Aber VR, Gharavi AE, and Hughes GR: Predictive value of the anticardiolipin antibody test. Arch Intern Med 146:2153-6, 1986.

168. Triplett DA, Brandt JT, and Maas RL: The laboratory heterogeneity of lupus anticoagulants. Arch Pathol Lab Med 109:946-51, 1985.

169. Exner T, Rickard KA, and Kronenberg H: Studies on phospholipids in the action of a lupus coagulation inhibitor. Pathology

7:319-28, 1975.

170. Thiagarajan P, Shapiro SS, and de Marco L: Monoclonal immunoglobulin M lambda coagulation inhibitor with phospholipid specificity. Mechanism of lupus anticoagulant. J Clin Invest 66:397-405,1980.

171. Triplett DA: The platelet: a review. In Platelet Function-Laboratory Evaluation and Clinical Application. Ed. by Triplett DA, Educational Products Division, Am Society of Clinical Pathologists Inc., Chicago, P.1-33, 1978.

172. Zucker MB, and Nachmias VT: Platelet activation. Arteriosclerosis 5:2-18, 1985.

173. Hourdillé P, Hasitz M, Belloc F, and Nurden AT: Immunocytochemical study of the binding of fibrinogen and thrombospondin to ADP- and thrombin-stimulated human platelets. Blood 65:912-20, 1985.

174. Aiken ML, Ginsberg MH, and Plow EF: Identification of a new class of inducible receptors on platelets. Thrombospondin interacts with platelets via a GPIIb-IIIa-independent mechanism. J Clin Invest 78:1713-6, 1986.

175. Vicic WJ, Lages B, and Weiss HJ: Release of human platelet Factor V activity is induced by both collagen and ADP and is inhibited by aspirin. blood 56:448-55, 1980.

176. Harris KW, and Esmon CT: Protein S is required for bovine platelets to support activated protein C binding and activity. J Biol Chem 260:2007-10, 1985.

177. Fujimoto T, Ohara S, Hawiger J: Thrombin-induced exposure and

prostacyclin inhibition of the receptor for Factor VIII-von Willebrand factor on human platelets. J Clin Invest 69:1212-20, 1982.

178. George JN, Lyons RM, and Morgan RK: Membrane changes associated with platelet activation. Exposure of actin on the platelet surface after thrombin-induced secretion. J Clin Invest 66:1-9, 1980.

179. Miles LA, and Plow EF: Binding and activation of plasminogen on the platelet surface. J Biol Chem 260:4303-11, 1985.

180. Sinha D, Seaman FS, Koshy A, Knight LC, and Walsh PN: Blood coagulation Factor XIa binds specifically to a site on activated human platelets distinct from that for Factor XI. J Clin Invest 73:1550-6, 1984.

181. Miletich JP, Kane WH, Hofmann SL, Stanford N, and Majerus PW: Deficiency of Factor Xa-Factor Va binding sites on the platelets of a patient with a bleeding disorder. Blood 54:1015-22, 1979.

182. Greenberg CS, and Shuman MA: Specific binding of blood coagulation Factor XIIIa to thrombin-stimulated platelets. J Biol Chem 259:14721-7, 1984.

183. Marguerie GA, Plow EF, and Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254:5357-63, 1979.

184. Bennett JS, and Vilaire G: Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393, 1979.

185. Kornecki E, Tuszynski GP, and Niewiarowski S: Identification of human platelet membrane fibrinogen receptors by immunochemical

techniques. Haematologia 17:387-98, 1984.

186. Broekman MJ, Ward JW and Marcus AJ: Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid and lysophospholipids. J Clin Invest 66:275-83, 1980.

187. Hsu-Lin SC, Berman CL, Furie BC, August D, and Furie B: A Platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. J Biol Chem 259:9121-6, 1984.

188. Nieuwenhuis HK, van Osterhout JJG, Rozemuller E, van Iwaarden F, and Sixma JJ: Studies with a monoclonal antibody against activated platelets: Evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. Blood 70:838-45, 1987.

189. Solum NO: Platelet membrane. Semin Hematol 22:289-302, 1985.

190. Tuszynski GP, Daniel JL, and Stewart G: Association of proteins with the platelet cytoskeleton. Semin Hematol 22:303-12, 1985.

191. Fox JEB, and Phillips DR: Inhibition of actin polymerization in blood platelets by cytochalasins. Nature 292:650-2, 1981.

192. Gonnella PA, and Nachmias VT: Platelet activation and microfilament binding. J Cell Biol 89:146-51, 1981.

193. Jennings LK, Fox JEB, and Edwards HH: Changes in the cytoskeletal structure of human platelets following thrombin activation. J Biol Chem 256:6927-32, 1981.

194. Davies GE, and Palek J: The state of actin polymerization in

tetracaine-treated platelets. Thromb Haemost 48:153-5, 1982.

195. Markey F, Persson T, and Lindberg U: Characterization of platelet extracts before and after stimulation with respect to the possible role of profilactin as microfilament precursor. Cell 23:145-53, 1981.

196. Pribluda V, and Rotman A: Dynamics of membrane cytoskeleton interactions in activated blood platelets. Biochem 21:2825-32, 1982.

197. Hirschberg H, Bergh OJ, and Thorsby E: Antigen-presenting properties of human vascular endothelial cells. J Exp Med 152:249s-55s, 1980.

198. Hirschberg H, Braathen LR, and Thorsby E: Antigen presentation by vascular endothelial cells and epidermal Langerhans cells: the role of HLA-DR. Immunol Rev 65:57-77, 1982.

199. Miossec P, Cavender D, and Ziff M: Production of interleukin 1 by human endothelial cells. J Immunol 136:2486-91, 1986.

200. Stevens SK, Weissman IL, and Butcher EC: Differences in the migration of B and T lymphocytes: organ selective localization in vivo and the role of lymphocyte-endothelial cell recognition. J Immunol 128:844-51, 1982.

201. Scott H, Brandtzaeg P, Hirschberg H, Solheim BG, and Thorsby E: Vascular and renal HLA-DR-like antigens. Tissue Antigens 18:195-202, 1981.

202. Cerilli J, and Brasile L: Antibodies against renal endothelial alloantigens. Transplant Proc 12 (Suppl.1):37-42, 1980.

203. Pober JS, Gimbrone MA Jr, Cotran RS, Reiss CS, Burakoff SJ,

Fiers W, and Ault KA: Ia expression by vascular endothelium is inducible by activated T cells and human interferon. J Exp Med 157:1339-53, 1983.

204. Zimmerman GA, and Klein-Knoeckel D: Human endothelial cells inhibit granulocyte aggregation in vitro. J Immunol 136:3839-47, 1986.

205. Vermylen J, Blockmans D, Spitz B, and Deckmyn H: Thrombosis and immune disorders. In Clinics in Haematology, Ed. by Chesterman CN, W.B. Saunders Company, Philadephia, P.391-411, 1986.

206. Hammerschmidt DE, Harris PD, Wayland JH, Craddock PR, and Jacob HS: Complement-induced granulocyte aggregation in vivo. Am J Pathol 102:146-50, 1981.

207. Jacob HS, Craddock PR, Hammerschmidt DE, and Moldow CF: Complement-induced granulocyte aggregation. An unsuspected mechanism of disease. N Engl J Med 302:789-94, 1980.

208. Craddock PR: Complement-mediated intravascular leukostasis and endothelial cell injury. In Pathobiology of the Endothelial Cell. Ed. by Nossel HL and Vogel HJ, Academic Press, New York, P369, 1982.

209. Fligiel SEG, Ward PA, Johnson KJ, and Till GO: Evidence for a role of hydroxyl radical in immune-complex-induced vasculitis. Am J Pathol 115:375-82, 1984.

210. Tzeng DY, Deuel TF, Huang JS, Senior RM, Boxer LA, and Baehner RL: Platelet-derived growth factor promotes polymorphonuclear leukocyte activation. Blood 64:1123-8, 1984.

211. Boogaerts MA, Yamada O, Jacob HS, and Moldow CF: Enhancement of

granulocyte-endothelial cell adherence and granulocyte-induced cytotoxicity by platelet release products. Proc Natl Acad Sci USA 79:7019-23, 1982.

212. Craddock PR, Hammerschmidt D, White JG and Dalmasso AP, and Jacob HS: Complement (C5a) - induced granulocyte aggregation in vitro. A possible mechanism of complement-mediated leukostasis and leukopenia. J Clin Invest 60:260-70, 1977.

213. Jaffe EA, Nachman RL, Becker CG, and Minick CR: Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. J Clin Invest 52:2745-56, 1973.

214. Gimbrone MA, Aster RH, and Cotran RS: Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. Nature 222:33-6, 1969.

215. Kitchens CS: Amelioration of endothelial abnormalities by prednisone in experimental thrombocytopenia in the rabbit. J Clin Invest 60:1129-34, 1977.

