SHORT TITLE: THE INTRAMOLECULAR HETEROGENEITY OF CEA

IMMUNOCHEMICAL STUDIES OF THE INTRAMOLECULAR HETEROGENEITY OF THE CARCINOEMBRYONIC ANTIGEN (CEA) OF THE HUMAN DIGESTIVE SYSTEM

by

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ABSTRACT

The experimental findings reported here indicate that the Carcinoembryonic Antigen (CEA) of the human digestive system is heterogeneous with respect to the antigenic determinants which it contains. These determinants are: (1) a tumor-specific antigenic grouping recognized by heterologous antisera which is shown to cross-react with a molecular moiety present in preparations of human ovarian cyst-derived A, B, H and Le^a substances; (2) a tumor-specific site recognized by IgM antibodies in the sera of some digestive cancer patients and some pregnant women. It is not known if this site is identical with the site defined above by heterologous antisera; (3) a site recognized by human anti-blood group A antibodies and designated the "A-like" site.

Hapten-inhibition studies in radioimmunoassay systems for blood group A and CEA were performed to define the A-like site. These studies showed that N-acetyl-D-galactosamine specifically inhibited the binding of anti-A to ^{125}I-labeled CEA. Furthermore, a glycopeptide (MW 4000 Daltons), obtained by the enzymatic degradation of CEA possessed both the tumor-specific and the A-like sites despite a lack of measurable N-acetyl-D-galactosamine in the preparation.

RESUME

Les résultats expérimentaux de ce rapport nous indiquent que l'Antigène Carcino-embryonnaire du système digestif humain (ACE) est hétérogène quant aux déterminants antigéniques qu'il contient. Ces determinants sont: (1) un groupement antigénique, spécifique de tumeur, reconnu par des anticorps hétérologues. Ces études ont démontré que ce groupement présente une réactivité croisée avec un déterminant antigénique présent dans les préparations des substances de groupe sanguin A, B, H et Le^a, obtenues à partir de kystes ovariens humains, (2) un site spécifique de tumeur, reconnu par des anticorps IgM dans les sérums de patients avec cancer du système digestif et de femmes enceintes. On ne sait pas si ce site est identique à celui mentionné ci-haut qui est reconnu par des anticorps hétérologues, (3) un site reconnu par les anticorps du groupe sanguin A de l'humain et désigné sous le nom de "A-like".

Des études d'inhibition par haptènes des systèmes d'essais radioimmunologiques pour le groupe sanguin A et ACE ont été faits pour définir le site "A-like". Ces études ont démontré que la liaison du anti-A au ^{125}I-CEA est spécifiquement inhibée par le N-acetyl-D-galactosamine. De plus, un glycopeptide (P.M. 4000 Daltons) obtenu par la dégradation enzymatique du ACE, possède et un site spécifique de tumeur et un site "A-like" en dépit de l'absence de N-acetyl-D-galactosamine mesurable dans la preparation.

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CHAPTER I

INTRODUCTION

The established importance of cancer as a cause of human mortality has stimulated the development of a vast field of Cancer Research or Oncology. Although the techniques and concepts employed in attempts to gain a better understanding of malignant tumor transformation have come from almost every branch of the biological and biophysical sciences, the rapidly changing discipline of Immunology has, in recent years, served as a ready source of new ideas for application to the problems of Oncology.

The application of immunologic technology to the problems under consideration has, in some instances, resulted in the description of qualitative differences in the composition of cancerous and embryonic tissues on the one hand and normal adult tissues on the other.

The components responsible for the observed differences have been described by a variety of different terms. One such antigenic system, identified in man involves the Carcinoembryonic Antigen (CEA) of the human digestive system.

The present thesis represents a further attempt to utilize immunological techniques to study the carcinoembryonic antigen of the human digestive system and its relation to the biological mechanisms associated with the development of cancer in the human host.

The Identification of Tumor-Specific Antigens in Animals

In 1953, Foley produced the first clear demonstration of tumor-specific antigenicity in a class of experimental animal tumors (1). These studies revealed that transplantable, methylcholanthrene-induced sarcomas were antigenic in a strain of highly inbred, or syngeneic, mice. Tumor recipients subjected to a second tumor isograft after the initial lesion had been made necrotic by ligation demonstrated an immune resistance to tumor growth.

In the years since Foley's experiments, the search for tumor-specific antigens in animal tumors have utilized syngeneic rodent strains almost exclusively. By definition, all of the nucleated body cells of such animals contain identical genetic histocompatability loci and express the same transplantation antigens. Host rejection of a tumor of syngeneic donor orgin must, then, be due to the development of new, tumor-specific transplantation antigens (TSTA) within the tumor tissue. Furthermore, certain other neoantigens which are not directly involved in transplantation immunity may be detected by examining the host's serum for circulating antibodies by immunological techniques other than graft rejection.

Utilizing this type of experimental design, it has been possible to demonstrate the existence of TSTA in a wide variety of murine tumors induced either by oncogenic viruses or by chemical carcinogens (2,3).

The work dealing with experimental animal tumors has been extensively reviewed (4,5,6,7). Only the most pertinent findings will be summarized here.

Tumor-Inducing Agents

Murine tumors may be induced by oncogenic viruses of either the DNA or RNA type (2,3,8) or by a variety of chemical carcinogens (1,9,10). Evidence has accumulated that these mutagens are also able to modify the immunologic status of the host. The resulting condition appears to obviate the immune response which could either inactivate the inducing agent or cause a rejection of the TSTA-bearing transformed cells (11).

Vertically transmitted oncogenic RNA viruses, such as the murine leukemia and mammary tumor agents, produce a full and specific immunologic tolerance in their natural host. The immunologic unresponsiveness is presumably associated with the perinatal exposure of the host to the agent. Certain of the oncogenic DNA viruses and some of the chemical carcinogens are capable of producing either non-specific immunologic depression or immunologic inertia. The latter state is characterized by the absence of either tolerance or sensitization to the TSTA.

Under some circumstances the presence of circulating tumor-specific antibodies may accelerate both the rate of tumor enlargement and the demise of the host. The mechanism underlying this phenomenon, termed immunologic enhancement, remains uncertain (12,13). It has, however, been proposed that enhancing antibodies may act, either centrally or peripherally, to inhibit a cytolytic cell-mediated immune response against the tumor. Furthermore, humoral anti-tumor antibodies may result in antigenic modulation, manifested as a reversible loss of certain of the antigenic specificities expressed by the tumor cells (14).

Characteristics and Range of Reactivity of the TSTA

The TSTA are constituents of tumor cell membranes, and behave very much like weak transplantation antigens (11). Little is known of the chemical nature of these components. If their similarity to normal transplantation antigens may be extrapolated, they are probably lipid-protein-polysaccharide complexes in which the protein moiety likely serves as the determinant grouping (15). Their chemical structure notwithstanding, the TSTA are capable of stimulating both humoral and cell-mediated responses on the part of the tumor host (11,14). The humoral antibodies might take part in tumor enhancement as already described. Host protection against tumor growth is, however, served primarily by the cell-mediated immune mechanism (16).

Tumors induced by the same virus in different animals, and even in different strains and species of animals, demonstrate virtually universal cross-reactive tumor-specific antigenicity (11). Preliminary observations (18,20) have suggested that at least some of these antigens are fetal in origin and are immunologically similar in fetal cells of widely different species (e.g. mouse, hamster and man). These observations have been utilized to support the view that adenovirus 31 and SV-40 viral oncogenesis involves the retrogenic activation of early fetal antigen genes in adult cells. The observation of the similarity of such antigens across wide species barriers also appears to support the concept that ontogeny repeats phylogeny at the molecular level. On this premise, fetal proteins from different vertebrates should resemble each other

more closely than do the corresponding proteins of the adult. These implications will probably be clarified in the near future.

Huebner et al have proposed another hypothesis to account for the observed sharing of antigens by embryonic and tumor cells; i.e. the group-specific antigen(s) of C-type RNA virus in embryonic and tumor (RNA viral and chemical-carcinogen induced) tissues of feral mice (21). They postulate that the cells of all vertebrates possess vertically transmitted (inherited) RNA-virus information that serves as an endogenous source (oncogenes) of oncogenic activity. Tumorigenesis depends upon the presence of specific host regulatory genes which favor oncogene expression.

Although the description of reverse transcriptase (RNA-dependant DNA polymerase) activity in a variety of tumor tissues suggests the possibility of chromosomal incorporation of the viral genomes, as in lysogeny, the absence of precise (immunochemical) data on the intra- and inter-species antigen(s) involved indicates the speculative nature of this theory.

Recently, Baldwin et al studied the reactivity of sera and lymph node cells from multiparous rats by a variety of in vitro techniques (22). They found common, embryonic antigens on the surface of carcinogen-induced rat hepatoma and sarcoma cells. Further studies of these antigens, and their possible relationships to the virally induced TSTA, may yield further insights into the mechanisms of tumor development.

In contrast to the common TSTA of virally-induced tumors described above, each tumor induced by a single carcinogen, even in different sites of the same animal, contains a different TSTA which

is specific for the individual tumor (9,10,17). The relationship of these individual tumor-specific transplantation antigens to the fetal components described is presently unknown.

Host Resistance to Tumor Growth

It has already been noted that the TSTA of virus-induced and carcinogen-induced tumors have been demonstrated by studies involving rejection of transplantable tumors between syngeneic hosts. Nevertheless, the same tumors in comparable animals may lead to the death of the tumor-bearing individuals. The ultimate result of the host-tumor interaction is completely dependent upon the experimental design.

The development of a state of protective immunity against a specific type of tumor can be achieved by any of the following procedures:

1. Ligation or surgical removal of the initial tumor after it has reached a certain minimal size, but before it has spread.

2. Injection of the host with cells that have been rendered incapable of growth and division such as by exposure to x-irradiation or ultraviolet light, but that otherwise maintain expressive antigenic and certain other biologic functions.

3. Injection of the host with a dose of malignant cells too small to result in a tumor.

Subsequent challenge of such an animal with an adequate tumor graft of the same origin as the cells employed in pretreatment,

reveals an immunologic resistance to tumor growth. At present almost all of the available evidence indicates that the type of immunity primarily involved in tumor rejection is that mediated by immunologically competent cells of the lymphoid series, rather than by humoral antibodies (16). This situation is, therefore, entirely comparable to that seen during normal allograft rejection (23).

When an animal is initially faced with a large mass of viable transformed cells, tumor growth is almost invariably established. Since they are only weakly immunogenic, the TSTA evoke a correspondingly weak immune reaction. The emerging response, which seems able to deal adequately with a small number of tumor cells, develops too slowly to eliminate the larger quantity of malignant tissue. Consequently, in order to achieve a state of protection against cancer growth, the immunologic system must be given a "head start" on the expanding tumor mass. Failing this, a fatal outcome is assured.

Tumor-Specific Antigens in Human Cancers

Even with moral and ethical considerations aside, the syngeneic donor-host relationship employed in the antigenic analysis of animal tumors is virtually precluded in the study of human cancers. The alternative experimental approaches utilized in human cancer immunology have been reviewed by Southam (24). The most extensively employed procedure has centered on the immunization of xenogeneic animals with preparations of human cancer tissue in order to stimulate the production of anti-tumor antisera. Unfortunately, such studies have often been difficult to interpret for

the following reasons:

1. All human tumor extracts contain large quantities of normal tissue "contaminants" from both the cellular and subcellular levels. Heteroimmunization with preparations of this type invariably results in a predominately anti-normal tissue antibody response on the part of the immunized animal.

2. The use of "normal control tissue", taken from non-cancerous patients for comparative studies, has given rise to serious problems of alloantigenic differences between the donors of the normal and tumor tissue material. It has, therefore, frequently been impossible to determine whether certain of the antigens considered unique to a particular cancer were truly tumor-specific, or simply individual-specific components.

In recent years, more carefully designed studies have resulted in well-founded demonstrations of tumor-specific antigens in a few human cancers. Regardless of its clinical prevalence, each of the tumors in this group is of importance in revealing a particular aspect of human cancer immunology.

Human Allogeneic Tumors

At least two clinical situations exist in which cells of a human cancer contain genes, and hence gene products, which are foreign to the host. The continued growth of such obviously immunogenic tumors is most reminiscent of the situation observed with transplanted, allogeneic experimental animal tumors which cross histocompatability barriers and also contain TSTA.

Human Tumor Allografts: Although small quantities of cancerous tissue have been transferred between patients for diagnostic (24), therapeutic (25), or investigative (24) reasons, the most significant allografts have occurred accidentally in association with kidney transplants (26). In these cases the donor has invariably been a cadaver whose death was occasioned by the cancerous condition.

The interaction of the foreign, allogeneic malignant tissue and the immune defence mechanisms of the host are perhaps best illustrated by a comparison of two cases in which the tumor tissue transplanted with each kidney was derived from a primary bronchogenic carcinoma (27,28).

In both instances, the recipients developed a metastatic cancer from the renal source several months after successful kidney transplantation. Both patients had also received immunosuppressive therapy in order to prevent rejection of the renal graft.

In one patient, metastatic disease was widespread when discovered and death ensued five months after the graft. In the other patient, cessation of immunosuppressive therapy resulted in rejection of the kidney, but not of the tumor tissue. When the rejected kidney, which contained a large mass of tumor tissue was removed, the residual metastases gradually disappeared. Furthermore, there was no evidence of tumor reappearance when a second successful kidney transplant was performed nine months later, even though the patient was placed on a full program of immunosuppressive therapy. Thus, complete destruction of the allografted cancer tissue would appear to have been accomplished by the host's immune system during

a relatively brief period of normal function, but only after the bulk of tumor tissue had been reduced.

Choriocarcinoma: A comparable situation to that which exists in the case of accidental tumor transplantation occurs in women who develop choriocarcinomas. Choriocarcinoma is a malignant growth arising from trophoblastic elements of the placenta during a molar pregnancy, and very occasionally following a normal pregnancy. The tumor proliferates, invades the uterine wall and undergoes dissemination to remote organs. The interval between the pregnancy and the development of clinical evidence of choriocarcinoma is usually about four to six months, but may vary from a few days to some years.

Because of its derivation, choriocarcinoma contains components whose synthesis are directed by gene loci of both maternal and paternal origin. The presence of paternal gene products, including transplantation antigens, renders the tumor an allograft. Since choriocarcinomas are associated with a high mortality, this form of malignant tumor provides an excellent example of an autochthonous human tumor which contains transplantation antigens foreign to the host, but which is not necessarily rejected. Nevertheless, a number of regressions of metastatic choriocarcinomas have been reported following hysterectomy and the removal of a large tumor mass (16,29). Moreover, choriocarcinomas are very sensitive to chemotherapeutic agents, and appropriate chemotherapy has resulted in a five year survival rate of well over fifty per cent. It seems likely that these

two observations are interdependent, in that both primary tumor removal and chemotherapy alter the tumor-host relationship in such a fashion as to favor the host. When this occurs, an effective immune response may promote appropriate tumor allograft rejection.

The very delicate balance which exists between tumor growth and the immune defense mechanism of the host is illustrated by a reported case of metastatic choriocarcinoma which had failed to regress while the patient was receiving chemotherapy (30). Nevertheless, apparent cure was achieved when the patient was immunized with her husband's leukocytes and was given antiserum prepared in rabbits against her husband's seminal fluid.

Possible Virus Directed Tumor-Specificity in Human Cancers

Burkitt's Tumor: In 1958 Burkitt described a tumor syndrome in African children which has subsequently been observed, but much more sporadically, in other parts of the world (31). Although frequently a widespread disease which runs its fatal course in a few months, the tumor manifests a peculiar predilection for growth in the jaw and facial bones. Burkitt's tumor has been classified as a malignant lymphoma and has aroused keen interest amongst chemotherapists because of its relative sensitivity to alkylating agents (32). Appropriate treatment has induced long remissions in up to 20 per cent of cases and a number of such patients are said to have been cured (33).

The epidemiologic aspects of this condition have recently been summarized by MacMahon (34). Briefly, the evidence indicates that the etiology of Burkitt's tumor is intimately related to an arthropod-borne infectious agent. The infection itself is probably a common one, of which the tumor is an uncommon manifestation. An intensive search has been made for the suspected infectious material. A number of ubiquitous agents, including reovirus, type 3, herpes simplex virus and mycoplasma, have been isolated on several occasions from biopsies of Burkitt's tumors (35).