216. Saba SR, and Mason RG: Effects of platelets and certain platelet components on growth of cultured human endothelial cells. Thromb Res 7:807-12, 1975.

217. Clemmons DR, Isley WL, and Brown MT: Dialyzable factor in human serum of platelet origin stimulates endothelial cell replications and growth. Proc Natl Acad Sci USA 80:1641-5, 1983.

218. King GL, and Buchwald S: Characterization and partial purification of an endothelial cell growth factor from human platelets. J Clin Invest 73:392-6, 1984.

219. Kaplan KL: Interactions of platelets with endothelial cells. In Pathobiology of the Endothelial Cell. Ed. by Nossel HL & Vogel HJ, Academic Press, New York, P.337-49, 1982.

220. Esmon CT, and Own WG: Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. Proc Natl Acad Sci USA 78:2249-52, 1981.

221. Moncada S, Higgs EA, and Vane JR: Human arterial and venous tissues generate prostacyclin (prostaglandin X), a potent inhibitor of platelet aggregation. Lancet 1:18-21, 1977.

222. Hemler ME: Adhesive protein receptors on hematopoietic cells. Immunol Today 9:109-13, 1988.

223. Hynes RO: Integrins: A family of cell surface receptors. Cell 48:549-54, 1987.

224. Takada Y, Strominger JL, and Hemler ME: the very late antigen family of heterodimers is part of a superfamily of molecules involved in adhesion and embryogenesis. Proc Natl Acad Sci USA 84:3239-43, 1987.

225. Charo IF, Bekeart LS, and Phillips DR: Platelet glycoprotein IIb-IIIa-like proteins mediate endothelial cell attachment to adhesive proteins and the extracellular matrix. J Biol Chem 262:9935-8, 1987.

226. Fitzgerald LA, Charo IF, and Phillips DR: Human and bovine endothelial cells synthesize membrane proteins similar to human platelet glycoproteins IIb and IIIa. J Biol Chem 260:10893-6, 1985.

227. Pischel KD, Bluestein HG, and Woods VL Jr: Lymphocytes bear

molecules (VLA) that are antigenically and structurally similar to platelet GPIa, GPIc and GPIIa. Blood 66:311a, 1985 (abstract).

228. Nieuwenhuis HK, Akkerman JQN, Houdijk WPM, and Sixma JJ: Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. Nature 318:470-2, 1985.

229. De Marco L, Girolami A, Zimmerman TS, and Ruggeri ZM: Interaction of purified type IIb von Villebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb-IIIa complex and initiates aggregation. Proc Natl Acad Sci USA 82:7424-8, 1985.

230. Harmon JT, and Jamieson GA: The glycocalicin portion of GPIb expresses both high and moderate affinity receptor sites for thrombin: A soluble radioreceptor assay for the interaction of thrombin with platelets. J Biol Chem 261:13224-9, 1986.

231. Francotti FG, Languino LR, Zanetti A, Peri G, Tarone G, and Dejana E: Platelets express a membrane protein complex immunologically related to the fibroblast fibronectin receptor and distinct from GP IIb/IIIa. Blood 69:1535-8, 1987

232. Tandon NM, and Jamieson GA: Isolation and characterization of GPIV and its role as a receptor for primary platelet: collagen interaction. Thromb Haemost 58:1107a, 1988.

233. Phillips DR, and Agin PP: Platelet plasma membrane glycoproteins. Identification of a proteolytic substrate for thrombin. Biochem Biophys Res Commun 75:940-7, 1977.

234. Wicki AN, and Clemetson KJ: Structure and function of platelet

membrane glycoproteins Ib and V: Effects of leukocyte elastase and other proteases on platelet response to von Willebrand factor and thrombin. Eur J Biochem 153:1-11, 1985.

235. Jaffe EA, and Nachman RL: Factor VIII binding protein in human platelets. Clin Res 23:276A, 1975 (abstract).

236. Weiss HJ, Hoyer LW, Rickles FR, Varma N, and Rogers J: Quantitative assay of a plasma factor deficient in von Willebrand's disease that is necessary for platelet aggregation: relationship to Factor VIII procoagulant activity and antigen content. J Clin Invest 52:2078-16, 1973.

237. Sakariassen KS, Bolhuis PA, and Sixma JJ: Human blood platelet adherence to artery subendothelium is mediated by Factor VIII-von Villebrand factor bound to the subendothelium. Nature 279:636-9, 1979.

238. Bunting S, Moncada S, and Vane JR: The prostacyclin-thromboxane A_2 balance: pathophysiological and therapeutic implications. Br Med Bull 39:271-6, 1983.

239. Moncada S, Herman AG, Higgs EA, and Vane JR: Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium. Thromb Res 11:323-44, 1977.

240. Eldor A, Falcone DJ, Hajjar DP, Minick CR, and Weksler BB: Recovery of prostacylin production by re-endothelialized rabbit aorta. Critical role of neointimal smooth muscle cells. J Clin Invest 67:735-41, 1981.

241. Gorman RR, Bunting S, and Miller OV: Modulation of human

platelet adenylate cyclase by prostacyclin (PGX). Prostaglandins 13:377-88, 1977.

242. Tateson JE, Moncada S, and Vane JR: Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. Prostaglandins 13:389-97, 1977.

243. Graber SE, and Hawiger J: Evidence that changes in platelet cyclic AMP levels regulate the fibrinogen receptor on human platelets. J Biol Chem 257:14606-9, 1982.

244. Niewiarowski S, and Holt JC: Platelet α-granule proteins: Biochemical and Pathological Aspects. In The Platelets: Physiology and Pharmacology, Academic Press, New York, P.49-69, 1985.

245. Day HJ, Holmsen H, and Hovig T: Subcellular particles of human platelets. Scand J Haematol 7 (Suppl.):3-35, 1969.

246. Kjellstrom BT, Ortenwall P, and Risberg B: Effects of 5-hydroxytryptamine and related agent on endothelial cell oxidative metabolism in vitro. Acta Phys Scand 125:501-4, 1985.

247. Richman AV, and Kasnic GJr: Endothelial and platelet reactions in the idiopathic nephrotic syndrome. An ultrastructural study. Human Pathol 13:548-53, 1982.

248. Fujimoto T, Suzuki H, Tanoue K, Fukushima Y, and Ymazaki H: Cerebrovascular injuries induced by activation of platelets in vivo. Stroke 16:245-50, 1985.

249. Budman DR, and Steinberg AD: Hematologic aspects of systemic lupus erythematosus. Current concepts. Ann Intern Med 86:220-

9, 1977.

250. Rick ME, and Hoyer LW: Hemostatic abnormalities in systemic lupus erythematosus. J Clin Rheum Dis 1:583-96, 1975.

251. Lee SL, and Miotti AB: Disorders of hemostatic function in patients with systemic lupus erythematosus. Semin Arthritis Rheum 4:241-52, 1975.

252. Regan MG, Lackner H, and Karpatkin S: Platelet function and coagulation profiles in lupus erythematosus. Studies in 50 patients. Ann Intern Med 81:462-8, 1974.

253. Dorsch CA, and Meyerhoff J: Mechanisms of abnormal platelet aggregation in systemic lupus erythematosus. Arthritis Rheum 25:966-73, 1982.

254. Corrigan JJ, Patterson JH, and May NE: Incoagulability of the blood in systemic lupus erythematosus. Am J Dis Child 119:365-9, 1970.

255. Rapaport SI, Ames SB, and Durall BJ: A plasma coagulation defect in systemic lupus erythematosus arising from hypoprothrombinemia combined with antiprothrombinase activity. Blood 15:212-27, 1960.

256. Simone JJ, Cornet JA, and Abildgaard CF: Acquired von Willebrand's syndrome in systemic lupus erythematosus. Blood 31:806-12, 1968.

257. Feinstein DI, and Rapaport SI: Acquired inhibitors of blood coagulation. In Progress in Hemostasis and Thrombosis, Ed. by Spaet TH, Grune & Stratton Inc, New York, 1:75-95, 1972.

258. Krieger H, Leddy JP, and Breckenridge RT: Studies on a

circulating anticoagulant in systemic lupus erythematosus: evidence for inhibition of the function of activated plasma thromboplastin antecedent (Factor XIa). Blood 46:189-97, 1975.

259. Galanakis DG, Ginzler EM, and Fikrig SM: Monoclonal IgG anticoagulants delaying fibrin aggregation in two patients with systemic lupus erythematosus (SLE). Blood 52:1037-46, 1978.

260. Byron MA: The clotting defect in SLE. Clin Rheum Dis 8:137-51, 1982.

261. Gladman DD, and Urowitz MB: Venous syndromes and pulmonary embolism in systemic lupus erythematosus. Ann Rheum Dis 39:340-3, 1980.