The most pertinent information has, however, come from electron microscopic examination of the steadily increasing number of continuous cell cultures of Burkitt's tumor origin. Such studies have revealed that a small proportion of cells of most lines harbor virus particles indistinguishable from members of the herpes group of viruses (35). For convenience, this agent is at present designated as the EB (Epstein-Barr) virus after the EB-1 line of Burkitt's cells in which it was first observed. Great controversy exists regarding the relationship between Burkitt's tumor, so-called conventional childhood lymphosarcoma, and acute leukemia in children. If the EB virus could be established as causative in Burkitt's tumor, it would not only serve as the first evidence of a human oncogenic virus, but would also bolster the search for similar etiologic agents in other human lymphoreticular malignancies. This is particularly pertinent since herpes-like virus particles have been observed in a number of continuous suspension cultures of blast-type cells derived from lymph nodes, bone marrows or buffy coats

of patients with leukemia and other malignancies (36). Similar particles have, however, also been observed in cells cultured from patients with apparently non-malignant diseases, such as infectious mononucleosis (37), as well as from peripheral leukocytes of healthy donors (38).

Hence, it remains uncertain whether the EB virus is the etiologic agent in Burkitt's tumor, a passenger within the tumor tissue, or merely a common contaminant of long-term leukocyte cultures.

On the basis of the information that has been obtained concerning the immunologic manifestations of virus-induced tumors in animals, similar data have been sought in Burkitt's tumor as potential evidence for a viral etiology of the condition. The results of the most concerted efforts along these lines indicate that the plasma membrane of the Burkitt's cell may well contain tumor-specific antigenic constituents capable of inducing a corresponding antibody response on the part of the host (39,40). Furthermore, the sera from a significant number of patients demonstrate cross-reactions with lines of Burkitt's cells other than their own, suggesting the presence of the same tumor-specific antigen(s) in tumors of different individuals (39,40). These observations are, therefore, analogous to those which have been made with virus-induced tumors. The results, however, have not always been clearcut and, at present, cautious interpretation is necessary.

Tumor Immunity as an Indicator of Tumor-Specific Antigenicity

The demonstration that a tumor-bearing patient is able to mount an immune response against his cancerous growth is highly suggestive of tumor-specific antigenicity. Tumor immunity may be manifested by the development of humoral anti-tumor antibodies, by cell-mediated immune reactivity, or both. Such findings have recently been made in a number of human cancer systems.

Malignant Melanoma: Indirect evidence of an immune reaction in patients with malignant melanoma has come from a number of sources. This tumor is one which occasionally undergoes spontaneous regression and which may remain localized for long periods before becoming disseminated (29,41). Moreover, even in patients with widespread disease, some metastatic deposits may regress at the same time as new ones appear and others continue to grow (42).

A number of studies, employing such techniques as immunofluorescence and cytotoxicity, have revealed that the sera of patients with melanoma often contain antibodies reactive against their own tumor cells and which may demonstrate cross-reactivity with melanoma cell preparations from other patients with the same condition (43,45). In a report of 103 melanoma patients, Lewis et al found that over one-third of all the sera studied had antibodies to autologous melanoma cells (46). The melanoma autoantibodies were virtually confined to patients in whom the disease had not undergone metastatic dissemination. In over 80 per cent of

patients with whom serial studies were done over a relatively prolonged period of time, the autoantibodies disappeared as the disease progressed to become widespread. The reason for the observed disappearance of the autoantibodies is, presumably, not well understood (47).

The anti-tumor antibodies recognized were of two types. One was active against an antigen(s) of the cell surface membrane, was entirely specific for the autologous tumor and possessed cytotoxic activity. The other antibody constituent reacted with cytoplasmic antigens present in most melanoma cell lines, and would appear to account for the cross-reactivity of sera from different patients with malignant melanoma. It is of interest that the melanoma cells possess both individual tumor-specific antigens and common, cross-reacting tumor antigens. This situation appears to resemble that described for some experimental, chemical carcinogen-induced tumors which possess both individual, tumor-specific transplantation antigens as well as common, embryonic antigens (9,10,17,22). The significance of this apparent similarity, if any, is unknown.

Osteosarcoma: Morton and Malmgren, employing an immunofluorescence technique demonstrated the presence of antibodies against a common antigen present in human osteosarcomas in the sera of 9 patients with this condition (48). Subsequent studies utilizing a microcytotoxicity test demonstrated a complement-dependent cytotoxic antibody against a common sarcoma cell surface antigen (49). Identical antibodies were found in

serums of 70 per cent of sarcoma patients, 58 per cent of their family members and close associates and 8 per cent of randomly selected specimens from the blood bank.

The demonstration of osteosarcoma-reactive antibodies amongst family members suggests a genetic predisposition to the development of this malignant tumor. The presence of positive sera in a large number of close contacts of sarcoma patients favors an infectious etiology for this condition. Although other studies in the osteogenic sarcoma system as well as those in other forms of sarcomatous disease, seem to support the presence of a viral agent, no definite conclusions can be based on the available data (50).

Colony-Inhibition: Employing the tests of tumor cell colony-inhibition and cytotoxicity, the Hellströms (51) have demonstrated that the great majority of patients suffering from a wide variety of solid tumors are capable of mounting a cell-mediated immune response against their own tumors. In addition, the sensitized lymphocytes are able to react against the cancer cells of other patients suffering from the same type of tumor (51). Such data, again, suggest the presence of common tumor-specific antigens in human tumors of the same origin.

The data derived from the colony-inhibition and cytotoxicity techniques also indicate that the same antigenic sites which are capable of evoking a cell-mediated immune response on

the part of the tumor-bearing host are also able to stimulate the production of "blocking" antibodies of the humoral type (52) which are protective to the tumor cell and may well be responsible for the experimentally observed phenomenon of immunologic enhancement of tumor growth (53). Correlative studies between the clinical condition and serologic status of cancer patients suggest that blocking antibodies may abrogate the tumor destructive effects of the cell-mediated immune response in vivo. Preliminary studies of the so-called "blocking antibodies" in a murine sarcoma system, have yielded suggestive evidence that such moieties might be antigen-antibody complexes (54).

Recent studies (55) have suggested the existence of an "unblocking factor" capable of inhibiting the effect of the "blocking antibody". This "factor" was present in the sera of tumor patients who had become clinically tumor-free, and could abrogate the blocking effect of sera from patients bearing growing tumors of the respective type. These data suggest that the clinical status of the tumor-bearing host is the result of a critical balance of the three or more immunologic constituents measured in vitro. At present, however, it is impossible to know if they are the cause or merely the result of, as yet, undefined processes.

Antigenic Reversion in Human Cancers

Alpha$_1$-Fetoprotein (AFP)

Since 1944 (56) a variety of "fetoproteins" have been described in mammals, including humans (57). These species-specific constituents are distinguished by the fact that they are present in

fetal serum but absent from the circulation of corresponding adult animals. In virtually all of its physicochemical parameters, the AFP of any given species is very similar to the albumin of that species.

In 1963 it was reported that certain chemically-induced mouse hepatomas synthesized an alpha-globulin lacking in normal adult mouse organs, but which was antigenically identical to that found in embryonic and neonatal mouse serum (58). Just two years later, human $alpha_1$-fetoprotein was detected in the sera of patients harboring primary hepatomas (59). This observation has been repeatedly confirmed since that time (60,61).

Employing the classical methods of immunoprecipitation with a maximum sensitivity of 10 µg/ml, the frequency with which AFP reappears in the sera of patients with primary hepatomas has been reported to vary between 50 to 80 per cent. The incidence of positive reactivity is greater in Africa than in Europe, but varies a great deal between different parts of Africa (62).

The recent elegant purification of AFP by Nishi (63) has facilitated the development of a variety of radioimmunoassay techniques for AFP (64-66). These highly sensitive techniques (20 ng/ml) have already proven extremely useful and are capable of detecting virtually all hepatomas and testicular teratoblastomas (66). Small, but significant, elevations of AFP have also been measured in other malignancies, for example, some cases of bronchogenic carcinoma, cancer of the stomach, Hodgkin's disease, and leukemia. Positive results have been forthcoming in cases of viral

hepatitis, in a variety of non-malignant hepatobiliary disorders and inflammatory gastrointestinal diseases. It is clear that longitudinal studies are needed in order to evaluate the ultimate role of AFP in Clinical Medicine.

The presence of low levels of AFP in normal adults has been suggested (65) but these studies await confirmation. In addition, the role of the antigenicity of AFP in the autologous host remains completely unknown. Of particular interest is the fact that AFP represents one of the first recognized examples of antigenic reversion in human cancer.

Alpha 2H Ferroprotein in Sera of Children with Tumors

The alpha 2H-globulin is another protein of hepatic origin which is normally present exclusively in human fetal organs and serum. It is not found in the sera of normal children beyond the age of 2 months. Nevertheless, this ferritin-like protein has recently been detected, by the technique of radioimmunodiffusion, in the sera of children suffering from a variety of cancerous conditions. Among the tumors which have been described in association with the elaboration of this material are nephroblastoma, neuroblastoma, teratoma, hepatoma, lymphosarcoma, reticulum cell sarcoma, osteogenic sarcoma and cerebral tumors. In all, 81 per cent of the sera of 334 children with malignant tumors gave positive results. On the other hand, only 8 per cent of 122 sera from children with non-cancerous diseases were positive (67).

Placental Alkaline Phosphatase

An alkaline phosphatase isoenzyme, normally absent from adult human tissues, has been identified in the sera of 4 to 5 per cent of patients with various malignant tumors (68). The isoenzyme, which has been named for the patient in whom it was first detected (Regan), is biochemically and immunologically indistinguishable from placental alkaline phosphatase. This substance appears to be a product of the tumor itself, and has been extracted from human cancer tissue. In those cases where the isoenzyme has been found, it has proved clinically useful in monitoring the progression or regression of tumor growth. Since the enzyme continues to be elaborated from the seedings of serosal surfaces, it may serve in identifying malignant effusion. Moreover, the detection of an unexplained, but persistently elevated level of serum alkaline phosphatase in any patient, should stimulate a search for an occult, cancerous lesion.

Fetal Sulfoglycoprotein Antigen (FSA)

A fetal type of sulfoglycoprotein antigen, (FSA), has been demonstrated by double immunodiffusion in microplates in the gastric juice of 75 of 78 patients who had histologically verified gastric cancers (69). The same material was, however, found in 14 of 148 individuals who were either normal or had suffered from non-cancerous gastric pathology. A relationship of the FSA to carcinogenesis was suggested because secretion of the antigen apparently preceded the development of morphologically distinct

cancer cells. Recent studies have suggested that the FSA preparations might share a common antigenic determinant with the CEA (70). This determinant appears not to be identical with the tumor-specific CEA determinant of the CEA macromolecule. The precise immunochemical relation of FSA to CEA remains, however, to be defined.

Gamma-Fetoprotein

A fetal constituent of gamma mobility on electrophoresis, named γ-fetoprotein, has recently been detected immunologically, and reported to be present in the majority of malignant solid tumors tested (71). In addition to the tissue content of the antigen, this material was found in a small proportion of the sera from patients with solid tumors or leukemias. Less than 1 per cent of the sera from patients with various types of cancers were, however, found to contain the corresponding antibody. The gamma-fetoprotein antigen has been found in a number of benign tumors as well and, unlike the fetal antigens of human cancer tissue previously considered, this substance showed no species-specificity and was found in the sera of human, bovine, canine, and procine feline fetuses. This new fetoprotein, which remains to be further defined, has an electrophoretic mobility in the mid-gamma-globulin region and is serologically distinct from the $alpha_1$-fetoprotein, and the CEA of the human digestive system.

T-Globulin

The T-globulin is the name proposed for a constituent which has reportedly been detected in the sera of cancer patients

and pregnant women (72). It has been suggested that this material is a symbody or tumor-cell protective molecule (73) directed against a ceremide lactoside or cytolipin component of the tumor cell surface and the surface of embryonic and fetal cells. Further work in this system is required before the clinical or theoretical value of this observation can be assessed.

The Carcinoembryonic Antigen (CEA) of the Human Digestive System

The term, carcinoembryonic antigen (CEA), was introduced in 1965 to designate a constituent found in all adenocarcinomas of the human digestive system, but which is normally present only in embryonic and fetal digestive tissues in the first two trimesters of gestation (74). The initial demonstration of the CEA followed a series of experiments involving adenocarcinomas of the human colon (75). This lesion was specifically chosen for study because its growth pattern is such that it does not extend intramurally for more than 6 to 7 centimeters either proximal or distal to the site of the visible tumor in the gross. Mucosa taken from surgical specimens beyond these points was, therefore, available as normal control tissue from the same donors who supplied the cancer material. One of the most perplexing problems in the antigenic analysis of human cancers, that of distinguishing tumor-specific from individual-specific antigenic differences, was thereby circumvented.

Antitumor antisera were prepared in rabbits and rendered tumor-specific either by absorption with an excess of corresponding

normal tissue extracts, or by utilizing the phenomenon of acquired immunologic tolerance. The antisera prepared by both procedures were tested for their content of antibodies by a number of different serologic procedures.

The results of these and other investigations revealed that all of the colonic adenocarcinomas examined contained an identical, qualitatively tumor-specific antigen which was absent from the corresponding, autologous normal colonic tissue (75,76, 77). Appropriate studies excluded the possibility that the antibodies giving rise to these tumor-specific reactions, were directed either against the bacterial flora of the bowel or the usually high concentrations of fibrin frequently found in malignant tumors.

By employing the tumor-specific system of the colon as a model, it was then demonstrated that all human adenocarcinomas arising from the entodermally-derived digestive system epithelium (esophagus, stomach, small bowel, colon, rectum, pancreas and liver) contained the same tumor-specific constituent. Although this component could not be detected in any other normal, diseased, or neoplastic tissues, it was found in embryonic and fetal gut, pancreas and liver during the first two trimesters of gestation. For this reason, the material was named the carcinoembryonic antigen (CEA) of the human digestive system (74).

The Cellular Localization of CEA

Agglutination studies with tissue-cultured cells of colonic cancer origin suggested that the CEA was a constituent of the tumor cell surface (78). This observation was confirmed

by immunofluorescence microscopy employing frozen or alcohol-fixed sections of digestive system tumors and fetal intestines, as well as viable cells explanted from freshly resected colon cancers (78-81). In addition, a number of different specimens of viable colonic cancer tissue were incubated with a ferritin-anti-CEA conjugate and studied by electron microscopy for localization of the ferritin label (82). In this manner, it was found that a portion of the CEA at least is situated in the glycocalyx of the tumor cell immediately adjacent to the surface membrane (82). It would, therefore, appear that CEA is not a component of the trilaminar image usually referred to as the plasma membrane, but lies further to the cell periphery.

Physicochemical Properties of the CEA

The CEA is a relatively large, water soluble glycoprotein with an approximate carbohydrate to protein ratio of 2 or 3 to 1, a sedimentation coefficient of 7-8S, and a mobility in the beta-globulin range upon immunoelectrophoresis in agar gel. The antigen has been purified by extraction in perchloric acid and subsequent fractionation by preparative gel chromatography and gel block electrophoresis (83,84).

Amino acid analyses of such purified materials have revealed a fairly consistent pattern with some minor variations from preparation to preparation (83,84). No comparable material could be isolated from normal digestive system tissues by this technique. Fragments obtained by acid degradation of CEA which were free of amino acids by the techniques employed retained anti-CEA binding activity (85,86). These data suggested that

N-acetyl-D-glucosamine and D-mannose may well be important constituents of the tumor-specific site(s) of the CEA molecule. In addition to these two monosaccharides, D-galactose, L-fucose, D-glucose and sialic acid were also found in CEA. The importance of these constituents has not, as yet, been determined.

The observations relating the carbohydrate moiety of the CEA molecule to its tumor-specific antigenic determinants (85,86) are of particular interest. The ABH blood group substances are present in the same cellular location in embryonic and fetal gastrointestinal tissues (87-91), and occasionally in digestive system adenocarcinomas (92,93).

The carbohydrate moieties of the ABH blood group substances are responsible for their antigenicity and the key to the establishment of the structures of these carbohydrate moieties was the recognition that L-fucose, D-galactose and N-acetyl-D-galactosamine were important determinants of blood group H, B and A specificity respectively. The elucidation of much of the carbohydrate structures of the blood group antigens was a major advance in the study of naturally occurring immunogenic molecules (94). Although the structures of the blood group and CEA antigenic determinants might ultimately prove to be quite different, the conceptual and methodologic approaches taken in the studies of the former will certainly be of great value in elucidating the structure of the latter.

Immunologic Reactivity of CEA in Humans

Cell-Mediated Immunity: Skin reactions of the delayed hypersensitivity type were observed in 17 of 19 patients with carcinomas of the colon and rectum when they were challenged intradermally with soluble membrane fractions obtained from the autochthonous tumor cells (95). Negative reactions were observed when comparable normal tissue fractions were employed. The skin-reactive antigen was also found in the digestive tract cells of both first and second trimester fetuses. Moreover, CEA was detected in many of the preparations producing positive skin tests. Subsequent studies have shown, however, that although they were found in the same membrane fractions, the CEA and the skin-reactive antigen are distinct from one another (96).

In vitro correlates of cell-mediated immunity have also been sought in patients with colon cancer. The colony-inhibition technique was used to demonstrate that the peripheral lymphocytes from such patients possess cytocidal properties directed not only against their own tumor cells but also against those obtained from colon cancers of other patients (97). Whether the CEA is the factor responsible for the cross-reactivity observed remains to be determined. It is, however, noteworthy that the incubation of peripheral blood lymphocytes from patients with digestive system cancer in the presence of CEA failed to stimulate a significant degree of lymphocyte transformation as measured by ^{3}H-Thymidine incorporation into DNA (98).

Humoral Immunity: The sera of 212 individuals, both normal subjects and those suffering from a variety of diseases, were examined for the presence of circulating antibodies against the CEA by the bis-diazotized benzidine (BDB) hemagglutination technique (99). A specific IgM humoral anti-CEA antibody response was detected in 30 of 43 patients with non-metastatic digestive system cancer and 28 of 46 pregnant women in all trimesters of pregnancy and in the immediate post-partum period. It should be noted that in 4 out of 30 cancer patients, the preoperative serum specimens were negative, but subsequent post-operative specimens were positive. However, in those cases where the malignant tumor had spread from its primary site in the digestive system to other organs, the sera were invariably unreactive.

In 1969, a radioimmunoassay was described for the detection of circulating CEA in the sera of patients bearing digestive system cancers (100). These observations have subsequently been confirmed in other laboratories (101-110). In addition, investigators employing the modified radioimmunoassay techniques and different preparations of CEA and anti-CEA antisera have reported the detection of "CEA-like" substances, termed "tumor associated antigen(s)" (TAA), in the sera of patients suffering from non-digestive system cancers, certain inflammatory bowel diseases, and a few other pathologic entities (101-110).

Recent studies suggest that the TAA may be similar to a glycoprotein which can be extracted from normal lung, spleen,

liver, breast, prostate, gut and colonic cancer tissue (111-113). This material has a mobility in the beta-globulin region upon immunoelectrophoresis. The molecular weight of this material is less than that of CEA, as it sediments at 3-4 S in the analytical ultracentrifuge. Various investigators have designated this material Normal Glycoprotein, NGP (111), nonspecific cross-reacting antigen, NCA (112) and Beta External, β_E (113).

Immunization of rabbits with colonic cancer tissue extracted in 0.6M perchlonic acid appears to result in antibodies directed against the immunodominant regions on this material, as well as a comparable site on the CEA molecule, which is distinct from the tumor-specific area. Antibodies directed against the normal glycoprotein determinant(s) may be removed by absorption of the antiserum preparation with relatively large quantities (80 mg/ml) of normal colonic mucosa extracted in 0.6M perchlonic acid. This treatment, however, does not diminish the CEA-anti-CEA reaction in any way (113).

These data suggest that, in addition to the tumor-specific antigenic determinant, the CEA molecule contains one or more antigenic sites, present in some normal, adult tissues and which must be distinguished from the tumor-specific grouping. Tumors originating outside of the gastrointestinal epithelium might also possess the normal glycoprotein present in colonic tumor, or materials which are antigenically cross-reactive. It is also possible that each of these tumors contains other antigenic determinants present on both CEA and TAA. Clearly, a more

detailed investigation of these materials is needed.

It was with this body of information as a background that the present series of experiments were conceived and performed.

CHAPTER II

OBJECTIVES OF THE PRESENT INVESTIGATION

From the data presently available, the following problems remain unsolved:

1. Is the humoral anti-CEA antibody response, in fact, exclusively IgM in type, or was this result due to the use of the BDB hemagglutination technique which is biased toward the detection of IgM by a factor of 10^2 - 10^3 greater than for IgG (114)? Since the function of humoral antibodies in the possible enhancement of human tumor growth is virtually unknown (13) it is, therefore, of the utmost importance, to attempt to detect and quantitate anti-CEA antibodies of the major immunoglobulin classes.

2. Does the anti-CEA circulate as a free constituent, or is it bound to CEA in the form of a CEA-anti-CEA complex?

To attempt to resolve these problems, the following aims were proposed for the present study:

1. To determine whether radioimmunoassay techniques, utilizing purified CEA, could be employed for the detection of anti-CEA antibodies in the sera of patients with digestive system cancers and in the sera of pregnant women.

2. To determine the immunoglobulin class and quantity of such antibody moieties.

3. To investigate the possibility that the anti-CEA antibodies, where present, might exist as free constituents and/or as antigen-antibody complexes with CEA.

4. To utilize radioimmunoassay techniques involving hapten inhibition to examine the structure of the immunodominant groupings of the CEA molecule.

CHAPTER III

MATERIALS AND METHODS

The Preparation of Purified Carcinoembryonic Antigen (CEA) of the Human Digestive System

The CEA utilized in the following studies was prepared, as outlined below, by the method of Krupey et al (84). The designations, CEA, or standard CEA, specify preparations of the carcinoembryonic antigen of the human digestive system prepared exactly by this method.

Initial Preparation of Tumor Specimens

Whenever possible, hepatic metastases from primary adenocarcinomas of the colon or rectum were employed because of the relatively high concentrations of CEA in such lesions (75). The tumor tissue obtained at autopsy was dissected as cleanly as possible from any surrounding normal tissue. This material was stored in 1 kg aliquots, at -20°C, until it was to be extracted. In preparation for extraction, an aliquot of tumor tissue was thawed at room temperature and then minced in a motor-driven Toledo meat grinder (Model 5220-0-010). The mince was suspended in 4 l. of distilled water and homogenized in a water-cooled VirTis Chemixer at 15,000 rev/min for 1 hr. The demonstration of CEA activity in the initial homogenate, and at each stage of purification, was performed by Ouchterlony reaction against monospecific anti-CEA antiserum as previously described (75). The

minimum quantity of material required to produce a precipitin line after each phase also served as an indicator of the approximate degree of CEA enrichment achieved by that portion of the isolation technique.

Perchloric Acid Extraction

Equal volumes of the tumor tissue homogenate and 2.0M perchloric acid were mixed, and then stirred for 30 min at room temperature. The resulting suspension was centrifuged at 4000 g at 4°C, in a IEC centrifuge (Model B-20) in 1500 ml lots for 30 min. The sediment was discarded and the supernatant dialyzed in Visking tubing against cold running tap water for 48 hr. Dialysis was then continued against frequent changes of distilled water, at 4°C, for an additional 48 hr. The non-dialyzable residue was lyophilized, redissolved in 200 ml of distilled water, and centrifuged at 15,000 g, at 4°C, for 30 min. The supernatant was removed and filtered sequentially through Millipore membranes of pore sizes 1.2μ, 0.45μ, and 0.22μ. The final filtrate was then lyophilized.

Preparative Gel Filtration Chromatography

A solution of 0.05M NaH_2PO_4 in 0.15M NaCl at pH 4.5 was employed as the eluting medium throughout the chromatographic procedures.

An aliquot of 1.5 g of the lyophilized powder of the perchloric acid-extracted tumor tissue was dissolved in 50 ml of the saline-phosphate solution and applied to a previously equilibrated Sepharose 4B, Pharmacia column (Type K-100/100), with dimensions

89 x 10 cm. Elution was performed by upward flow at a rate of 150 ml/hr. The eluate was constantly monitored for its spectrophotometric absorption at 280nm, and was collected in 25 ml fractions. Those fractions possessing CEA activity were dialyzed against distilled water at 4°C for 48 hr and then lyophilized.

A 200 mg sample of powder derived from the Sepharose 4B column, and containing the CEA activity, was dissolved in 10 ml of the saline-phosphate solution. This solution was then applied to a 90 cm x 5 cm Pharmacia column (Type K 50/100) containing equilibrated Sephadex G-200. Chromatography was performed, at 4°C, by upward flow at a rate of 40 ml/hr. The eluate, again monitored at 280nm, was collected in 10 ml aliquots. Fractions containing CEA activity were then pooled, dialyzed, and lyophilized as described for the eluate from the Sepharose 4B column.

Preparative Gel Electrophoresis

Sephadex G-25 (fine) was washed and equilibrated with borate buffer (pH 8.6, 0.05M). A thick slurry of this material was poured into a leveled lucite mold (61 cm x 7.5 cm x 2 cm) so that it was evenly distributed along the plate at a depth of 1 cm. The surface of the gel was blotted with cotton gauze sponges until it had a firm consistency but was not dry. The gel block was fitted with Whatmann 3MM chromatography paper contacts and placed in an electrophoresis apparatus where the electrode chambers contained the same borate buffer as that used in washing the Sephadex G-25 gel.

Equilibration of the system was allowed to occur for 1 hr under the operating conditions of 400 V and approximately 20 mA at 4°C. A sample of 60 mg of the CEA-containing powder derived from the Sephadex G-200 chromatographic procedure was dissolved in 0.5 ml of the borate buffer. A 1 cm strip of gel was then removed from the center of the block and was thoroughly mixed with the solution of CEA. This slurry was then poured into the trough in the center of the block, formed when the gel strip was removed. Ferritin (0.01 mg in 0.005 ml of borate buffer), which served as a marker, was spotted 3 cm from the cathodal extremity of the block. Electrophoretic separation was carried out, under the conditions described, for 24 hr. After electrophoresis, 1 cm strips of gel were cut from the block and were suspended in 25 ml of normal saline. The liquid was removed from each aliquot of gel by filtration through a 0.45μ Nalgene grid membrane. The dried Sephadex cake was then washed, in the same filter unit, with an additional 25 ml of saline. The total filtrate was dialyzed, at 4°C, against pooled, lyophilized, and stored at 4°C *in vacuo*.

The Preparation of a Material Containing CEA Activity from Immune Precipitates of Anti-CEA and Aqueous Tumor Extract Utilizing Ion Exchange Chromatography with Acidic Buffers

The initial step in the purification of CEA involves the extraction of an aqueous tumor homogenate in 1.0M perchloric acid (83,84). An alternate procedure was sought to purify CEA

from an aqueous tumor homogenate, to eliminate the exposure of the CEA molecule to the extremely acidic, and possibly damaging conditions of the perchloric acid (pH less than 1, for 1 hour at 20°C).

The cellulose-bound ion exchangers, developed by Sober and Peterson (115a;115b) possess a high capacity for the binding of proteins on the basis of molecular charge. These materials can be made to selectively release the bound proteins under relatively mild conditions by alterations of elution buffer pH, salt concentration and temperature.

A procedure employing Carboxymethyl Cellulose (CMC) was developed to enrich the CEA activity from aqueous tumor homogenates. In this technique, immune precipitates, prepared by the addition of anti-CEA antibodies to aqueous tumor extracts in the zone of equivalence, were solubilized with acid buffer and the components of the immune complexes were then separated by the differences in their affinities for the CMC at the salt and hydrogen ion concentration employed.

Preparation of Immune Precipitates

A 65 g aliquot wet weight of liver metastases from a patient with a primary gastric carcinoma (belonging to blood group 0) was homogenized in 200 ml iced saline. The homogenate was subjected to centrifugation at 30,000 g for 30 min at 4°C in a Sorvall RC2-B centrifuge and the supernatant fraction was mixed with 200 ml of anti-CEA antiserum. The volumes used were calculated from Ouchterlony reactions to be in the zone of equivalence. The mixture was then incubated at 37°C for 24 hr and at 4°C for

48 hr. The precipitate was harvested by centrifugation at 10,000 g for 20 min at 4°C.

Dissociation and Washing of the Immune Precipitate

The immune precipitate was suspended in 40 ml of PBS pH 7.3 at 4°C and then sedimented at 10,000 g for 20 min at 4°C. This procedure was repeated once. The precipitate was then suspended in 20 ml of glycine HCl (0.1M, 270mmho conductivity), pH 3.0 at 20°C. Most of the precipitate went into solution within 10 min at which time the solution was subjected to centrifugation at 10,000 g for 20 min at 4°C. The acid-insoluble material was discarded and the supernatant fraction was removed and immediately neutralized to pH 7.0 with 0.4M, Na_2HPO_4 solution. After 4 hr at 37°C and 16 hr at 4°C, the precipitate had reformed and the process of acidification was repeated. The second acidification step resulted in complete dissociation of the precipitate. Neutralization-reassociation and precipitation were repeated and the immune precipitate was finally redissolved in 5 ml of the glycine HCl buffer immediately prior to ion exchange chromatography.

Preparative Ion Exchange Chromatography

Carboxymethyl cellulose (Whatman CM 52) was equilibrated with glycine HCl buffer (pH 3.0, 0.1M, 270mmho conductivity). The equilibrated CMC slurry was packed to a height of 40 cm in a 2.5 x 45 cm glass chromatographic column (Pharmacia K25/45) by descending flow at 180 ml/hr.

Descending ion exchange chromatography of the dissociated immune precipitate was performed at 25°C at 50 ml/hr. The eluate was

constantly monitored for its spectrophotometric absorption at 280 nm and was collected in 5 ml aliquots.

Elution was continued until the spectrophotometric absorption of the eluate had attained baseline levels (Pharmacia UV monitor, full scale, 0.040 OD units at 280 nm). The column was then eluted with a saline-phosphate buffer (0.4M NaCl, 0.03M phosphate buffer), pH 7.4.

The Preparation of Goat Anti-CEA Antibodies

An adult male goat weighing 10 kg was immunized with 1 mg of CEA dissolved in 1.0 ml of sterile deionized water and emulsified in an equal volume of complete Freund's adjuvant (Difco). The animal had received such injections intramuscularly in the flank areas at 2-4 week intervals for 2 years. Fourteen days after each injection, after the first three months of immunization, the animal was bled by jugular venipuncture and his sera stored at 4°C. The goat antiserum used in the experiments to be described is, therefore, designated G-81 anti-CEA antiserum. After appropriate absorption with normal human tissue extracts, as described below, the antiserum was termed G-81 abs.

Absorption of Goat Anti-CEA Antiserum with Normal Human Tissue Extracts

Pooled normal human bowel and normal human liver were extracted in 0.6M perchloric acid by the method of Winzler (116). After exhaustive dialysis against deionized water the extracts were lyophilized. G-81 anti-CEA antiserum was absorbed as follows:

Each 1.0 ml of serum was admixed with 80 mg of each extract and incubated at 37°C for 4 hr and 4°C for 16 hr with gentle agitation. The mixtures were then subjected to centrifugation at 100,000 g for 1 hr in a Spinco Model L Ultracentrifuge (SW 39 rotor). The supernatant was carefully removed, filtered through a 0.22μ Millipore filter into a sterile bottle and stored at 4°C without further treatment.

Blood Group Substances

Human ovarian cyst-derived blood group substances A, B, H and Le^A were the gracious gift of Dr. W.T.J. Morgan of the Lister Institute (London) (117). A preparation of Pneumococcal Polysaccharide Type XIV was kindly provided by Dr. M. Heidelberger (New York University School of Medicine).

The designation A or A substance used in this thesis refers to the human ovarian cyst fluid-derived blood group A substance described above.

Radioiodine Conjugation to Glycoproteins (CEA and A)

Both CEA (83) and human ovarian cyst fluid-derived blood group substance preparations have been shown to contain tyrosine residues (118,119). These materials were, therefore, radiolabelled with ^{125}I by the chloramine-T method (120), as follows:

1. The diluent utilized in all steps was phosphate buffer, pH 7.4, 0.05M.

2. The reaction was carried out in a 4.0 ml flat bottom

glass vial, containing a 1/16" x 1/4" teflon-coated magnetic stirring bar. These were discarded after each labelling procedure.

3. Eppendorf pipets (Brinkmann Instruments, Toronto) of appropriate capacities were utilized to measure and dispense all reagents.

4. Fresh solutions of chloramine-T (1.0 mg/ml, Eastman Chemicals, Rochester) and sodium metabisulfite (2.0 mg/ml) in the diluent buffer, were prepared before each radioiodination procedure.

5. The pH of the ^{125}I solution was measured by placing 1 µl aliquots onto narrow range alkaline pH paper and used only if the pH of the reagent was mildly alkaline.

6. I^{125} as $Na^{125}I$, carrier-free in NaOH solution, pH 8-11, free from reducing agent and containing less than 1% ^{126}I, was obtained from Amersham Searle (Don Mills, Ontario).

7. The glycoprotein to be labeled was diluted with borate-saline buffer (NaCl 0.05M, borate buffer 0.05M), pH 8.6, to a final concentration of 1.0 mg/ml. The conjugation procedure was performed as follows:

A 50 µl volume of the glycoprotein (1 µg/µl) was mixed with 4 mCi of ^{125}I and 100 µl (100 µg) of the chloramine-T solution, for 75 sec, at 25°C. 100 µl of sodium metabisulfite was then added and the reaction solution was vigorously mixed for 20 sec. The reaction mixture was applied to a 0.8 x 50 cm Sephadex G-100 column equilibrated with the diluent buffer. After the reaction mixture had soaked into the column, the reaction vessel was washed with 10% KI and the washings were applied to the

column, which was then eluted with the diluent buffer at a pressure head of 20 cm H_2O.