262. Kant K, Pollak VE, Weiss MA, Glueck HI, Miller AN, and Hess EV: Glomerular thrombosis in systemic lupus erythematosus: Prevalence and significance. Medicine 60:71-86, 1981.

263. Gladman DD, Urowitz MB, Tozman EC, and Glynn MFX: Hemostatic abnormalities in systemic lupus erythematosus. Quart J Med, New Series LII 207:424-33,1983.

264. Mintz G, Acevedo-Vazquez E, Gutiérrez-Espinosa G, and Avelar-Garnica F: Renal vein thrombosis and inferior vena cave thrombosis in systemic lupus erythematosus. Frequency and risk factors. Arthritis Rheum 27:539-44, 1984.

265. Clark WF, Linton AL, Cordy PE, Keown PE, Lohmann RC, and Lindsay RM: Immunologic findings, thrombocytopenia and disease activity in lupus nephritis. Can Med Assoc J 118:1391-3, 1978.

266. Harris EN, Asherson RA, Gharavi AE, Morgan SH, Derue G, and Hughes GR: Thrombocytopenia in SLE and related autoimmune

disorders: association with anticardiolipin antibody. Br J Haematol 59:227-30, 1985.

267. Asherson RA, Derksen RHWM, Harris EN, Bingley PJ, Hollbrand BI, Gharavi AE, Kater L, and Hughes GRV: Large vessel occlusion and gangrene in systemic lupus erythematosus and "lupus-like" disease. A report of six cases. J Rheumatol 13:740-7, 1986.

268. Carreras LO, and Vermylen JG: 'Lupus' anticoagulant and thrombosis: possible role of inhibition of prostacyclin formation. Thromb Haemost 48:38-40, 1982.

269. Remuzzi G, Misiani R, Maratore D, Marchesi D, Livio M, Schieppati A, Mecca G, de Gaetano G, and Donati MB: Prostacyclin and human foetal circulation. Prostaglandins 18:341-8, 1979.

270. Remuzzi G, Misiani R, Maratore D, Marchesi D, Livio M, Schieppati A, Mecca G, de Gaetano G, and Donati MB: Reduced umbilical and placental vascular prostacyclin in severe pre-eclampsia. Prostaglandins 20:105-10, 1980.

271. Mikhail MH, Heine MJ, Pengo V, and Shapiro SS: Thrombosis: platelet activation regulatory mechanisms. Circulation 78 (suppl.):II 514, 1988 (abstract).

272. Stern W, and Macdonald VE: Factors affecting the sensitivity of the APTT reagents to lupus inhibitors. Read before the 33rd Annual New York Seminar on Hemostasis, New York, Feb 9, 1985.

273. Shapiro SS, Thiagarajan P, and DeMarco L: Mechanisms of action of the lupus anticoagulant. Ann NY Acad Sci 370:359-65, 1981.

274. Shapiro SS, and Thiagarajan P: Lupus anticoagulants. Prog Haemost Thromb 6:263-85, 1982.

275. Angles-Cano E, Sultan Y, and Clauvel JP: Predisposing factors to thrombosis in systemic lupus erythematosus. Possible relation to endothelial cell damage. J Lab Clin Med 94:312-23, 1983.

276. Freyssinet JM, Wiesel ML, Gauchy J, Boneu B, and Cazenave JP: An IgM lupus anticoagulant that neutralizes the enhancing effect of phospholipid on purified endothelial thrombomodulin activity - a mechanism for thrombosis. Thromb Hemost 5:309,313, 1986 (abstract).

277. Comp PC, DeBault LE, Esmon NL, and Esmon CT: Human thrombomodulin is inhibited by IgG from two patients with non-specific anticoagulants. Blood 62 (Suppl. 1):299, 1983 (abstract).

278. McVerry BA, Machin SJ, Parry H, and Goldstone AH: Reduced prostacyclin activity in systemic lupus erythematosus. Ann Intern Med 39:524-5, 1980.

279. Marchesi D, Barbaru A, Frampton G, Livio M, Remuzzi G, and Cameron JS: Thrombosis tendency in systemic lupus erythematosus. Lancet 1:719, 1981.

280. Elias M, and Eldor A: Thromboembolism in patients with the "lupus" type circulating anticoagulant. Arch Intern Med 144:510-5, 1984.

281. Vila L, de Castellarnau C, Borrell M, Fontcuberta J, Fabra A, Sancho MJ, and Rutllant MCI: Lupus anticoagulant and vascular prostacyclin production. Thromb Haemost 54:38, 1985 (abstract).

282. Hasselaar P, Derksen RHWM, Blokzijl L, and de Groot PG: Thrombosis associated with antiphospholipid antibodies cannot be

explained by effects on endothelial and platelet prostanoid synthesis. Thromb Haemost 59:80-85, 1988.

283. Rustin MHA, Bull HA, Machin SJ, Isenberg DA, Snaith ML, and Dowd PM: Effects of the lupus anticoagulant in patients with systemic lupus erythematosus on endothelial cell prostacyclin release and procoagulant activity. J Invest Dermatol 90:744-8, 1988.

284. Dreyer WJ, and Bennett JC: The molecular basis of antibody formation: a paradox. Proc Natl Acad Sci USA 54:864-9, 1965.

285. Hozumi N, and Tonegawa S: Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc Natl Acad Sci USA 73:3628-32, 1976.

286. Tonegawa S: Somatic generation of antibody diversity. Nature 302:575-81, 1983.

287. Honjo T: Immunoglobulin genes. Ann Rev Immunol 1:499-528, 1983.

288. Kim S, Davis M, Sinn E, Patten P, and Hood L: Antibody diversity: somatic hypermutation of rearranged V_H genes. Cell 27:573-81, 1981.

289. Jerne NK: Toward a network theory of the immune system. Ann Immunol (Inst Pasteur) 125:373-89, 1974.

290. Schwartz RS, and Stollar BD: Origins of anti-DNA antibodies. J Clin Invest 75:321-7, 1985.

291. Kofler R, Perlmutter RM, Noonan DJ, Dixon FJ, and Theofilopoulos AN: Ig heavy chain variable region gene complex of lupus mice exhibits normal restriction fragment length polymorphism. J Exp Med 162:346-51, 1985.

292. Trepicchio W Jr, and Barrett DKJ: The Igh-V locus of MRL mice: restriction fragment length polymorphism in eleven strains of mice as determined with V_H and D gene probes. J Immunol 134:2734-9, 1985.

293. Kofler R, Noonan DJ, Levy DE, Wilson MC, MØller NP, Dixon FJ, and Theofilopoulos AN: Genetic elements used for a murine lupus anti-DNA autoantibody are closely related to those for antibodies to exogenous antigens. J Exp Med 161:805-15, 1985.

294. Reininger L, Ollier P, Poncet P, Kaushik A, and Jaton JC: Novel V genes encode virtually identical variable regions of six murine monoclonal anti-bromelain-treated red blood cell autoantibodies. J Immunol 138:316-23, 1987.

295. Kofler R, Noonan DJ, Strohal R, Balderas RS, MØller NP, Dixon FJ, and Theofilopoulos AN: Molecular analysis of the murine lupus-associated anti-self response: involvement of a large number of heavy and light chain variable region genes. Eur J Immunol 17:91-5, 1987.

296. Monestier M, Manheimer-Lory A, Bellon B, Painter C, Dang H, Talal N, Zanetti M, Schwartz R, Posetsky D, and Kuppers R: Shared idiotypes and restricted immunoglobulin variable region heavy chain genes characterized murine autoantibodies of various specificities. J Clin Invest 78:753-9, 1986.

297. Perlmutter RM, Kearney JF, Chang SP, and Hood LE: Developmentally controlled expression of immunoglobulin V_H genes. Science 227:1597-601, 1985.

298. Bellon B, Manheimer-Lory A, Monestier M, Moran T, Dimitriu-Bona

A, Alt F, and Bona C: High frequency of autoantibodies bearing cross-reactive idiotype among hybridomas using V_H 7183 genes prepared from normal and autoimmune murine strain. J Clin Invest 79:1044-53, 1987.

299. Lofler R, Dixon FJ, and Theofilopoulos AN: The genetic origin of autoantibodies. Immunol Today 8:374-80, 1987.

300. Naparstek Y, André-Schwartz J, Manser T, Wysocki LJ, Breitman L, Stollar BD, Gefter M, and Schwartz RS: A single germline V_H gene segment of normal A/J mice encodes autoantibodies characteristic of systemic lupus erythematosus. J Exp Med 164:614-26, 1986

301. Davidson A, Homme JLP, Solomon A, Chang MDY, Beede S, and Diamond B: Idiotypic analysis of myeloma proteins: anti-DNA activity of monoclonal immunoglobulins bearing an SLE idiotype is more common in IgG than IgM antibodies. J Immunol 138:1515-8, 1987.

302. Rudikoff S, Giusti AM, Cook WD, and Scharff MD: Single amino acid substitution altering antigen-binding specificity. Proc Natl Acad Sci USA 79:1979-83, 1982.

303. Giusti AM, Chien NC, Zack DJ, Shin SU, and Scharff MD: Somatic diversification of S107 from an antiphosphocholine to an anti-DNA autoantibody is due to a single base change in its heavy chain variable region. Proc Natl Acad Sci USA 84:2726-30, 1987.

304. Crews S, Griffin J, Huang H, Calame K, and Hood L: A single V_H gene segment encodes the immune response to phosphorylcholine:

somatic mutation is correlated with the class of the antibody. Cell 25:59-66, 1981.

305. Gearhart PJ, Johnson JD, Douglas R, and Hood L: IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. Nature 291:29-34, 1981.

306. Hood L, Loh E, Hubert J, Barstad P, Eaton B, Early P, Fuhrman j, Johnson N, Kronenberg M, and Schilling J: The structure and genetics of mouse immunoglobulins: an analysis of NZB myeloma proteins and sets of BALB/c myeloma proteins binding particular haptens. Cold Spring Harbor Symp. Quant Biol 41:817-36, 1976.

307. Davidson A, Shefner R, and Diamond B: Studies on the role of somatic mutation in the generation anti-DNA antibodies. Monogr Allergy 22:31-7, 1987.

308. Billingham RE, Brent L and Medawar PB: 'Acquired tolerance' of foreign cells. Nature 172:603-6, 1953.

309. Golub ES: Immune Tolerance: self-nonself discrimination. In: Immunology. A Systhesis. Ed. by Golub ES, Sinauer Associates, Inc., Sunderland, Massachusetts, P.219-20, 1987.

310. Miller RG: An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it. Nature 287:544-6, 1980.

311. Pullen AM, Marrack P, and Kappler JW: The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature 335:796-801, 1988.

312. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, Pritchard-Briscoe H, Wotherspoon JS, Loblay RH,

Raphael K, Trent RJ, and Basten A: Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature 334:676-82, 1988.

313. Strelkauskas AJ, Callery RT, McDowell J, Borel Y, and Schlossman SF: Direct evidence for loss of human suppressor cells during active autoimmune disease. Proc Natl Acad Sci USA 75:5150-4, 1978.

314. Golub ES: Know thyself: autoreactivity in the immune response. Cell 21:603-4, 1981.

315. Naparstek Y, Duggan D, Schattner A, Madaio MP, Goni F, Franglone B, Stollar BD, Kabat EA, and Schwartz RS: Immunochemical similarities between monoclonal antibacterial Waldenstrom' macroglobulins and monoclonal anti-DNA lupus autoantibodies. J Exp Med 161:1525-38, 1985.

316. Smith HR ,and Steinberg AD: Autoimmunity - a perspective. Ann Rev Immunol 1:175-210, 1983.

317. Lane D, and Koprowski H: Molecular recognition and the future of monoclonal antibodies. Nature 296:200-2, 1982.

318. Ghosh S, and Campbell AM: Multispecific monoclonal antibodies. Immunol Today 7:217-22, 1986.

319. Andrzejewski CJr, Rauch J, Lafer E, Stollar D, and Schwartz RS: Antigen-binding diversity and idiotypic cross-reactions among hybridoma autoantibodies to DNA. J Immunol 126:226-31, 1980.

320. Richards FF, Konigsberg WH, Rosenstein RWR, and Varga JM: On the specificity of antibodies. Biochemical and biophysical evidence indicates the existence of polyfunctional antibody combining

regions. Science 187:130-6, 1975.

321. Westhof E, Altschuch D, Moras D, Westhof E, Altschuch D, Moras D, Bloomer AC, Mondragon A, Klug A, and van Regenmortal MHV: Correlation between segmental mobility and the location of antigenic determinants in proteins. Nature 311:123-7, 1984.

322. Parham P: Changes in conformation with loss of alloantigenic determinants of a histocompatibility antigen (HLA-B7) induced by monoclonal antibodies. J Immunol 132:2975-83, 1984.

323. Avrameas S, Guilbert B, and Dighiero G: Natural antibodies against tubulin, actin, myoglobulin, thyroglobulin, fetuin, albumin, and transferrin are present in normal human sera, and monoclonal immunoglobulins from multiple myeloma and Waldenstrom's macroglobulinemia may express similar antibody specificities. Ann Immunol (Inst Pasteur) 132C:231-6, 1981.

324. Guilbert B, Dighiero G, and Avrameas S: Naturally occurring antibodies against nine common antigens in human sera. I. Detection, Isolation and Characterization. J Immunol 128:2779-87, 1982.

325. Dighiero G, Guilbert B, and Avrameas, S: Naturally occurring antibodies against nine common antigens in humans. II. High incidence of monoclonal Ig exhibiting antibody activity against actin and tubulin and sharing antibody specificities with natural antibodies. J Immunol 128:2788-92, 1982.

326. Madaio MD, Schattner A, Schattner M, and Schwartz RS: Lupus serum and normal human serum contain anti-DNA antibodies with the same idiotype marker. J Immunol 137:2535-40, 1986.

327. Dighiero G, Lymberi P, Holmberg D, Lundquist I, Coutinho A, and Avrameas S: High frequency of natural autoantibodies in normal newborn mice. J Immunol 134:765-71, 1985.

328. Holmberg D, Freitas AA, Portnoi D, Jacquemart F, Avrameas S, and Coutinho A: Antibody repertoires of normal BALB/c mice: B lymphocyte populations defined by state of activation. Immunol Rev 93:147-69, 1986.

329. Holmberg D, Forsgren S, Ivars F, and Coutinho A: Reactions among IgM antibodies derived from normal, neonatal mice. Eur J Immunol 14:435-41, 1984.

330. Vakil M, and Dearney JF: Functional characterization of monoclonal auto-anti-idiotype antibodies isolated from the early B cell repertoire of BALB/c mice. Eur J Immunol 16:1151-8, 1986.

331. Theofilopoulos AN, and Dixon FJ: Autoimmune disease: immunopathology and etiopathogenesis. Am J Pathol 108:319-65, 1982.

332. Izui S, Lambert PH, Fournie GJ, Turler H, and Merscher PA: Features of systemic lupus erythematosus in mice injected with bacterial lipopolysaccharides: identification of circulating DNA and renal localization of DNA anti-DNA complexes. J Exp Med 145:1115-30, 1977.

333. Portnoi D, Freitas A, Holmberg D, Bandeira A, and Coutinho A: Immunocompetent autoreactive B lymphocytes are activated cycling cells in normal mice. J Exp Med 164:25-35, 1986.

334. Underwood JR, Pedersen JS, Chalmers PJ, and Toh BH: Hybrids from normal germ free, nude and neonatal mice produce monoclonal autoantibodies to eight different intracellular structures.

Clin Exp Immunol 60:417-26, 1985.

335. Prabhakar BS, Junzo JS, Onodera T, and Notkins AL: Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. J Immunol 133:2815-20, 1984.

336. Strejan GH, Essani K, and Surlan D: Naturally occurring antibodies to liposomes. II. Specificity and eletrophoretic pattern of rabbit antibodies reacting with sphingomyelin-containing liposomes. J Immunol 127:160-5, 1981.

337. Cairns EJ, Block J, and Bell DA: Anti-DNA autoantibody-producing hybridomas of normal human lymphoid cell origin. J Clin Invest 74:880-7, 1984.

338. Cairns E, Komar R, and Bell DA: Cytoskeletal binding of monoclonal anti-DNA antibodies derived from tonsillar lymphoid cells of a normal subject. Arthritis Rheum 29:1351-8, 1986.

339. Logtenberg T, Kroon A, Gmelig-Meyling FHJ, and Ballieux RE: Analysis of the human tonsil B cell repertoire by somatic hybridization: occurrence of both "monospecific" and "multispecific" (auto)antibody-secreting cells. Eur J Immunol 17:855-9, 1987.

340. Denomme GA, and Bell DA: Anti-platelet antibodies to platelet glycoproteins are among the antibodies produced by normal human B cells. Arthritis Rheum 32 (suppl.):R18, 1989 (abstract).

341. Datta SK, Naparstek Y, and Schwartz RS: In vitro production of an anti-DNA idiotype by lymphocytes of normal subjects and patients with systemic lupus erythematosus. Clin Immunol

Immunopathol 38:302-18, 1986.

342. Bell DA, Cairns E, Cikalo K, Block VJ, and Pruzanski W: Antinucleic acid autoantibody responses of normal human origin: antigen specificity and idiotypic characteristics compared to patients with systemic lupus erythematosus and patients with monoclonal IgM. J Rheumatol 14:127-31, 1987.