Fractions which were 1.0 ml in volume, were collected into 1.0 ml of 3% BSA solution in diluent buffer. The radioactivity of 10 µl aliquots of the eluate was determined in the Nuclear Chicago Model 1185 Gamma Radiation Spectrometer calibrated for ^{125}I with a Counting Efficiency of 50%. All of the radioactive measurements, described in this thesis were performed on this Gamma Spectrometer, with a Counting Efficiency of 50%. Radioactivity in the void volume peak, as determined by prior calibration with Blue Dextran 2000 (Pharmacia) represented the labeled glycoprotein, while the radioactivity eluted in the column volume peak represented free ^{125}I. The calculation of the specific activity of the labeled glycoprotein was based on the assumption that 50% of the glycoprotein was recovered in the void volume peak.

Collection of Human Sera

The sera studied were obtained from patients with localized and disseminated tumors of digestive system and with non-digestive system cancers. Sera were also obtained from pregnant women in all trimesters of gestation. Sera employed as controls for those studied from cancer patients were obtained from laboratory personnel, young orthopedic patients with no evidence of systemic disease and from patients with inflammatory diseases of all organ systems.

Blood was obtained by venipuncture and collected into sterile empty 20 ml BD vacutainers. Clotting occurred at room

temperature and after incubation at 37°C for 4 hr, and 4°C for 16 hr, the clots were removed and the sera spun at 2000 g for 20 min at 25°C. The sera were then divided into two equal 5.0 ml aliquots and stored at -20°C.

Absorption of Human Sera with Human Erythrocytes

A 1.0 ml sample of each serum under study was first incubated at 56°C for 30 min. A 200 µl aliquot of each sample so prepared was added to a 200 µl sample of washed, packed blood group A, B or O erythrocytes. After gentle mixing and incubation for 1 hr at 37°C and 1 hr at 4°C, the samples were centrifuged at 10,000 g for 2 min and the supernatants removed. Any sample with visible evidence of hemolysis was discarded. Aliquots of these sera were then studied for residual antibody content by passive hemagglutination.

The Preparation of High Titer Human Anti-A Antibody from Individuals not Actively Immunized

The designation, anti-A antibody, refers henceforth, to human isoagglutinins directed against blood group substance A. Purified, high titer human anti-A antibodies were prepared by the method of Kochwa and Rosenfield (121) with some modifications, as follows:

Preparation of Blood Group A Erythrocyte Membranes

Blood samples from blood group A donors were collected into Acid Citrate Dextrose (ACD). The blood was centrifuged at

1750 g for 8 min at 25°C and the plasma and buffy coat were removed. The cells were washed three times in 1% saline and, after the final wash, were resuspended with 1% saline to a hematocrit of 25%. Five ml of this suspension was pipetted into each of eight 45 ml Sorvall centrifuge tubes and 35 ml of 30 mosm-phosphate buffer, pH 7.2, was added to each tube. The cell suspension was mixed thoroughly and allowed to stand for 20 min at room temperature, and then sedimented in the Sorvall RC 2-B centrifuge at 4°C for 10 min at 9000 g. The supernatant was removed and the cells suspended in 45 ml of 60 mosm phosphate buffer, pH 7.2, mixed, and allowed to stand for 5 min, at room temperature. The erythrocyte suspensions were then centrifuged as before and supernatant removed. The cells were resuspended in 45 ml of the 30 mosm buffer, were allowed to stand for 5 min at room temperature, and were then centrifuged. The last step was repeated two more times and the erythrocyte "ghosts" were then ready for use.

Adsorption and Elution of Anti-A Antibodies from Erythrocyte Ghosts

The anti-A antibodies were obtained from the sera of blood group O or B donors whose hemagglutination titers were greater than 1 in 64. Antibodies were adsorbed to the A-erythrocyte stroma as follows: A three volume aliquot of whole serum was incubated for 24 hr at 37°C, with 1 volume of the erythrocyte ghosts. The ghosts, so treated, were sedimented at 9000 g at 4°C and the supernatant was removed. If no anti-A antibody was detectable in the supernatant

by hemagglutination, the procedure was repeated with the addition of fresh serum to the same preparation of red cell membranes, until anti-A antibodies were detectable in the supernatant fluid. The red cell membranes were then washed a total of five times in approximately 40 volumes of 0.15M saline (4°C) with centrifugation each time at 9000 g at 4°C for 20 min. The supernatant fluid was discarded after each wash. The adsorbed antibody was then eluted from the membranes by the addition of one volume of 0.1M, pH 3.0, Glycine HCl buffer for each volume of packed ghosts. The membranes were then sedimented by centrifugation at 9000 g at 4°C for 20 min, the supernatant fluid removed and dialysed exhaustively against phosphate buffered saline (PBS) (0.01M phosphate buffer, pH 7.3, 0.14M NaCl) for 48 hr at 4°C. The membranes were treated with six such cycles of elution. The eluates were pooled and concentrated in a Diaflow ultrafiltration cell employing a PM-30 membrane (Amicon Corp., Lexington, Mass.). Aliquots of this preparation were studied for anti-A antibody content by passive hemagglutination.

Techniques of Antibody Demonstration

General Considerations of Antibody Measurement

With the tremendous burst of activity in immunological research over the past two decades, the body of knowledge concerning humoral antibodies has become complex. However, it is currently possible to make four statements about antibodies as a class.

First, under normal circumstances, antibodies are produced in response to antigenic stimulation. In terms of function,

this property separates antibodies from other binding proteins in a serum such as haptoglobin and transferrin. It should be borne in mind that immunogenic stimulation can come from very subtle exposures as, for example, the bacteria in the intestinal tract stimulating the production of isoagglutinins (122).

Secondly, much has been learned about the structure of the several classes of immunoglobulins, and despite the differences between the various heavy, or H chains, all known antibody molecules have either kappa or lamda light, or L chains (123).

The third characteristic of antibody populations raised following exposure to even the purest antigens is their heterogeneity. They are heterogeneous not only as regards their structure as alluded to above, but also with respect to the strength of the bond which they can form with the corresponding antigenic sites, and with respect to their function in vivo and in vitro.

The fourth generalization that can be made is that all antibodies have the capacity to bind specifically with their respective antigens through the antigen binding sites of the F(ab) portions of the molecule.

Notably absent from these statements are the classic, and now defunct, generalizations that all antibodies precipitate with antigen, fix complement in the presence of antigen, or that all antibodies have the capacity to agglutinate antigen-coated erythrocytes.

The binding of antigen to antibody may be represented by the following equation (124):

$$Ag + Ab \underset{kd}{\overset{ka}{\rightleftharpoons}} Ag - Ab.$$

In this prototype reaction, Ag represents one of the several antigenic sites usually present on a given antigen molecule, Ab represents 1 of 2 or more antigen binding sites present on a given antibody molecule, and Ag - Ab represents the combined state after these two molecules interact with one another. Like every other chemical reaction this one has an association constant (ka) and a dissociation constant (kd). The combined effect of the two will yield an equilibrium constant. It is only after the primary interactions of antigen and antihody has occurred that a secondary manifestation, as described below, may or may not become apparent.

Examples of primary techniques of antibody detection which may be quantitated are equilibrium dialysis (125) and the precipitation of antigen-antibody complexes by 50% saturated ammonium sulfate (126). Other tests of primary antigen-antibody interactions, such as radioimmunoelectrophoresis (RIEP) are qualitative only. Examples of secondary manifestations of antigen-antibody interactions in vitro are the various types of precipitin reactions, agglutination of particulate antigen or antigen-coated particles, and complement-fixation. Tertiary manifestations of antigen-antibody interaction are seen in the in vivo phenomena of the Prausnitz-Kustner test for reagins or IgE antibodies, passive cutaneous anaphylaxis and the Arthus reaction. Secondary and tertiary tests frequently fail to detect significant amounts of antibody demonstrable by primary binding tests (127).

In the experiments to be described, the following techniques were utilized to demonstrate the presence of antibodies in the sera tested.

Techniques for the Detection of Primary Binding of Antigen with Antibody

1. Molecular Sieve Radioimmunoassay.
2. Radioimmunoelectrophoresis Technique employing
   (a) Cellulose Acetate Membranes.
   (b) Agar Gel.
3. The Detection of Antibody by the Coprecipitation of Soluble Immune Complexes in 50% Saturated Ammonium Sulfate.
4. The Detection of Antibody in Acid-Treated Serum Preparations by the Coprecipitation of Soluble Immune Complexes in 50% Saturated Ammonium Sulfate.
5. Radioimmunoassay Employing Antibody Coupled to a Solid Phase.

Techniques for Detecting Secondary Manifestation of Antigen-Antibody Interaction

1. Precipitation in Agar Gel — Ouchterlony Technique, Immunoelectrophoresis.
2. Passive Hemagglutination Technique.

Description of Techniques

Molecular Sieve Radioimmunoassay

Quantitative radioimmunoassay techniques, employing gel filtration to separate free, radiolabeled antigen from antibody-bound radiolabeled antigen have been described (128-131) for a variety of antigens. This separation is effected by differences in molecular volume between the free antigen and the corresponding antigen-antibody complex. In this study, a technique was devised to study the quantitative binding of serum immunoglobulin moieties to ^{125}I-CEA.

Preparation of Columns: Sepharose 6B (Pharmacia, Uppsala, Sweden) was washed in a coarse, sintered glass filter funnel under suction, with 100-fold the volume of the gel with phosphate-buffered saline (PBS), pH 7.3, (0.01M phosphate buffer, 0.14M NaCl). The Sepharose gel so prepared, was packed under a constant pressure head of 20 cm H_2O into 0.9 cm x 60 cm columns (Pharmacia K9/60). The gel was packed above the top of the column into the column reservoir (Pharmacia R9) which also held 20 ml of PBS above the gel. The reservoir was then tightly capped and 10 ml of air was injected into the reservoir above the buffer compressing the packed gel. After 60 sec under this pressure, the reservoir was removed from the top of the column and replaced by a regular column end-piece, which had been filled with gel prepared in the same fashion as that in the column.

Ascending chromatography was performed with PBS at a rate of 2.0 ml per hr utilizing a LKB Perpex pump (No. 10200), a 1 : 500 ratio gear set (102-4-7) and 1.10 mm I.D. pump tubing.

The columns were calibrated with Blue Dextran 2000 (Pharmacia) for void volume, and ^{125}I-CEA as a marker for CEA.

Method of Assay: An aliquot of 500 µl of the serum under investigation was incubated with 50 µl of ^{125}I-CEA (0.5 ng CEA, 40,000 dpm, specific activity approximately 7×10^6 mCi/mM) for 4 hr at 37°C and 16 hr at 4°C with gentle agitation. The resulting mixture was applied to the column and 0.5 ml samples were collected. The radioactivity in each fraction was measured in a Nuclear Chicago Model 1185 gamma radiation spectrometer calibrated for ^{125}I.

Antigen-antibody inhibition studies in this system were performed by adding the preparation under investigation directly to the mixture of serum and ^{125}I-CEA; the rest of the procedure was performed as described.

The columns were calibrated before use with Blue Dextran 2000 (Pharmacia) and ^{125}I-CEA (mw 2×10^5). Results were displayed graphically by plotting the radioactivity of each fraction on the ordinate and the column elution volume on the abscissa. The binding of an individual serum to ^{125}I-CEA was calculated as follows:

$$\frac{\text{Area under Bound } ^{125}\text{I-CEA Peak}}{\text{Area under Bound } ^{125}\text{I-CEA Peak} + \text{Area under Free } ^{125}\text{I-CEA Peak}}$$

Radioimmunoelectrophoresis

Radioimmunoelectrophoresis procedures have proven to be powerful tools for the study of complex antigen or antibody mixtures. This sensitive method has been successfully applied to study the

antigen binding characteristics of immunoglobulins and immunoglobulin subunits (132-145).

Radioimmunoelectrophoresis was performed by first incubating 50 µl of the serum under investigation with 25 µl of a solution of ^{125}I-CEA, containing approximately 20 ng of purified CEA conjugated to ^{125}I at a specific activity of approximately 7×10^6 mCi/mM for 48 hr at 37°C.

Radioimmunoelectrophoresis Technique Employing Cellulose Acetate Membranes: An aliquot of each of the resulting mixtures prepared as described above was subjected to electrophoresis in a Millipore Fourcell Electrophoresis Cell (XE21 004 15) employing Mylar-backed cellulose acetate membranes (Immuno-phoroslides single trough no. ESWM 031 1A for studies of IgM antibodies and double trough ESWM 031 1B for studies of IgG and IgA antibodies). Aliquots of 2.0 µl of each preparation were applied with 2 µl Drummond microcaps to the wells of the single trough slides and 4 µl aliquots were applied to the wells of the double trough slides. Electrophoresis was carried out at 100 volts for 25 min at 25°C. A 35 µl aliquot of a goat antiserum, directed against one of human IgG, IgA or IgM (Meloy Laboratories, Springfield, Va.), was applied to the trough of each slide and repeated once after the first volume applied had soaked in. In the initial studies, goat antiserum, directed against whole human serum, was utilized in some instances.

Precipitin patterns were developed in a moist chamber for 48 hr at 37°C.

After washing the slides for 4 days in 12 changes of normal saline and 3 changes of deionized water, the membranes were stained with 2% nigrosine (Millipore) in 2% acetic acid, rinsed twice in deionized water, and air dried at room temperature overnight.

Autoradiographs of the immunoelectrophoretic patterns were subsequently developed on Ilford Ilfex 25 EP X-ray film. After incubation with the film for 21 days, the autoradiographs were developed and read as 0 if no autoradiograph of the precipitin line was visible, 1+ if a faint but definitely visible autoradiograph was present, 2+ if the lines were quite dark, and 3+ if the lines were thick and dark. The results of duplicate determinations on the same aliquot of serum were consistent in all cases evaluated.

Radioimmunoelectrophoresis Technique Employing Agar Gel: 3¼ by 4" glass slides were coated with 0.5% Difco Noble agar in deionized water. After drying for 16 hr at 37°C, the slides were layered with 15 ml of 1% Difco Noble agar in Tris buffer (0.075M, pH 8.6). A 5 µl aliquot of each of the solutions of serum and ^{125}I-CEA prepared as described above was subjected to electrophoresis at 15 volts/cm for 90 min at 4°C in the same buffer system used to prepare the agar gel. Troughs were filled with the Meloy antisera as previously indicated and washed in the same way as the cellulose acetate membranes. The patterns were stained with 0.5% Ponceau S in 5% TCA and then washed in deionized water. Autoradiographs were prepared in the same

manner as described for the cellulose acetate membrane RIEPs but were developed after 5 days of exposure to the film.

The Detection of Antibody by the Coprecipitation of Soluble Immune Complexes in 50% Saturated Ammonium Sulfate

To measure the binding of anti-A antibody and goat anti-CEA antiserum to ^{125}I-CEA, a radioimmunoassay based upon the phenomenon of the coprecipitation of soluble immune complexes in 50% saturated ammonium sulfate was employed, as first described by Farr (126). The method, described below, will, therefore, be designated The Farr Technique.

During the procedure, normal human serum (NHS) was diluted 1 : 100 with borate-saline buffer (0.05M borate buffer, 0.1M NaCl), at pH 8.6, and this preparation was employed as a diluent for the high titer human anti-A antibody, the G-81 abs anti-CEA antiserum and the ^{125}I-CEA.

The titration curves were obtained as follows: doubling dilutions of the anti-A antibody or the anti-CEA antiserum in 500 µl of the borate-saline NHS diluent were added to a series of tubes. 500 µl of ^{125}I-CEA was added to each of the tubes, and the resultant mixtures incubated for 4 hr at 37°C and 4 hr at 4°C. At the end of this period, 1.0 ml of saturated ammonium sulfate at 4°C was added to each of the tubes. After centrifugation at 20,000 g for 30 min at 4°C, the supernatant fluid was decanted. The ^{125}I-CEA content of the supernatant was determined in a Nuclear Chicago Model 1185 Gamma Radiation Spectrometer calibrated for ^{125}I.

The Detection of Antibody in Acid-Treated Serum Preparations by the Coprecipitation of Soluble Immune Complexes in 50% Saturated Ammonium Sulfate

The dissociation of antigen-antibody complexes, usually in the form of precipitates or as antibody bound to erythrocyte surfaces, has been effected non-specifically by procedures such as change of pH, increase of ionic strength (146), addition of the so-called chaotropic ions (SCN^-, I^- etc) (147,148) or unfolding agents (urea and guanidine) (149). Specific dissociation can also be achieved by addition of the corresponding hapten (146).