343. Klinman DM, Ishigatsubo Y, and Steinberg AD: Acquisition and maturation of expressed B cell repertoires in normal and autoimmune mice. J Immunol 141:801-6, 1988.

344. Cancro MP, and Klinman NR: B cell repertoire ontogeny-heritable but dissimilar development of parental and F1 repertoires. J Immunol 126:1160-4, 1981.

345. Roux GL, Wautier MP, Guillevin L, and Wautier J-L: IgG binding to endothelial cells in systemic lupus erythematosus. Thromb Haemost 56:144-6, 1986.

346. Aauci AS, Haynes BF, and Katz P: The spectrum of vasculitis. Clinical pathologic, immunologic, and therapeutic considerations. Ann Intern Med 89:660-76, 1978.

347. Cines DB, Lyss AP, Bina M, Corkey R, Kefalides NA, and Friedman HM: Fc and C3 receptors induced by herpes simplex virus on cultured human endothelial cells. J Clin Invest 69:123-8, 1982.

348. Hansson GK, Bjornheden T, Bylock A, and Bondjers G: Fc-dependent binding of monocytes to areas with endothelial injury in the rabbit aorta. Exp Mol Pathol 34:264-80, 1981.

349. Sordat B, Hess MW, and Cottier H: IgG immunoglobulin in the wall

of post-capillary venules: possible relationship to lymphocytes recirculation. Immunology 20:115-8, 1971.

350. Fillit HM, Jaffe EA, and Zabriskie JB: In vitro correlates of endothelial injury and repair. Lab Invest 46:1-9, 1982.

351. Shingu M, Hashimoto Y, Johnson AR, and Hurd ER: The search for Fc receptor on the surface of human endothelial cells. Proc Soc Exp Biol Med 167:147-55, 1981.

352. Andrews BS, Shadforth M, Cunningham P, and Davis IVJS: Demonstration of a C1q receptors on human tissues and human endothelial cells in culture. J Immunol 127:1075-80, 1981.

CHAPTER X APPENDICES

Table 1. Hybridoma Antibody Induced Chromium-51 Release from Platelets in the Absence and Presence of Complement

Table 2. Reactivities of Antibodies with Defined Specificities with Platelet Proteins in Western Blotting Analysis

Table 3. Binding of Rabbit Anti-Factor VIII Antibodies to Human Endothelial Cells and Fibroblasts

Table 4. Binding of Hybridoma Antibodies to Fibrinogen by ELISA

Table 5. Cloning Efficiencies of Human Hybridoma Cells Using GM4672 Cells or ECGS as Feeder Layer

Table 6. Comparison of Cloning Efficiencies Using GM4672 Cells or GM4672 Cell Conditioned Medium as Feeder Layer

Figure 1. Spontaneous Release of chromium-51 from Platelets and Endothelial Cells

Figure 2. Time-Dependent Binding of Hybridoma Antibodies to Live Platelets

Figure 3. Western Blotting Analysis of Platelet Proteins using 9604 Hybridoma Supernatants of Cells Kept in Culture for Different Periods of Time

Figure 4. Western Blotting Analysis of Isolated Platelet Proteins and Endothelial Cell Proteins with Antibody 9604

Method 1. Preparation of Hybridoma Antibody Affinity Columns

Method 2. Detection of the Binding of Hybridoma Autoantibodies or Rabbit Anti-Factor VIII Antibodies to Live Human Endothelial Cells by ELISA

Method 3. Cloning of Hybridomas Using the GM4672 Cells as Feeder

Legend to Table 1

[a] The ^{51}Cr-release assay procedure is described in Chapter VI.

[b] Freshly reconstituted rabbit serum (GIBCO, Grand Island, NY)) was used as the complement source.

[c] Each value represents the mean of duplicate samples.

[d] The values shown in bold print represent positive reactions.

[e] ND = not done.

[f] The cut-off value, above which a result was considered positive, was calculated from the mean plus 2 SD of 4 control samples, including S.R. (spontaneous release), HIgM (human IgM), PBS-A buffer and HT medium, and was 18.3 for hybridoma supernatants and 7.2 for affinity-purified antibodies.

Objective and Conclusion:

This experiment was performed to examine whether increased hybridoma antibody-induced ^{51}Cr release from platelets was due to antibody-dependent complement-mediated cytotoxicity or to complement-independent mechanisms, such as release of platelet granule contents.

The results demonstrate that the effects of the antibodies on platelets occurred only in the presence of complement, indicating that the increased ^{51}Cr release was caused by complement-mediated cytotoxic effects of these antibodies.

Table 1. Hybridoma Antibody Induced Chromium-51 Release from Platelets in the Absence and Presence of Complement[a]

Hybridoma	^{51}Cr-Release from Platelets (%)			
	Supernatants		Purified Antibodies	
	Complement[b]		Complement	
	+	−	+	−
9200	**25.7**[c,d]	16.1	**12.1**	6.9
9604	**27.5**	14.6	**14.2**	7.2
9700	**28.9**	14.9	**11.1**	6.0
9703	**19.7**	16.3	ND[e]	ND
9705	**28.4**	15.8	5.4	6.9
9603	**20.1**	13.9	**7.9**	6.5
9500	ND	ND	**13.3**	6.2
9502	18.1	16.5	**15.9**	7.0
1200	18.3	15.9	ND	ND
B3123	16.9	14.5	7.3	6.0
B109	16.3	14.5	6.4	6.9
B110	17.1	15.4	6.7	6.7
B111	17.1	16.3	6.8	5.8
10031	ND	ND	5.9	6.1
S.R.	18.3	14.2	4.8	5.2
PBS-A	ND	ND	6.9	6.8
HT medium	16.4	15.3	ND	ND
HIgM	15.0	15.5	6.6	6.5
Mean ± 2 SD[f]	16.6 ± 1.7		6.1 ± 1.1	

Legend to Table 2

[a] Platelets were solubilized in SDS sample buffer containing 2-mercaptoethanol and run under reducing conditions in an 8-12% gradient SDS PAGE. The procedure for Western blotting was the same as that described in Chapter VI. The antibodies used in these experiments were diluted according to instructions provided with the purchased antibodies or suggestions from their laboratories of origin.

[b] Relative mobilities were calculated from a standard curve of high and low molecular weight standards (Bio Rad, Richmond, CA) run in the same gel. The letters following the mobilities represent the strength of the bands: s for strong, m for medium, and w for weak.

[c] Antibodies were kindly provided by Dr. J.-L. Senecal.

[d] Antibodies and lectins were purchased from Sigma (St. Louis, MO). The cytoskeletal proteins used as antigens to prepare these antibodies were isolated from chickens.

[e] Antibody was kindly provided by Dr. P. Bockenstedt.

[f] Antibody was purchased from Serotec (Blackthorn, England).

[g] Antibodies were kindly provided by Drs. K. Crae and D. Cines.

[h] When platelet proteins were run under non-reducing conditions, reactive bands were seen with approximate M.W. of 80,000 (s) and 120,000 (m).

Objective and Conclusion:

Western blotting analyses were performed using various antibodies of known specificities in order to identify platelet proteins with mobilities similar to those reactive with the human hybridoma autoantibodies.

The results show that most of the defined platelet protein bands were not similar to the hybridoma antibody reactive protein bands, with the exception of actin, to which many of the hybridoma antibodies bound.

Table 2. Reactivities of Antibodies with Defined Specificities with Platelet Proteins in Western Blotting Analysis[a]

Defined Specific Antibodies/Reagents	Mobilities of Reactive Bands[b]
Anti-Cytoskeletal Proteins:	
Mouse anti-actin[c] 1:1000	45,000(s)
Mouse anti-tubulin[c] 1:250	47,500(w), 55,000(m)
Mouse anti-vimentin[c] 1:150	64,000(w), 97,000(w), 120,000(m), 170,000(m), >200,000(w)
Guinea pig anti-vimentin[c] 1:250	54,500 - 64,000(m)
Mouse anti-cytokeratin[c] 1:75	120,000(w), 170,000(w)
Mouse anti-desmin[c] 1:100	No visible band
Human anti-microfilament[c] 1:40	No visible band
Rabbit anti-actin[d] 1:50	53,000 - 62,000(m)
Rabbit anti-tropomyosin[d] 1:200	31,000 - 41,500(m)
Rabbit anti-α-actinin[d] 1:500	97,000(w)
Rabbit anti-spectrin[d] 1:400	No visible band
Goat anti-filamin[d] 1:100	89,000(m)
Mouse anti-vinculin[d] 1:100	78,500(w), 86,500(s), 94,000(w), 115,000(s)
Mouse anti-myosin (Fast)[d] 1:500	No visible band
Mouse anti-myosin (L chain)[d] 1:400	No visible band
Anti-Platelet-Associated Proteins:	
Goat anti-Protein S[e] 1:1000	55,000(w), 85,000(s), 105,000(w)
Mouse anti-GPIb (MCA226)[f] 1:200	25,000(s), 120,000(m)
Mouse anti-GPIIb (B1B5)[g] 1:400	27,000(s), 28,000(s)
Mouse anti-GPIIIa (SSA-6)[g] 1:20	No visible band[h]
Wheat Germ Agglutinin[d] 1:100	No visible band
Concanavalin A[d] 1:100	80,000(s), 82,000(m)

Legend to Table 3

[a] The assay was performed on live endothelial cell monolayers and the procedure is described in Method 2 of the Appendices.