The use of acidic media to dissociate antigen-antibody complexes was first described in 1923 when Ramon (150) used acetic acid to dissociate diptheria toxin-antitoxin floccules. Subsequently, this type of procedure has been widely used, notably in the recent elegant purification of human Alpha Fetoprotein by Nishi (63).

An attempt was made to detect the existence of circulating CEA and anti-CEA complexes in the sera of patients bearing digestive system cancers, particularly in those patients in whom dissemination of the primary lesion had occurred.

Aliquots of the sera under investigation were treated with acid glycine-HCl buffer in the presence of nanogram quantities of ^{125}I-CEA. It was postulated that CEA-anti-CEA complexes, when treated with acid, would dissociate and that ^{125}I-CEA would be incorporated into these complexes when the solutions were neutralized. Precipitation in 40% saturated ammonium sulfate was then

employed to measure the amount of ^{125}I-CEA incorporated into the immune complexes. This technique is therefore designated The Farr Technique After Acid Treatment of the Serum Preparation.

Gammaglobulin enriched fractions from 4.5 ml aliquots of sera were prepared by precipitation at 4°C in 50% saturated ammonium sulfate. This should have served to precipitate both free and antigen-bound anti-CEA antibodies, while free CEA should have been removed in the supernatant fractions. This step should have prevented a decrease in the specific activity of the added ^{125}I-CEA in those sera in which antigen-antibody complexes were present in CEA excess.

The redissolved precipitates, reconstituted to the original serum volume (4.5 ml) with phosphate buffered saline, PBS (0.01M phosphate buffer, 0.14M NaCl) pH 7.3, were dialyzed to equilibrium against three changes of 450 ml of this buffer. Four 0.5 ml aliquots were taken from the dialyzed residue. A 0.5 ml volume of ^{125}I-CEA (containing 0.5 ng and 40,000 dpm, specific activity 7 x 10^6 mCi/mM) was added to each of the four samples (No. B 7060b, Brand 2.0 ml dispenser, Carl Zeiss, West Germany). The ^{125}I-CEA was diluted with 0.5% BSA (Pentex, Kankakee, Ill. in PBS). Two of the 4 aliquots were then acidified to pH 3.0 with 1.3 ml Glycine HCl buffer (0.4M, pH 2.6), and were incubated at 37°C for 2 hr with intermittent agitation. At the end of this period, the assay mixtures were titrated to pH 8.0 ± 0.2 by the addition of 0.7 ml of 0.4M Tris in deionized

water. The remaining 2 aliquots were treated with a volume of Tris-Glycine buffer, pH 8.0, to reach the same volume and ionic strength as the acid-treated aliquots. All 4 samples were subsequently incubated for 4 hr at 37°C and 4 hr at 4°C with agitation. A 2.0 ml volume of saturated ammonium sulfate was added to each tube to reach a final concentration of 40% ammonium sulfate. After incubation for 30 min at 4°C, the samples were subjected to centrifugation at 30,000 g for 20 min at 4°C in the Sorvall RC2-B centrifuge. The supernatants were decanted and the radioactivity in the precipitates was measured in a Nuclear Chicago Model 1185 Gamma Radiation Spectrometer calibrated for ^{125}I. The amount of radioactivity in the precipitates (after background subtraction) divided by the total number of counts added was calculated and recorded as the per cent binding for each of the serum aliquots studied.

Radioimmunoassay Employing Antibody Coupled to a Solid Phase

Radioimmunoassay techniques, in which the antibodies are chemically coupled to an insoluble polymer, have been applied to the measurement of a variety of different antigens, ranging from polypeptide hormones to immunoglobulin molecules (151-156). Antigen bound to antibody is easily separated from free antigen by centrifugation of the solid phase coupled antibody reagent. Consequently, such systems need not rely upon any other specific physicochemical properties of the radiolabeled antigen.

Utilizing the principles of such systems, a method was devised to quantitate the interactions between anti-A and anti-CEA preparations with ^{125}I-CEA and ^{125}I-A.

The Conjugation of G-81 abs Anti-CEA Antibodies to Sephadex: G-81 abs was coupled to Sephadex G-25 superfine (Pharmacia, Uppsala, Sweden) by the method of Wide (151) as follows: 10 g of Sephadex G-25 superfine was allowed to swell in deionized water. After washing with 2 l. of deionized water, the gel was activated with 100 g of cyanogen bromide (Aldrich, Milwaukee) in 400 ml of deionized water at pH 11.0 ± 0.2 for 5 min at 25°C. The pH was maintained with 1.0 NaOH throughout the reaction. The activated gel was washed with 1500 ml of ice cold $NaHCO_3$ (0.1M, pH 8.0) on a Buchner funnel fitted with a fine, sintered glass disk. The washed gel was finally suspended in 40 ml of the $NaHCO_3$ solution which contained 1.0 ml of G-81 abs anti-CEA antiserum. The mixture was incubated, with gentle stirring, for 24 hr at 4°C. Preparation of the conjugate was completed by washing it with 7500 ml of PBS (0.01M, phosphate buffer, 0.14M NaCl) at pH 7.3, with 500 ml of Glycine HCl (pH 3.0, 0.2M) and finally with another 7200 ml of PBS.

The Conjugation of Anti-A Antibodies to Sepharose: Anti-A antibodies, prepared by adsorption and acid elution from A erythrocyte ghosts were coupled to Sepharose 2B (Pharmacia, Uppsala, Sweden) by a modification of the method of Cuatrecasas (157,158,159), as follows:

A 25 ml volume of packed Sepharose 2B gel was washed with 5 liters of deionized water. The gel was then activated with 6.25 g cyanogen bromide (Aldrich, Milwaukee) at pH 11.0 ± 0.1 for 10 min at 25°C. The pH was maintained at 11 with 2N NaOH throughout

the reaction. The activated gel was immediately washed with 5 liters of deionized water at 4°C and then with 2 liters of sodium citrate buffer (0.2M, pH 6.5) at 4°C. The washed gel was added to a solution of the anti-A antibody preparation (hemagglutination titer 1 : 1024) in 20 ml of 1% BSA in the citrate buffer.

The mixture was incubated, with gentle stirring, for 72 hr at 4°C. Preparation of the conjugate was completed by washing it, in the same manner, as the Sephadex conjugate described above.

The Construction of Antibody Titration Curves for the Sepharose and Sephadex-Coupled Antibody Studies: Antibody titration curves were prepared by reacting varying quantities of the Sepharose or Sephadex-coupled antibody suspension with constant concentrations of the ^{125}I-conjugated antigens. The diluent utilized in assays designated "A-dil", consisted of 0.5% BSA in PBS pH 7.3. The solid-phase coupled antibody preparations were first diluted 1 : 10 with A-dil and then over a range of doubling dilutions to a final concentration of 1 : 640.

Control studies utilized either Sepharose or Sephadex gels which had BSA or high titer human anti-blood group B antibodies coupled to them or the untreated gels.

500 µl of each dilution of the solid phase-coupled antibody preparations were added, in duplicate to 16 x 125 mm plastic tubes (Falcon Plastics, Oxnard, Calif.). A 500 µl aliquot of either ^{125}I-CEA or ^{125}I-A, containing 0.5 ng and 40,000 dpm (7 x 10^6 mCi/mM) was added to each tube with a 2.0 ml dispenser (B 7060b, Brand, Carl Zeiss, West Germany). The tubes were capped tightly and rotated at 20 rpm at 25°C in a tissue culture tube rotater.

After 48 hr, the assay mixtures were washed 3 times by adding 5 ml of PBS, centrifuging at 1500 g and the supernatant fluid was carefully removed. Radioactivity bound to the washed solid phase-coupled antibody was measured in a Nuclear Chicago Model 1185 Gamma Radiation Spectrometer calibrated for ^{125}I.

Assays, which contained no solid phase-coupled antibody gave a measurement of the binding of the ^{125}I-conjugated glycoprotein to the walls of the plastic counting tubes. This background measurement, which was subtracted from all data, was usually 0.3 - 0.5% of the total radioactivity used for each measurement. Results were recorded as the percentage of the ^{125}I-glycoprotein bound at each antibody dilution.

Solid Phase Antibody — Radiolabeled Antigen Binding — Inhibition Studies: Binding-inhibition studies in the solid phase system were performed with a concentration of solid phase-coupled antibody and radiolabeled antigen in the zone of antigen excess. The carbohydrate or glycoprotein under study, in 375 µl of A-dil, was added to the dilution of solid phase-coupled antibody, in 125 µl of A-dil, to reach a total volume of 500 µl. After incubation for 2 hr at 25°C with constant agitation, the appropriate radiolabeled glycoprotein, in 500 µl of A-dil, was added, and the rest of the assay performed as described above. Results of these studies were expressed graphically as the percentage of ^{125}I-conjugated glycoprotein bound in the presence of the putative inhibitor compared to the amount bound in the absence of any added inhibitory substances.

Techniques for Detecting Secondary Manifestations of Antigen-Antibody Interaction

Ouchterlony Technique

Double diffusion in two dimensions in agar gel, described first by Ouchterlony is a well known and widely used technique (160). In the present study the Ouchterlony reactions were performed in 1% agar gel prepared in 0.9% saline with merthiolate added as a preservative to a final concentration of 1 in 10,000. The agar sol was prepared by heating powdered Difco Noble agar in saline to the boiling point with continuous stirring. 10 ml of the clear sol was then poured into 60 x 15 mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.) and allowed to gel at room temperature. The patterns were cut in the gel plates so that the central and peripheral wells were spaced 1.0 cm apart from center to center. Each well was filled with 0.15 ml of test material on one occasion only. Initial incubation of the plates was carried out in a moist environment for 24 hr at 37°C and then for 24 hr at 4°C, after which time the plates were photographed. In some instances, Immuno-plate^{R} immunodiffusion plates (085-073 pattern C, Hyland, Costa Mesa, Calif.) with center to center distance between central and the peripheral wells of 5 mm, were used.

Passive Hemagglutination Technique

The titration of antibodies and reverse grouping of blood groups A and B were performed exactly according to standard blood bank technique (161,162).

CEA Fragments

The CEA was treated with Nagarse, a non-specific proteolytic enzyme for 24 hours at 40°C (163). The digest was chromatographed on Sephadex G-25, and the CEA-equivalent antigenic activity was determined by the Z-gel radioimmunoassay for CEA (101). Four active fragments designated GP-1, GP-2, GP-3, and GP-4 possessing CEA activity were examined.

CHAPTER IV

RESULTS

Molecular Sieve Radioimmunoassay

Figure 1 shows the pattern of radioactivity of the eluate obtained when ^{125}I-CEA was chromatographed alone, or when a serum specimen which apparently contained no antibodies capable of binding CEA was incubated with ^{125}I-CEA, and then chromatographed on Sepharose 6B.

Figure 2 shows the pattern of radioactivity of the eluate obtained when serum from an individual containing antibodies apparently capable of binding CEA was incubated with ^{125}I-CEA and the mixture chromatographed on a Sepharose 6B column. The serum specimen shown bound 14% of 0.5 ng of ^{125}I-CEA. The column void volume, measured with Blue Dextran 2000 (B.D.) is indicated by Vo in Figures 1 and 2. The molecular weight of moieties eluted in the first peak is of the order of 10^6 (based on 2×10^6 for B.D. and 2×10^5 for ^{125}I-CEA).

Figure 3 shows the binding with serum constituents in 48 subjects studied. Of this group of 48 sera, 17 were obtained from patients with digestive system cancers, 3 were from patients with cancers arising in other locations, 17 were from patients with inflammatory and degenerative diseases of all organ systems (and designated as "others") and 11 were from apparently healthy medical students (designated "normals").

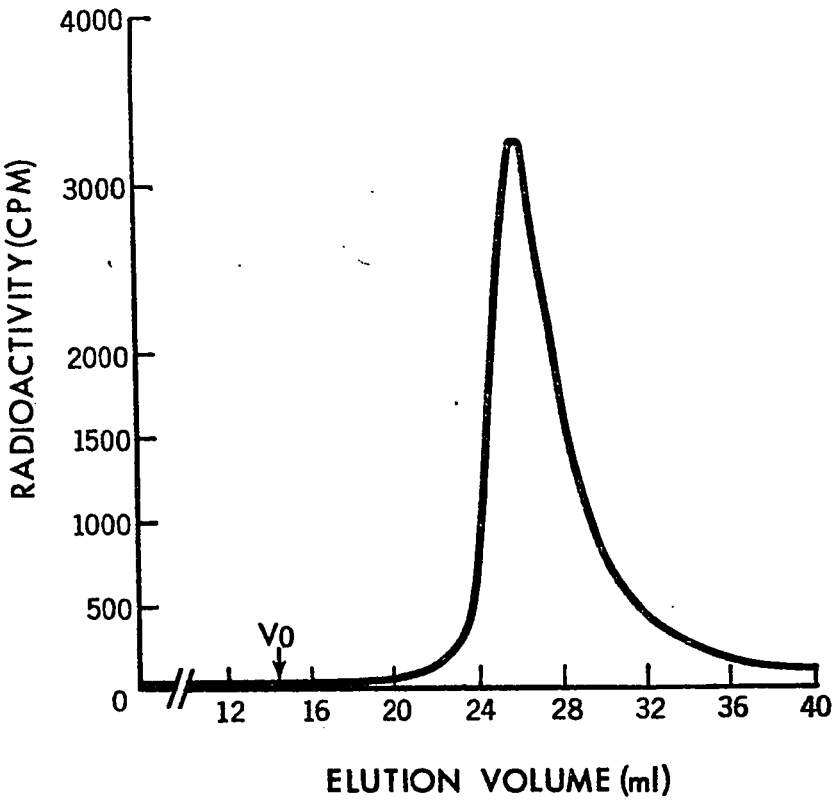

Figure 1 Molecular Sieve Radioimmunoassay. Pattern of eluate radioactivity obtained when 500 µl of serum apparently containing no molecular moieties capable of binding CEA was incubated with 0.5 ng ^{125}I-CEA and chromatographed on a 0.9 x 60 cm Sepharose 6B column. The radioactive peak eluted at a column volume of 26 ml represents free ^{125}I-CEA. Hence, this serum specimen did not demonstrate any binding to ^{125}I-CEA. An identical pattern was obtained when ^{125}I-CEA alone was chromatographed.

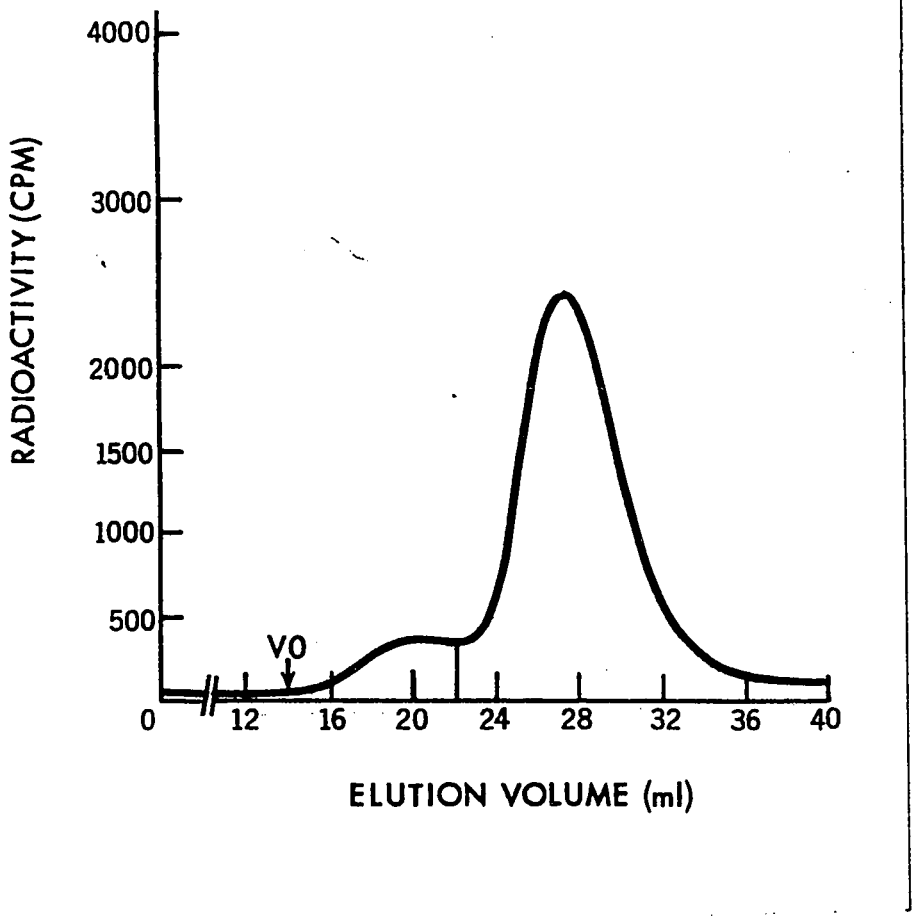

Figure 2 Molecular Sieve Radioimmunoassay. Pattern of radioactivity eluted when 500 µl of serum capable of binding ^{125}I-CEA was incubated with 0.5 ng ^{125}I-CEA and chromatographed on a 0.5 x 60 cm Sepharose 6B column. Vo indicates void volume, the first peak (20 ml) represents ^{125}I-CEA bound by molecular moieties present in this serum specimen and the last peak (26 ml) represents free ^{125}I-CEA. This serum specimen demonstrated a binding of 14% (0.07 ng) of the ^{125}I-CEA.

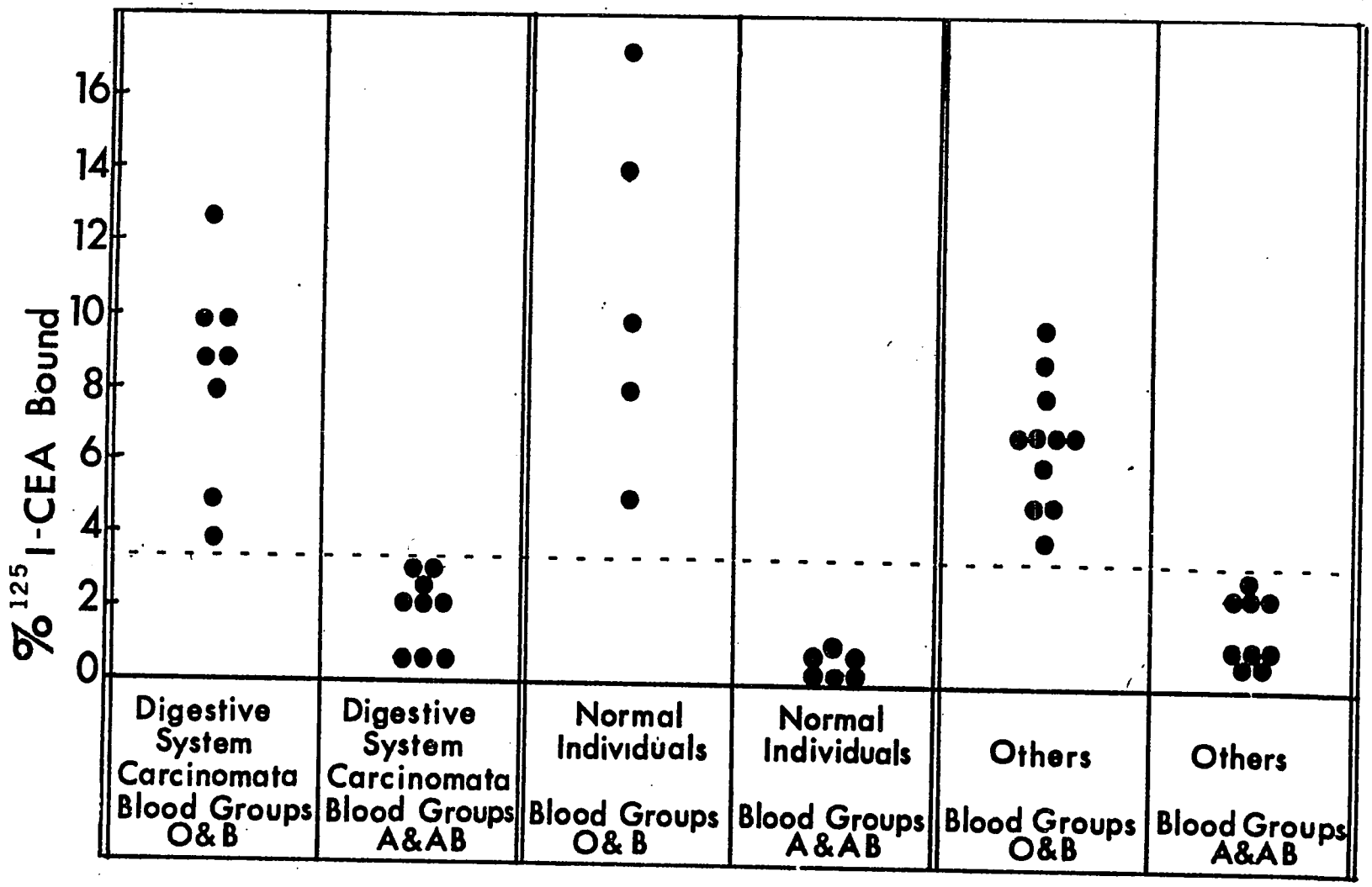
% 125 I-CEA Bound
16
14
12
10
8
6
4
2
0
Digestive System Carcinomata Blood Groups O&B
Digestive System Carcinomata Blood Groups A&AB
Normal Individuals Blood Groups O& B
Normal Individuals Blood Groups A&AB
Others Blood Groups O&B
Others Blood Groups A&AB

Figure 3 Molecular sieve radioimmunoassay. The quantity of ^{125}I-CEA bound by 500 µl aliquots of serum obtained from patients bearing digestive system cancers, from normal individuals and from patients with disease other than digestive system cancer. The capacity of sera containing anti-A antibodies to bind the radiolabeled CEA is clearly greater than that of sera without anti-A antibodies.

Anti-CEA antibodies appeared at first to be randomized amongst the cancer patients and the control subjects. When the data were tabulated according to the ABO blood groups of the individuals studied, however, an interesting pattern became apparent. The 24 sera which contained anti-A antibodies bound 8.2 ± 3.1% of 0.5 ng of ^{125}I-CEA. The 24 sera which did not contain anti-A antibodies bound 1.3 ± 1.2% of 0.5 ng of ^{125}I-CEA. The difference between the group of sera containing anti-A antibodies, from the group which did not, was significant at $p < 0.001$.

Figure 4 shows the diminution of binding measured when human ovarian cyst-derived A substance or CEA was included in the incubation mixture of anti-A and ^{125}I-CEA. Under the conditions described for Figure 2, an inhibition of 50% of the binding of ^{125}I-CEA was obtained with 60 µg of A and 120 µg of CEA.

To demonstrate that the binding in the sera containing anti-A antibodies was due to immunoglobulin moieties, the sera which had demonstrated binding to ^{125}I-CEA were incubated with either goat antiserum directed against human IgM or against human IgG. These antisera were absorbed extensively on blood group A erythrocytes in order to remove any anti-A activity. The preparation finally employed was free of such activity as demonstrated by hemagglutination. Moreover, these antiserum preparations were shown to be free of any trace of anti-CEA activity by the molecular sieve radioimmunoassay technique. As expected, treatment of the anti-A ^{125}I-CEA mixture with anti-IgM caused a displacement of

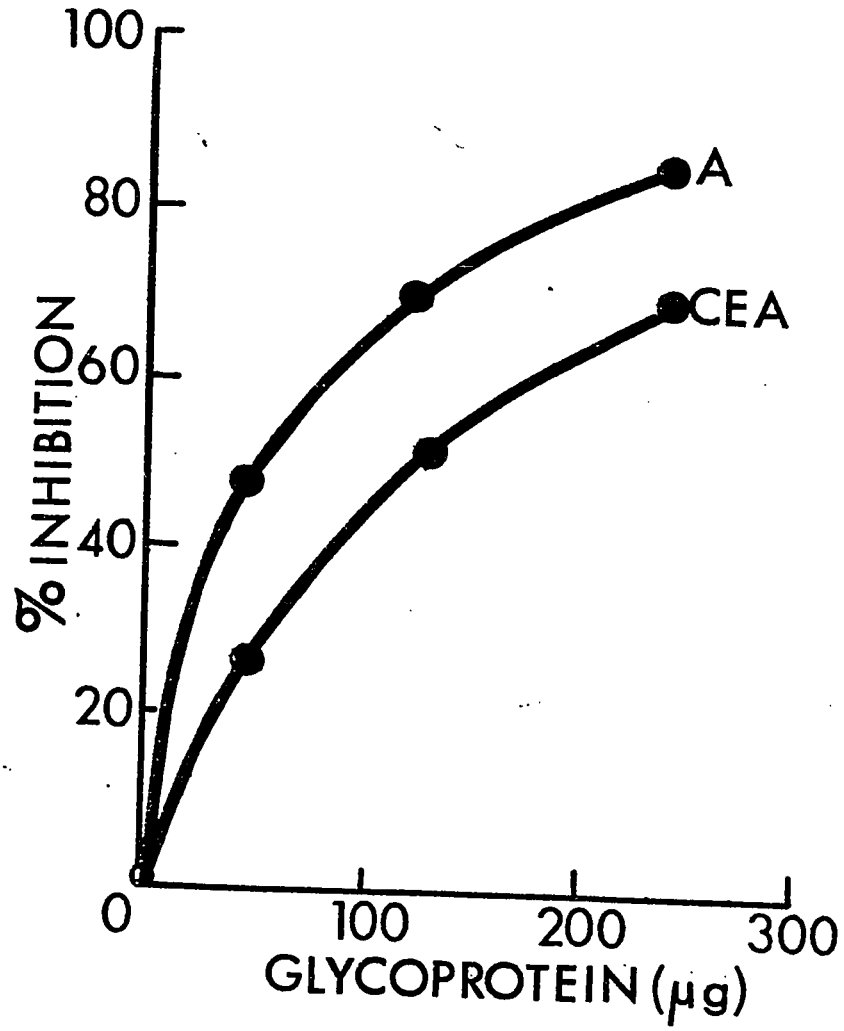

Figure 4 Molecular Sieve Radioimmunoassay. The inhibition of binding of serum containing anti-A antibodies to 0.5 ng ^{125}I-CEA by ovarian cyst-derived A substance and by CEA.

the higher molecular weight peak (Figure 2) towards the void volume of the column by 2.0 to 4.0 ml. Treatment of the mixture of serum from a patient capable of binding ^{125}I-CEA with anti-IgG, caused a broadening of the ^{125}I-CEA peak when that patient had been previously immunized with A substance. No such change was demonstrable with the IgG fractions obtained from O or B individuals who had not been actively immunized with A substance.

Blood group substance binding activity has been reported with a number of the plant lectins (164). Hence, Ulex europeus, Dolichos bifloris and Concanavalin A were studied in this system. None of these materials were capable of binding ^{125}I-CEA by this technique.

Radioimmunoelectrophoresis (RIEP)

To confirm the data obtained by the molecular sieve radioimmunoassay, and to look for anti-CEA antibodies by another method, the radioimmunoelectrophoresis technique was employed.

Initial studies utilizing whole anti-normal human serum antibodies indicated that the binding observed was due to immunoglobulin moieties in the sera under investigation. These studies demonstrated that the technique employing cellulose acetate membranes gave less radioactive background than that employing agar gel. Therefore, the studies were performed utilizing the cellulose acetate membrane technique with monospecific anti-IgA, IgG and IgM antisera. An example of the RIEP technique with the cellulose acetate membranes is shown in Figure 5.

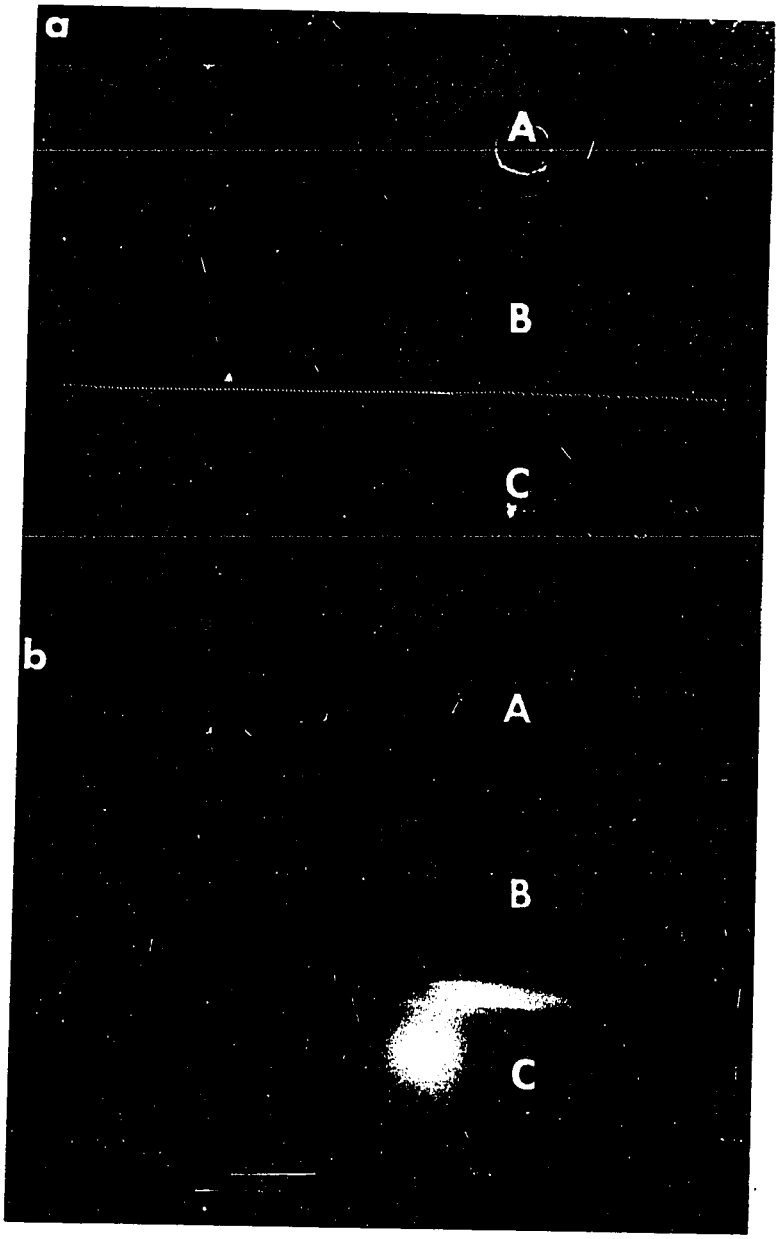

Figure 5 (a) Immunoelectrophoretic pattern in which Well A contained serum from a patient of blood group O which had been thoroughly absorbed on blood group A erythrocytes, Well C contained the same serum before absorption, and the Central Trough B contained goat anti-human IgM. Despite the immunospecificity of the anti-IgM antiserum, fragmentation of the single band which developed was invariably seen on the supporting medium employed.

(b) Radioautograph of the immunoelectrophoretogram shown in (a).

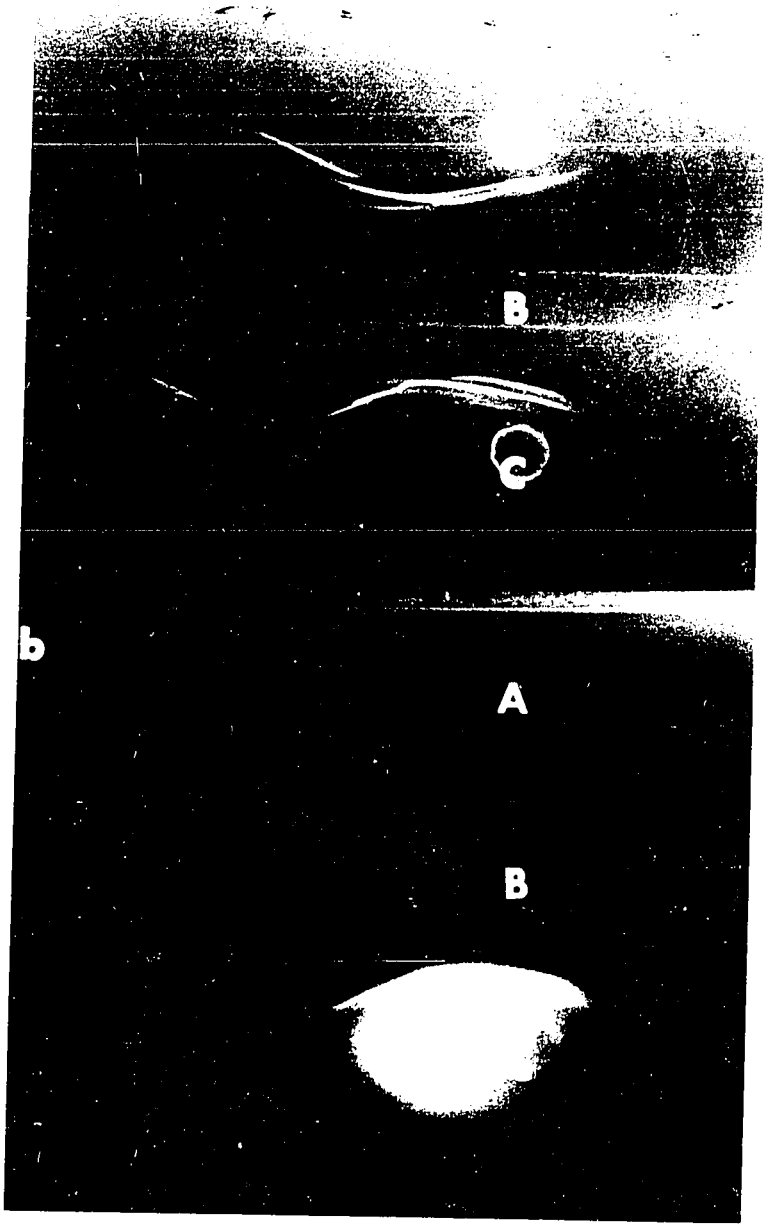

Figure 5 (a) Immunoelectrophoretic pattern in which Well A contained serum from a patient of blood group O which had been thoroughly absorbed on blood group A erythrocytes, Well C contained the same serum before absorption, and the Central Trough B contained goat anti-human IgM. Despite the immunospecificity of the anti-IgM antiserum, fragmentation of the single band which developed was invariably seen on the supporting medium employed.