[b] Rabbit anti-Factor VIII antiserum was kindly provided by David Bell and Dr. H. Goldsmith.

[c] The procedure for preparing human umbilical EC and fibroblast monolayers in microtiter plates is described in Chapter VI. The fibroblast cell line, GM10-TS, from fetal skin was kindly provided by Dr. E. Golds.

[d] Each value represents the mean of duplicate samples.

Objective and Conclusion:

This binding assay was performed to demonstrate that ECs and not other adherent cells were coated to the plate in our EC ELISA. Factor VIII antigen is present only on ECs, platelets, and megakaryocytes.

The results demonstrate that the cells prepared from human umbilical veins and used to coat the ELISA plates in these experiments are ECs. Rabbit anti-Factor VIII antibodies bound to ECs in a dose-dependent manner, but did not bind to the fibroblast control cells. The normal rabbit serum control did not react with either of these cells.

Table 3. Binding of Rabbit Anti-Factor VIII Antibodies to Human Endothelial Cells and Fibroblasts[a]

Serum[b] Dilution	Binding to Cells (OD_{410})	
	Endothelial Cells[c]	Fibroblasts[c]
Rabbit Anti-Factor VIII		
1:20	0.21[d]	0.04
1:40	0.16	0
1:80	0.11	0
1:160	0.01	0
1:320	0.01	0
1:640	0	0
1:1280	0	0
Normal Rabbit Serum		
1:20 - 1:1280	0	0
PBS Buffer	0	0
0.5% BSA in RPMI 1640	0	0

Legend to Table 4

[a] Immulon-2 plates (Dynatech, Alexandria, Virginia) were coated with fibrinogen (5 µg/ml) in 0.05 M sodium borate buffer, pH 8.6 for 18 hours at 4°C. The assay was performed using the same procedure as that described for the platelet-binding ELISA (Chapter VI).

[b] Each value represents the mean of duplicate samples.

[c] All of the samples were hybridoma culture supernatants, except for 9604 IgM, which was affinity purified antibody.

Objective and Conclusion:

This experiment was performed to exclude the possibility that the platelet-binding activity of the hybridoma antibodies, in particular, 9604, was due to with reactivity with fibrinogen, which binds to activated platelets.

The data shows that most antibodies, including 9604, did not react with fibrinogen in a direct binding ELISA.

Table 4. Binding of Hybridoma Antibodies to Fibrinogen by ELISA[a]

Hybridoma	Binding to Fibrinogen (OD_{410})
Buffer	0.01[b]
B109	0.01
B110	0.01
B105	0.13
B122-2	0.12
4003	0.71
134	0.10
600	0.01
1104	0.03
1200	0.05
1305	0.29
1407	0.02
1420	0.04
9603	0.02
9604	0.02
9604 IgM[c]	0.02
9702	0.01
9703	0.06
9705	0.02
9706	0.03
B3123	0.02
B3700	0.02

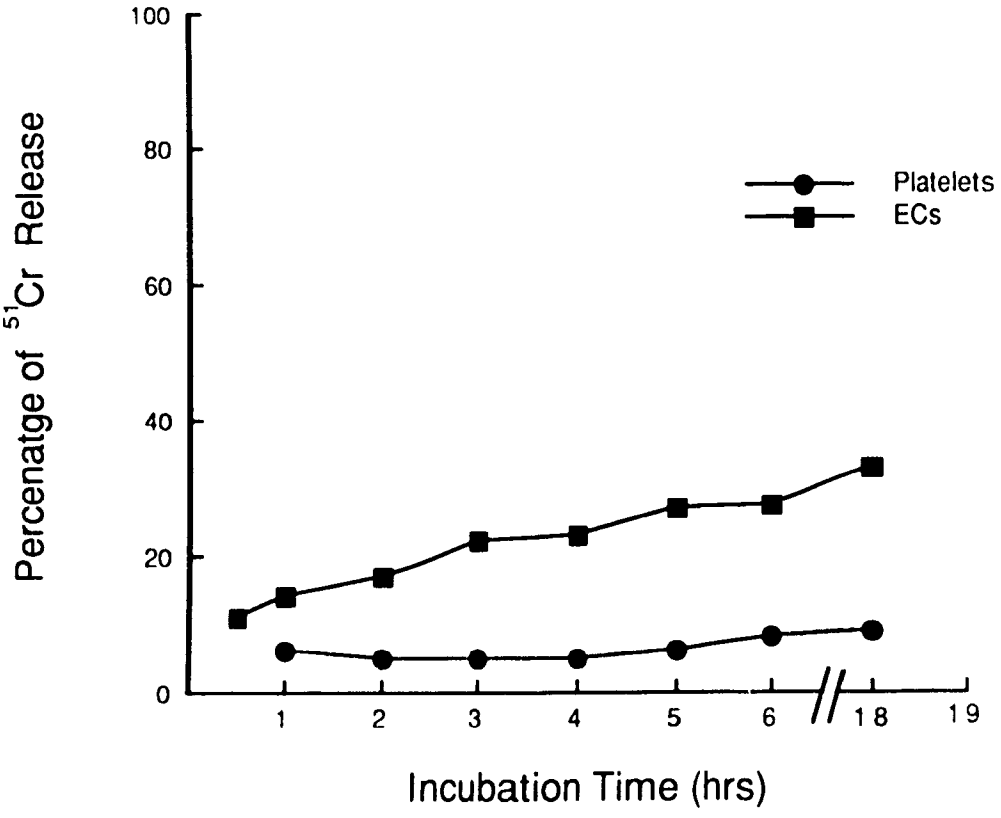


Figure 1. Spontaneous Release of Chromium-51 from Platelets and Endothelial Cells

Objective and Conclusion:

These are results of preliminary experiments done prior to testing the cytotoxicity of the hybridoma antibodies to platelets and ECs. These experiments were performed to determine the optimal incubation time for ^{51}Cr-release assays on platelets and ECs. The ^{51}Cr-release assay procedures on platelets and ECs are described in Chapters IV and VI, respectively.

The results show that the optimal incubation period was between 1 to 2 hours for both platelets and ECs, as the spontaneous ^{51}Cr-release did not significantly increase during this time. Spontaneous ^{51}Cr-release was much greater and increased more rapidly for ECs than platelets at all time points.

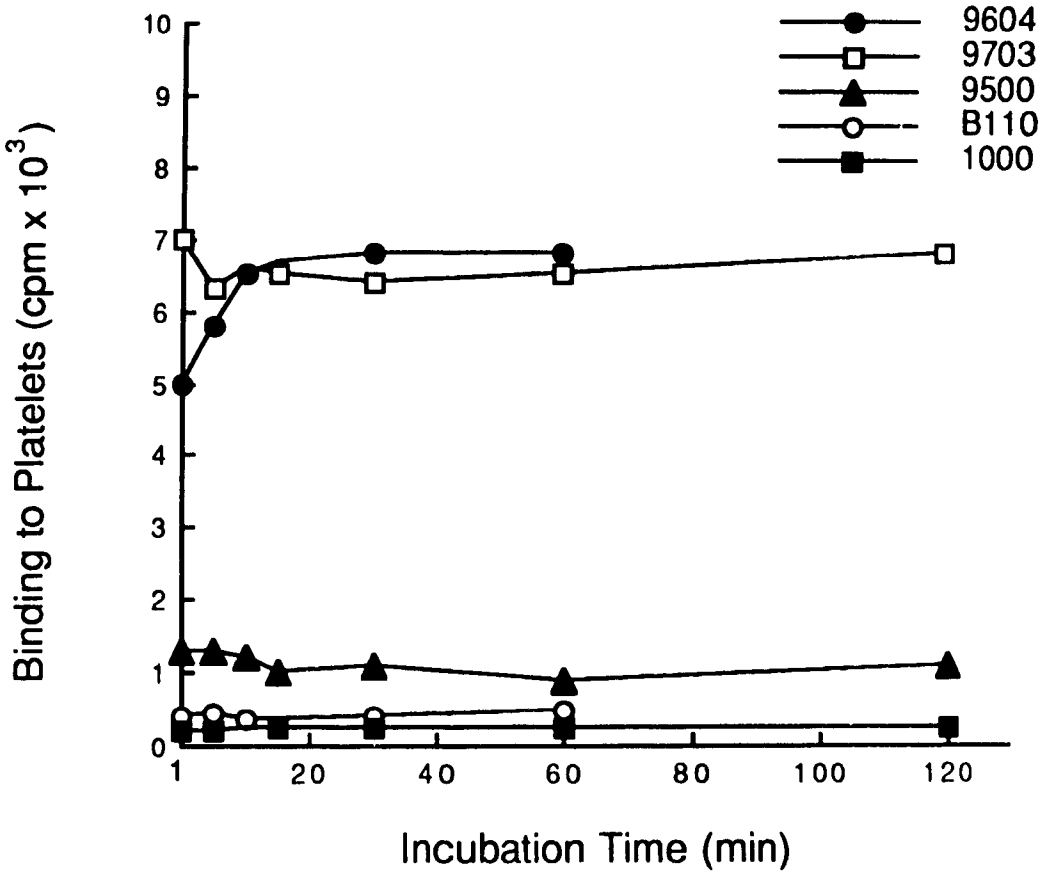


Figure 2. Time-Dependent Binding of Hybridoma Antibodies to Live Platelets

Objective and Conclusion:

The platelet-binding RIA was performed on live platelets and is described in Chapter III.

The results of this experiment demonstrate that different hybridoma antibodies showed different kinetics of binding to live platelets. Nonplatelet-binding antibodies B110 and 1000 did not bind to live platelets after any length of incubation. Platelet-binding antibody 9500 showed highest binding to live platelets between 1-15 minutes and 9703 showed highest binding during the first minute, and both showed a slight decrease thereafter, while antibody 9604 showed a time-dependent increase in binding between 1-15 minutes.

Figure 3. Western Blotting Analysis of Platelet Proteins using 9604 Hybridoma Supernatants of Cells Kept in Culture for Different Periods of Time

SDS PAGE was run under reducing conditions and the procedure for Western blotting was the same as that described in Chapter VI. Lanes 1 and 2: M.W. standards and whole platelet proteins, respectively, stained with amido black; Lanes 3 to 18: whole platelet proteins blotted with hybridoma 9604 culture supernatants harvested from cells kept in culture for different periods of time.

Objective and Conclusion:

The binding properties of 9604 supernatants harvested from hybridoma cells after being kept in culture for different periods of time were examined by Western blotting in order to attempt to explain the increased polyspecificity of some 9604 hybridoma supernatants.

The results demonstrate that the binding characteristics of the antibody secreted by hybridoma 9604 did become more polyspecific with time in culture. An aliquot of the original 9604 cells frozen in 1986, was thawed in October, 1988, and that supernatant (10/21/88) showed comparable binding properties to the early supernatants harvested in 1986.

Figure 3

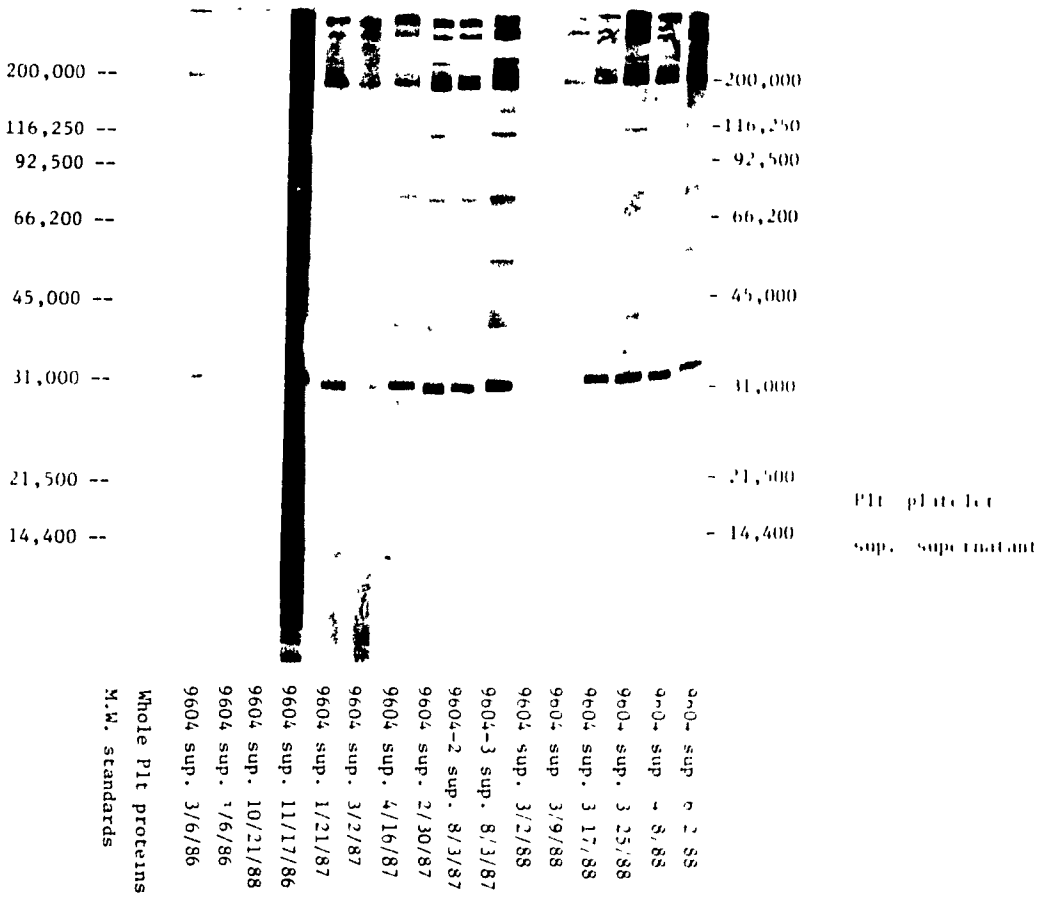

200,000 --
116,250 --
92,500 --
66,200 --
45,000 --
31,000 --
21,500 --
14,400 --
-200,000
-116,250
- 92,500
- 66,200
- 45,000
- 31,000
- 21,500
- 14,400
Plt platelet
sup. supernatant
Whole Plt proteins
M.W. standards
9604 sup. 3/6/86
9604 sup. 1/6/86
9604 sup. 10/21/88
9604 sup. 11/17/86
9604 sup. 1/21/87
9604 sup. 3/2/87
9604 sup. 4/16/87
9604 sup. 2/30/87
9604-2 sup. 8/3/87
9604-3 sup. 8/3/87
9604 sup. 3/2/88
9604 sup 3/9/88
9604 sup. 3 17/88
9604 sup. 3 25/88
9604 sup. 4 8/88

Figure 4. Western Blotting Analysis of Isolated Platelet Proteins and Endothelial Cell Proteins with Antibody 9604

Platelet proteins were isolated by an electroelution procedure which is described in Chapter V. Briefly, platelet proteins were separated by SDS PAGE. Gel bands with mobilities of M.W. 200,000, 32,000 and 31,000 (i.e. those bands with which antibody 9604 might be reactive) were cut and electroeluted. The electroeluted materials were dialyzed against PBS and concentrated. Then, the concentrated protein solutions were centrifuged at 9,990 x g and the precipitates (insoluble components) and supernatants (soluble components) were used in this experiment.

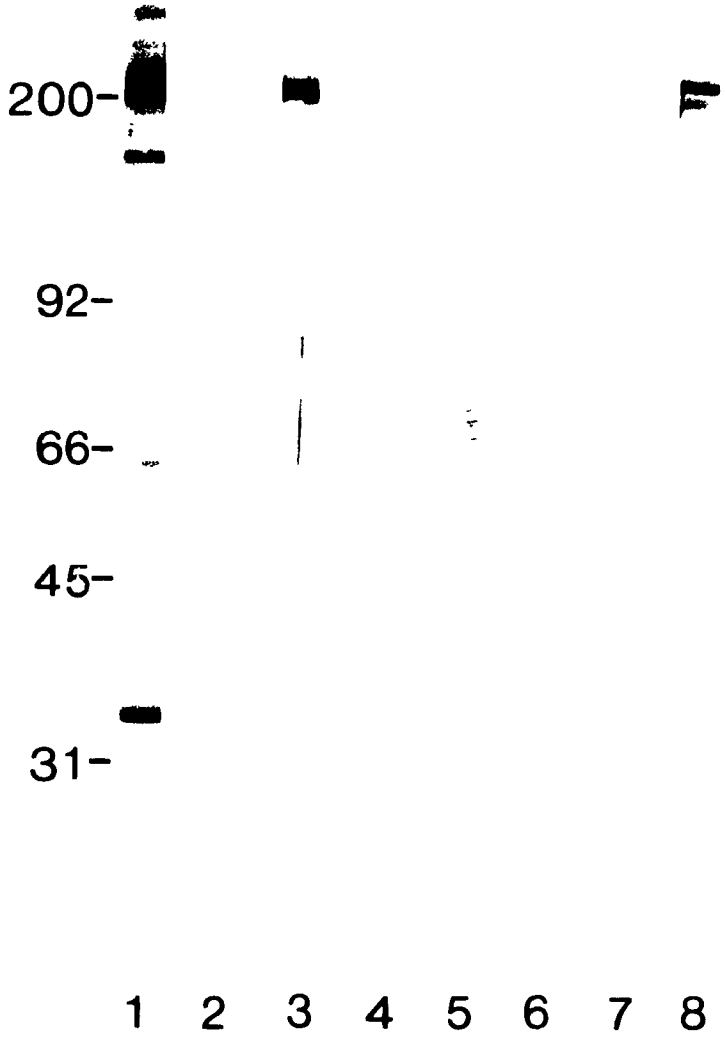
In this experiment, SDS PAGE was run under reducing conditions and Western blotting analysis was performed as in Chapter V.
Lane 1: M.W. standards stained with amido black; Lanes 2 to 7 were all blotted with antibody 9604. In lanes 2 and 3, supernatant and precipitate, respectively, of the electroeluted preparation with a mobility of approximately 200,000 M.W.; Lanes 4 and 5: supernatant and precipitate, respectively, of the electroeluted preparation with a mobility of approximately 32,000 M.W.; Lane 6 and 7: supernatant and precipitate, respectively, of the electroeluted preparation with a mobility of approximately 31,000 M.W.; Lane 8: whole EC proteins blotted with antibody 9604.

Objective and Conclusion:

The identification and isolation of 9604-reactive platelet proteins was done to further characterize the biological function of these proteins and the precise effect of antibody 9604 on platelets.

The data obtained here confirm that these 9604-reactive platelet components were successfully isolated and retained their reactivity after electroelution. The 9604-reactive polypeptide of 200,000 M.W. is present in the precipitated fraction (insoluble in PBS), and the polypeptide of 32,000 M.W. in the supernatant (soluble in PBS). No reactivity was seen with the 31,000 M.W. band.

Figure 4



Method 1. Preparation of Hybridoma Antibody Affinity Columns

Affinity columns were prepared for the isolation of platelet proteins reactive with hybridoma autoantibodies.

1) Human hybridoma IgM, purified on an affinity column of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) conjugated to rabbit anti-human IgM, was dialyzed against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).

2) CNBr-activated Sepharose 4B gel was swollen in 0.001 M HCl and then washed with distilled water and coupling buffer.

3) The gel was mixed with hybridoma IgM (5 mg protein per ml of gel) in coupling buffer in a 50 ml plastic tube and incubated on an end-over-end rotator for 2 hours at room temperature.

4) After centrifugation for 10 minutes at 1500 x g, the supernatant was removed and the residual protein in the supernatant was determined by OD_{280}.

5) The gel was then incubated with 1 M glycine, pH 8.0 on an end-over-end rotator for 15 hours at 4°C.

6) The gel was then washed twice with 0.01 M PBS, pH 7.3 by centrifugation at 1,500 x g, packed into a column, and washed with PBS until the OD_{280} of the buffer wash was zero.

7) The column was eluted with 0.1 M glycine-HCl, pH 2.3 and then washed with PBS until the OD_{280} of the effluent was zero.

8) The elution and wash steps were repeated once and the column was kept at 4°C until needed.

Method 2. Detection of the Binding of Hybridoma Autoantibodies or Rabbit Anti-Factor VIII Antibodies to Live Human Endothelial Cells by ELISA

1) EC monolayers were grown in microtiter plates as described in Chapter VI and cultured human fetal fibroblast monolayers were grown under similar conditions as the ECs.

2) The cell monolayers were gently washed 3 times with 3% BSA in RPMI 1640 medium (BSA-RPMI).

3) The cells were incubated with 0.5% BSA in RPMI 1640 medium for 1 hour at 37°C and the medium was discarded.

4) The cells were then incubated with 75 µl/well of hybridoma antibody to be tested or rabbit anti-Factor VIII antiserum (diluted serially in EC medium) for 2 hours at 37°C.

5) The cell monolayers were washed 5 times with BSA-RPMI.

6) The cells were then incubated with alkaline phosphatase-conjugated goat anti-human Ig or goat anti-rabbit Ig antibody diluted in EC medium (100 µl/well) for 90 minutes at 37°C.

7) The cell monolayers were washed 5 times with BSA-RPMI.

8) One hundred microliters of substrate solution (1 mg/ml p-nitrophenyl phosphate disodium in 0.05 M $NaCO_3$ buffer, pH 9.5) was added to each well and incubated for 30 minutes at room temperature and the plates were read at OD_{410} using a Dynatech MR600 ELISA reader.

Method 3. Cloning of Hybridomas Using the GM4672 Cells as Feeder

Feeder Cells

The GM4672 human lymphoblastoid cell line, obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, New Jersey), was used as feeder. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum.

GM4672 Cell Conditioned Medium

The supernatants of GM4672 cells were harvested at log growth phase (4-8 x 10^5 cells/ml) and used as cell conditioned medium. The supernatant was filtered through a 0.45um Millipore filter unit (Nalge, Rochester, New York) to remove cells and cell debris, kept at 4°C and used undiluted within two weeks.

Endothelial Cell Growth Supplement (ECGS)

ECGS was purchased from Collaborative Research Inc. (Lexington, Massachusetts). The lyophilized ECGS powder was dissolved in HAT medium at a concentration of 5mg/ml, frozen in 0.5ml aliquots at -20°C, and thawed only once. The ECGS solution was added to the medium at 100, 125 or 150ug/ml immediately prior to use.

Cloning of Hybridoma Cells

The human-human hybridoma cells, obtained from fusions of GM4672 cells and human peripheral blood lymphocytes from 5 patients with systemic lupus erythematosus and 2 normal individuals as previously described (22), were diluted in HAT medium and seeded at 1, 2 or 5 cells per well in microtiter wells (Costar, Cambridge, MA) containing 5 x 10^3 GM4672 cells in 0.1ml of HAT medium, 0.1 ml of GM4672 cell conditioned medium, 0.1ml of ECGS at a final concentration of 100,

125 or 150ug/ml or 0.1ml of HAT medium as control. The cells were incubated at 37°C in 5% CO_2 and fed once a week with HAT medium, HAT medium with GM4672 conditioned medium, or HAT medium with ECGS, depending on the feeder layer used. In most instances, growth of clones was apparent at 2 to 3 weeks, but plates were kept for 4-5 weeks.

Table 5. Cloning Efficiencies of Human Hybridoma Cells Using GM4672 Cells or ECGS as Feeder Layer

Hybridoma Cells	Feeder	Cell Number Per Well	Subclone Number Per 96 Wells	Cloning Efficiency(%)
9700	GM4672[a]	5	26	27.1
		1	11	11.5
	ECGS[b]	5	14	14.6
		1	3	3.1
B122	GM4672	5	3	3.1
		1	0	0
	ECGS[b]	5	3	3.1
		1	0	0
9603	GM4672	2	39	40.6
	ECGS[c]	2	0	0
1420	GM4672	2	11	11.5
	ECGS[c]	2	0	0
9502	GM4672	2	2	2.0
	ECGS[c]	2	0	0
9706	GM4672	2	1	1.0
	ECGS[c]	2	0	0

[a] GM4672 cells at 5×10^4/ml.
[b] ECGS at 100ug/ml.
[c] ECGS at 125ug/ml.

Table 6. Comparison of Cloning Efficiencies Using GM4672 Cells or GM4672 Cell Conditioned Medium as Feeder Layer

Hybridoma Cells	Feeder	Cell Number Per Well	Subclone Number Per 48 Wells	Cloning Efficiency(%)
9703	GM4672[a]	5	12	25.0
		2	6	12.5
	GCM[b]	5	0	0
		2	0	0
9700	GM4672	5	21	43.8
		2	16	33.3
	GCM	5	0	0
		2	0	0
9703-5	GM4672	5	8	16.7
		2	5	10.4
	GCM	5	0	0
		2	0	0
	medium	5	0	0
		2	0	0
4003	GM4672	5	11	22.9
		2	9	18.8
	GCM	5	0	0
		2	0	0
	medium	5	0	0
		2	0	0
9604	GM4672	5	21	43.8
		2	12	25.0
	GCM	5	0	0
		2	0	0
	medium	5	0	0
		2	0	0

[a] GM4672 cells at $5x10^4$/ml.
[b] GCM = GM4672 cell conditioned medium at 100ul/well.