(b) Radioautograph of the immunoelectrophoretogram shown in (a).

Again the data, shown in Tables I and II, were tabulated according to the ABO blood group of the individuals studied. Of the 91 sera examined, 54 of the donors were of either blood group O or B and contained anti-A antibodies as measured by hemagglutination and 37 of the sera were from patients of blood groups A or AB and did not contain anti-A antibodies. Using the RIEP technique described above, 43 of the 54 sera which contained anti-A antibodies showed IgM binding to ^{125}I-CEA, but only 8 of the 37 sera without anti-A antibodies demonstrated this phenomenon. Of the 8 sera without anti-A antibodies which demonstrated IgM binding to ^{125}I-CEA, 4 were from patients with metastatic digestive system cancers, 3 were from pregnant women and 1 was from a patient with widely disseminated cancer of the urinary bladder.

IgG binding to ^{125}I-CEA was observed in only 20 sera, all of which contained anti-A antibodies and which had demonstrated IgM binding to ^{125}I-CEA. No IgA binding to ^{125}I-CEA was observed in any of the sera studied.

Sufficient serum was available for further study of 25 of the 45 sera which contained anti-A antibodies and had demonstrated IgM binding to ^{125}I-CEA. Following absorption with blood group A erythrocytes, only 2 of the 25 samples demonstrated the persistence of IgM antibodies capable of binding ^{125}I-CEA. Both of these active, post-absorption sera were from patients suffering from widely disseminated digestive system cancers. Absorption of the still available sera from the 8 which did not have anti-A antibodies but which gave positive results by RIEP failed to reduce

TABLE I

Radioimmunoelectrophoresis with ^{125}I-CEA: Patients of Blood Type O, B. (Sera Containing Anti-A)

Patients	Unabsorbed Sera		Sera Absorbed on Blood Group A Erythrocytes*	
	Number Studied	Number Demonstrating IgM Binding to ^{125}I-CEA	Number Studied	Number Demonstrating IgM Binding to ^{125}I-CEA
Digestive system cancers	12	11	10	2
Pregnant women	3	2	2	0
Others	39	30	13	0
TOTALS	54	43	25	2

*Sera absorbed with blood group O or B erythrocytes gave results similar to unabsorbed sera.

TABLE II

Radioimmunoelectrophoresis with ^{125}I-CEA: Patients of Blood Type A, AB. (Sera Not Containing Anti-A)

Patients	Unabsorbed Sera		Sera Absorbed on A Erythrocytes*	
	Number Studied	Number Demonstrating IgM Binding to ^{125}I-CEA	Number Studied	Number Demonstrating IgM Binding to ^{125}I-CEA
Digestive system cancers	7	4	2	2
Pregnant women	6	3	N.D.	N.D.
Controls	24	1	1	1
TOTALS	37	8	3	3

*Sera absorbed with blood group O or B erythrocytes gave results similar to unabsorbed sera.

N.D. = not done

the capacity of the IgM fraction of these sera to bind ^{125}I-CEA. Of these 3 sera, 2 were from patients with widely disseminated digestive system cancer and 1 was from the patient with disseminated cancer of the urinary bladder.

Similar treatment of active sera with blood group O or B erythrocytes failed to alter the RIEP results from those obtained prior to absorption.

Farr Technique after Acid Treatment of the Serum Preparations

Sufficient serum was available from 74 of the patients investigated by RIEP to allow study by means of the Farr technique after acid treatment (Figure 6). When the data were divided according to the presence or absence of anti-A antibodies in the sera studied, a familiar pattern emerged.

The 46 sera which contained anti-A antibodies bound 8.6 ± 4.7 per cent of 0.5 ng of ^{125}I-CEA. The 28 sera which contained no anti-A antibodies bound 2.4 ± 2.3 per cent of 0.5 ng of ^{125}I-CEA. The difference between the two groups was significant at $p < 0.005$.

Of the 46 sera which contained anti-A antibodies, 32 (70%) demonstrated binding greater than or equal to 5% of the ^{125}I-CEA. Twenty-six of these sera showed IgM binding to ^{125}I-CEA by RIEP and 6 did not. Of the remaining 14 sera which contained anti-A antibodies, but which bound less than 5%, of the ^{125}I-CEA, 12 showed IgM binding by RIEP and 2 did not.

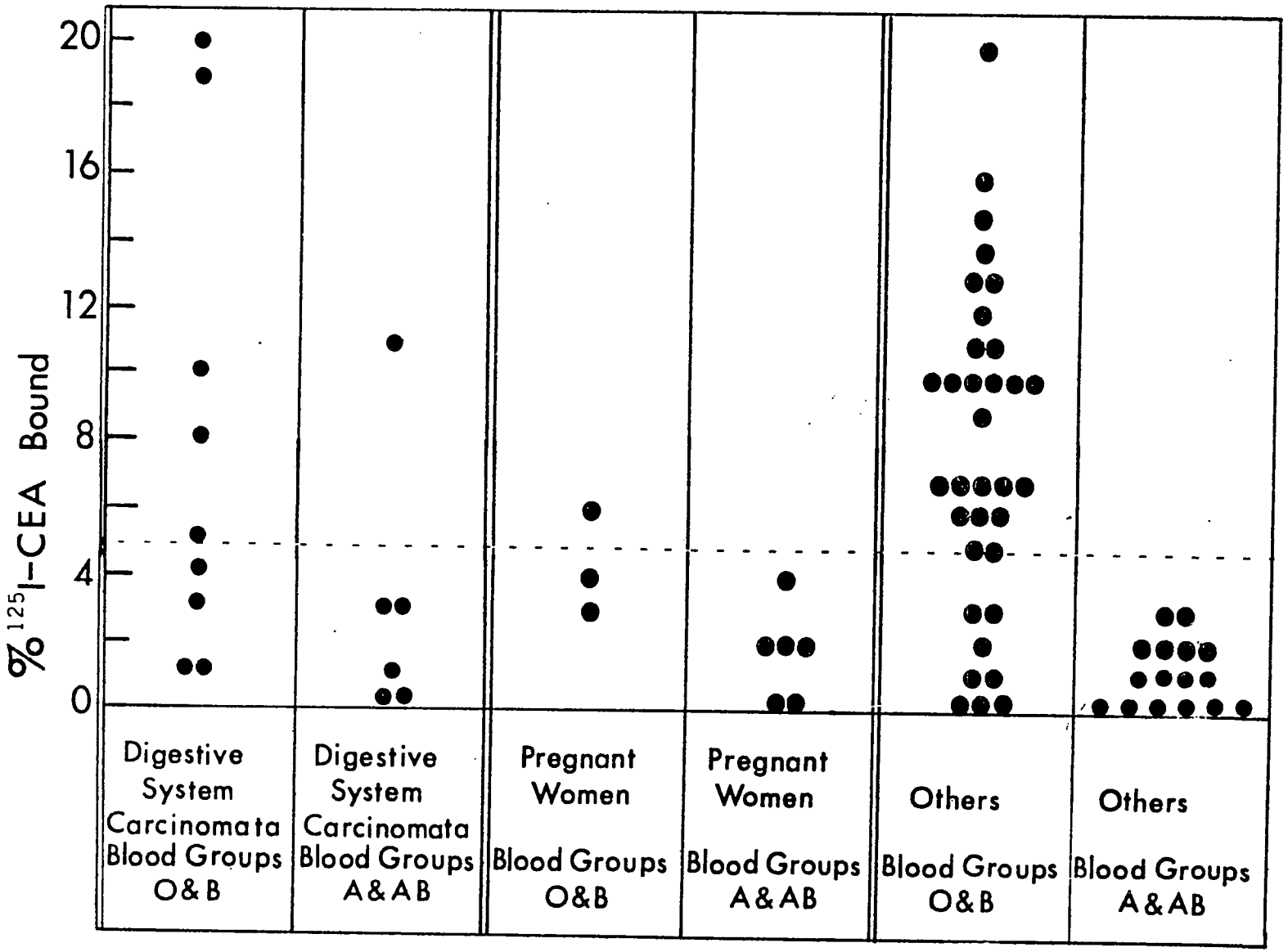
20
16
12
8
4
0
% 125I-CEA Bound
Digestive System Carcinomata Blood Groups O&B
Digestive System Carcinomata Blood Groups A&AB
Pregnant Women Blood Groups O&B
Pregnant Women Blood Groups A&AB
Others Blood Groups O&B
Others Blood Groups A&AB

Figure 6 Farr Technique after acid treatment. The quantity of ^{125}I-CEA bound (% of 0.5 ng) by 500 µl aliquots of prepared serum obtained from patients bearing digestive system cancers, from normal individuals, and from patients with diseases other than digestive system cancer. A binding value of 5% separates sera with anti-A activity from those without such activity.

Of the 28 sera which contained no anti-A antibodies, only 1, obtained from a patient with metastatic pancreatic carcinoma, bound more than 5% of the ^{125}I-CEA. This serum specimen demonstrated IgM binding to ^{125}I-CEA by RIEP. Furthermore, 7 of the 8 sera which contained no anti-A antibodies, but showed IgM binding by the RIEP, did not demonstrate this phenomenon when the Farr technique after acid treatment was employed.

The results of the Farr technique without acid treatment, performed to measure free antibody binding to ^{125}I-CEA and as a control for the preceding study, are shown in Figure 7. These data show that the sera containing anti-A antibodies bound 5.5 ± 4.8% of the 0.5 ng of the ^{125}I-CEA and the sera which contained no anti-A antibodies bound 2.5 ± 1.3% of the 0.5 ng of ^{125}I-CEA. The difference between the two groups was significant at $p < 0.05$.

The Binding of Purified Human Anti-A Antibodies to ^{125}I-CEA

Anti-A antibodies, prepared by adsorption to and elution from human blood group A erythrocyte ghosts, had a hemagglutination titer of 1:1024 against A erythrocytes. When this preparation was studied for binding to ^{125}I-CEA by the Farr technique, a maximal net binding of 45% of 0.5 ng of ^{125}I-CEA was demonstrated (Figure 8). The failure to reach a plateau at the upper level of binding indicates that the maximal level of binding achieved was antibody-limited.

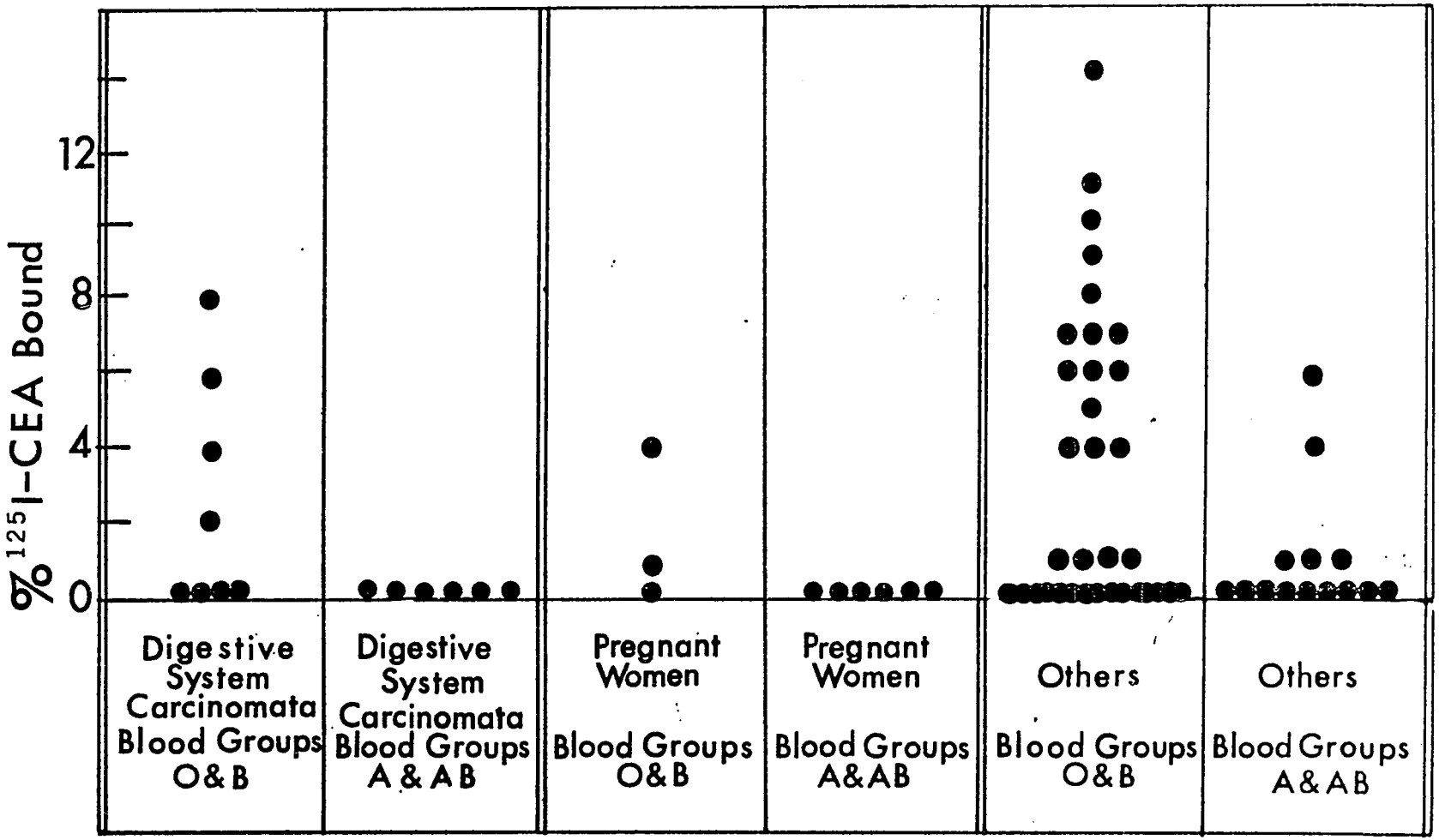
% 125I-CEA Bound
12
8
4
0
Digestive System Carcinomata Blood Groups O&B
Digestive System Carcinomata Blood Groups A&AB
Pregnant Women Blood Groups O&B
Pregnant Women Blood Groups A&AB
Others Blood Groups O&B
Others Blood Groups A&AB

Figure 7 Farr Technique without acid treatment. To measure free antibodies capable of binding to ^{125}I-CEA, the Farr technique was performed without the procedure of acid treatment of the serum specimens. The quantity of ^{125}I-CEA bound by a 500 µl aliquot of prepared serum was, in each instance, less than the quantity of ^{125}I-CEA bound when the serum preparation was acid treated.

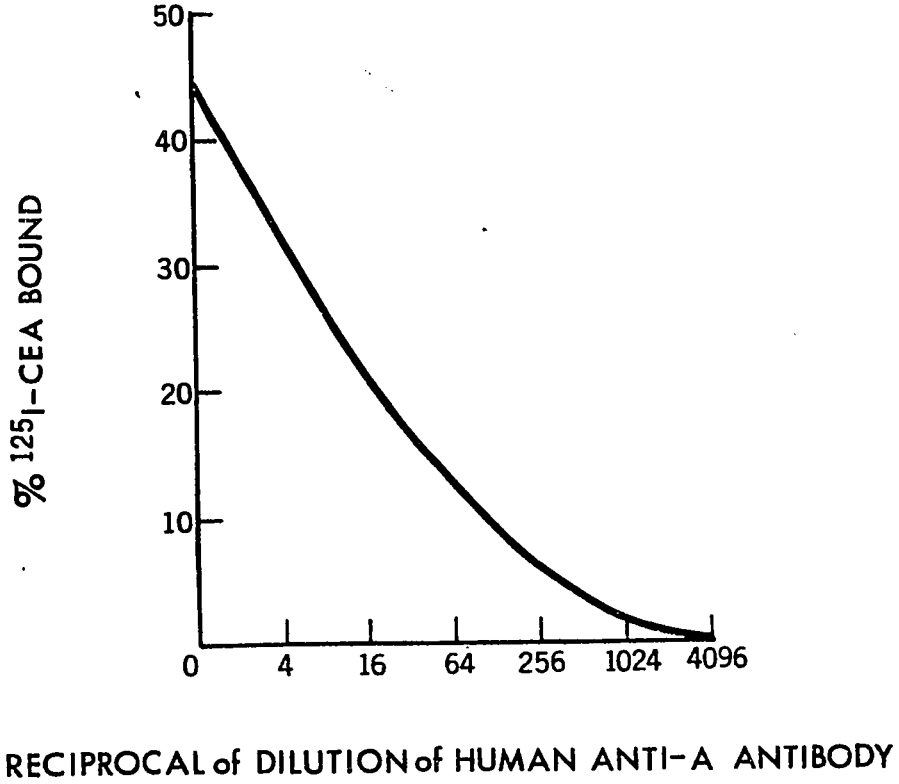

Figure 8 Titration curve of the concentrated human anti-A antibody preparation with 0.5 ng ^{125}I-CEA. A maximal net binding of 45% was obtained. The failure to reach a plateau at the upper level of binding indicates that the titration was antibody-limited, or that the limiting factor in the titration was a deficiency of anti-A antibody rather than a deficiency of binding sites on the ^{125}I-CEA.

Solid Phase-Coupled Antibody Radioimmunoassay

The Titration of Sepharose-Coupled Anti-A with ^{125}I-A and ^{125}I-CEA

The binding of Sepharose-coupled anti-A antibodies to ^{125}I-A, and ^{125}I-CEA is shown in Figures 9 and 10 respectively.

The maximal binding of anti-A to ^{125}I-A was 50% of 0.5 ng of ^{125}I-A (specific activity approximately 7 x 10^6 mCi/mM). The maximal binding of anti-A to ^{125}I-CEA, at 11% of 0.5 ng of ^{125}I-CEA (specific activity approximately 7 x 10^6 mCi/mM) appeared to be antibody-limited.

Inhibition of anti-A-^{125}I-A and anti-A-^{125}I-CEA Binding by A and CEA

Inhibition studies of the anti-A-^{125}I-A systems were performed in the zone of antigen excess, at the point of 30% binding of 0.5 ng of ^{125}I-A (Figure 11). The comparable studies of the anti-A-^{125}I-CEA system (Figure 12), also performed in antigen excess, utilized a level of 6% binding of 0.5 ng of ^{125}I-CEA. One hundred-fold more CEA than A, on a weight per volume basis was required to inhibit the binding of anti-A to ^{125}I-A. By contrast, the inhibition of binding of anti-A to ^{125}I-CEA by either A or by CEA was similar at all concentrations of these materials on a weight basis.

Inhibition of Anti-A-^{125}I-A and Anti-A-^{125}I-CEA Binding by Simple Sugars

The inhibition of binding of anti-A to ^{125}I-A (0.15 ng)

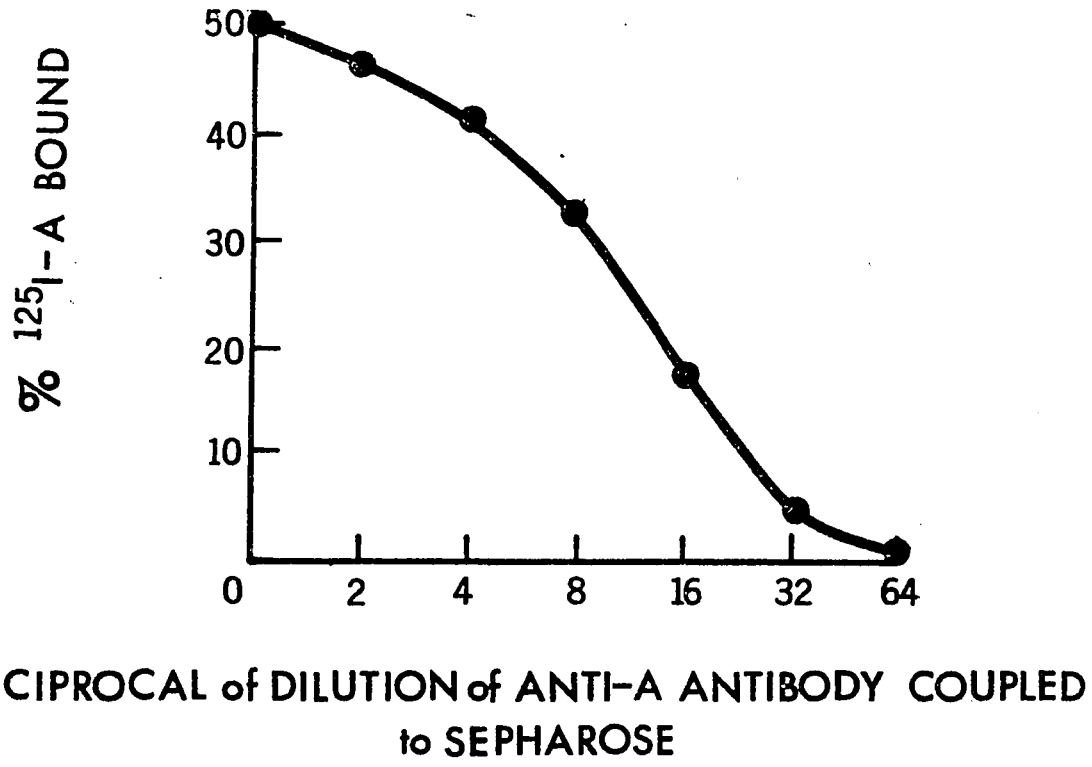

Figure 9 Titration curve of the Sepharose-coupled, high-titer human anti-A antibody preparation with 0.5 ng ^{125}I-A substance (human ovarian cyst-derived). A maximal net binding of 50% was obtained. Although the slope of the curve diminished as the concentration of anti-A antibodies increases, the maximal binding appears to be antibody-limited.

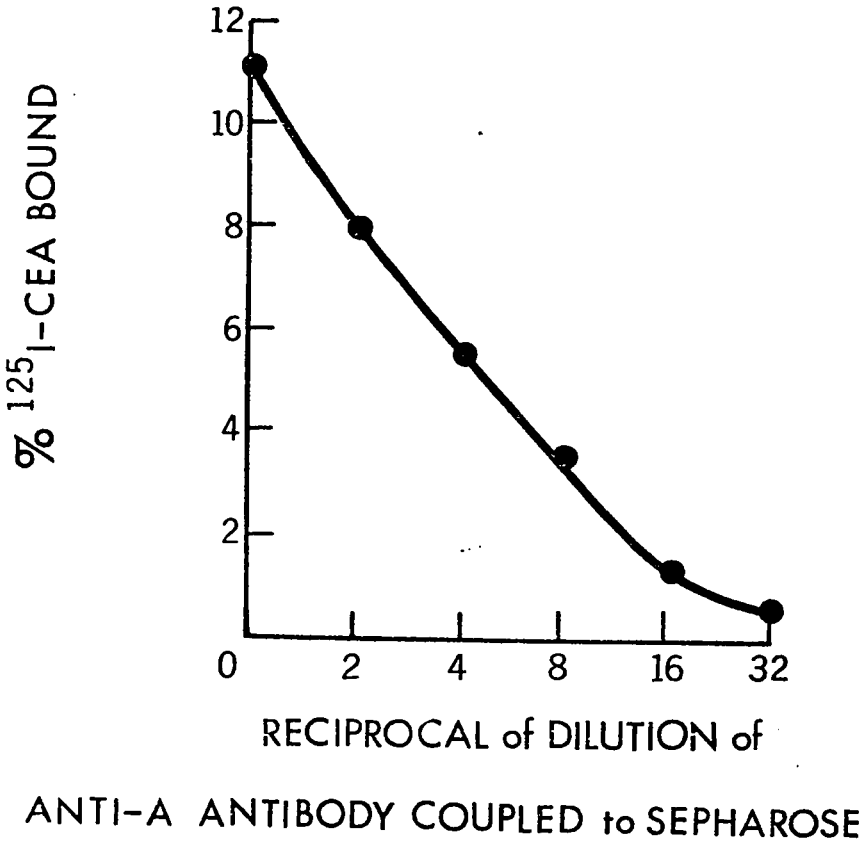

Figure 10 Titration curve of the Sepharose-coupled, high titer human anti-A antibody preparation with 0.5 ng ^{125}I-CEA. The maximal net binding attained was 11% and was antibody-limited. At this concentration, however, the antibody preparation was capable of binding 50% of 0.5 ng of ^{125}I-A substance.

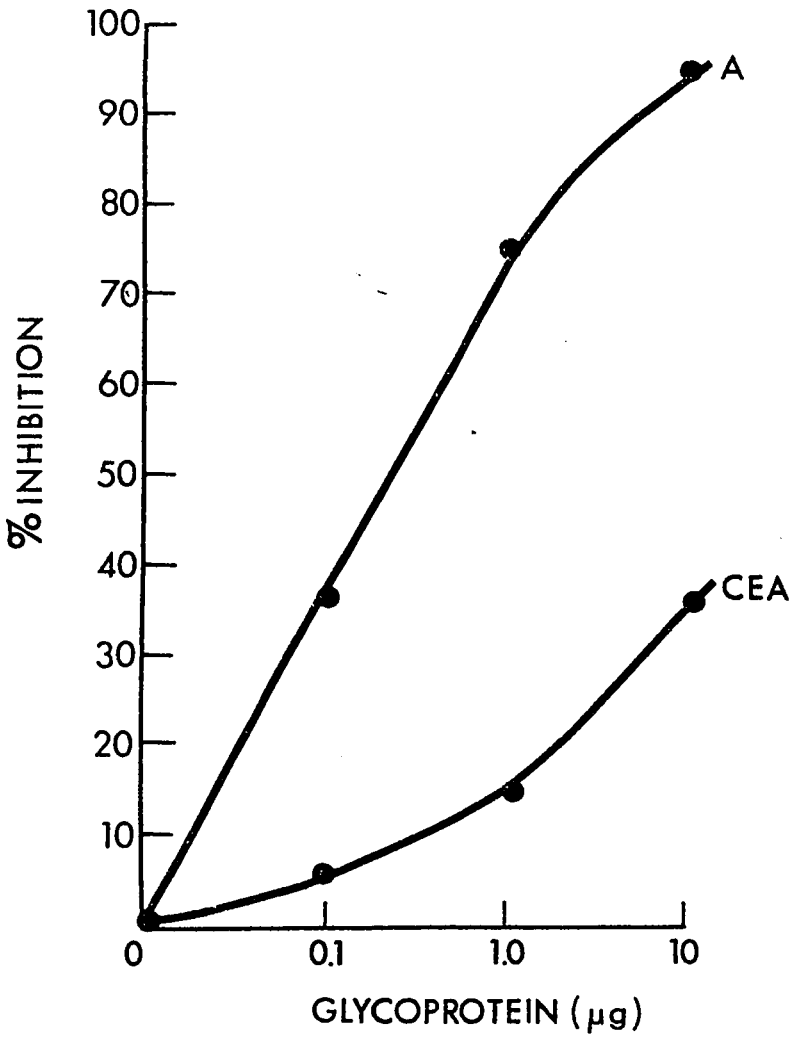

Figure 11 The inhibition of binding of the Sepharose-coupled anti-A antibody preparation to ^{125}I-A by CEA and A. These studies were performed at 30% binding of 0.5 ng ^{125}-A (see Fig. 9). One hundred-fold more CEA than A substance by weight is required to inhibit binding to an equivalent extent in the ^{125}I-A-anti-A system.

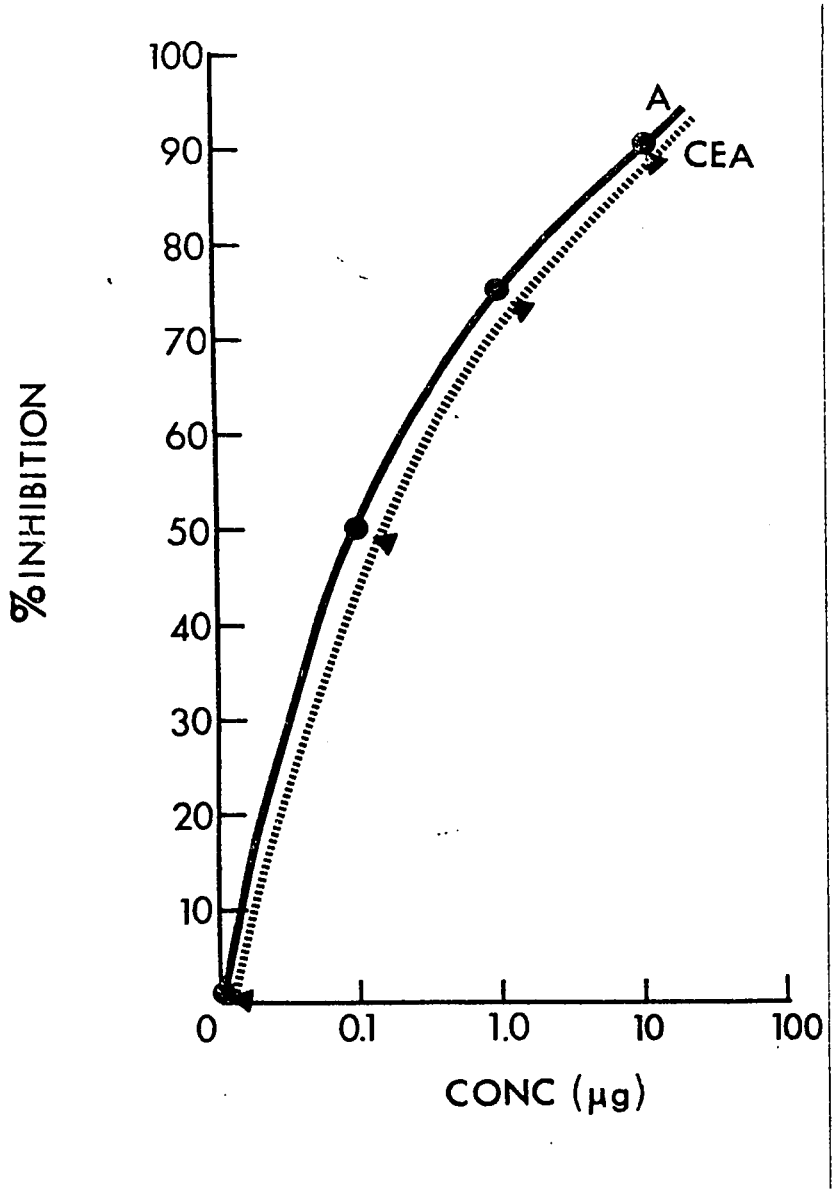

Figure 12 The inhibition of binding of the Sepharose-coupled anti-A antibody preparation to ^{125}I-CEA by A substance and CEA. These studies were performed at 6% binding of 0.5 ng ^{125}I-CEA (see Fig. 10). This inhibition by equal weights of CEA and A substance was similar in the range tested.

and anti-A to ^{125}I-CEA (0.03 ng) by a variety of monosaccharides and disaccharides is shown in Figures 13 and 14, respectively. The sugars which did not significantly inhibit binding are shown in Table III. In both studies, N-acetyl-D-galactosamine gives a sigmoid-shape inhibition curve over the range of 10^{-2} to 10^{2} mM. The maximum inhibition measured in the anti-A-^{125}I-A system was 80% and in the anti-A-^{125}I-CEA system was 70%. D-glucosamine and D-galactosamine also inhibited binding in both these systems, however, 100- to 1000 times the concentration of these sugars was necessary to produce the same degree of inhibition as that obtained with N-acetyl-D-galactosamine.

Inhibition of Anti-A-^{125}I-A Binding by CEA Fragments

The inhibition of binding of anti-A to ^{125}I-A by CEA fragments, obtained from Nagarse digestion (86) of CEA and subsequent Sephadex G-25 chromatography is shown in Figure 15. The assay system differed from the one employed above in that the anti-A antibodies were coupled to Sephadex and the inhibition studies were performed under conditions where the binding of ^{125}I-A by anti-A was 6% of 0.5 ng of ^{125}I-A. These studies demonstrated that the CEA fragment GP-1, having a molecular weight of approximately 4000 Daltons was capable of inhibiting the binding of anti-A to ^{125}I-A by 45% at a molar concentration of 1000 to 10,000 times less than that required for equivalent inhibition by N-acetyl-D-galactosamine. The maximal inhibition achieved with GP-1, however, was 46%. The other fragments tested demonstrated significantly less maximal inhibition,

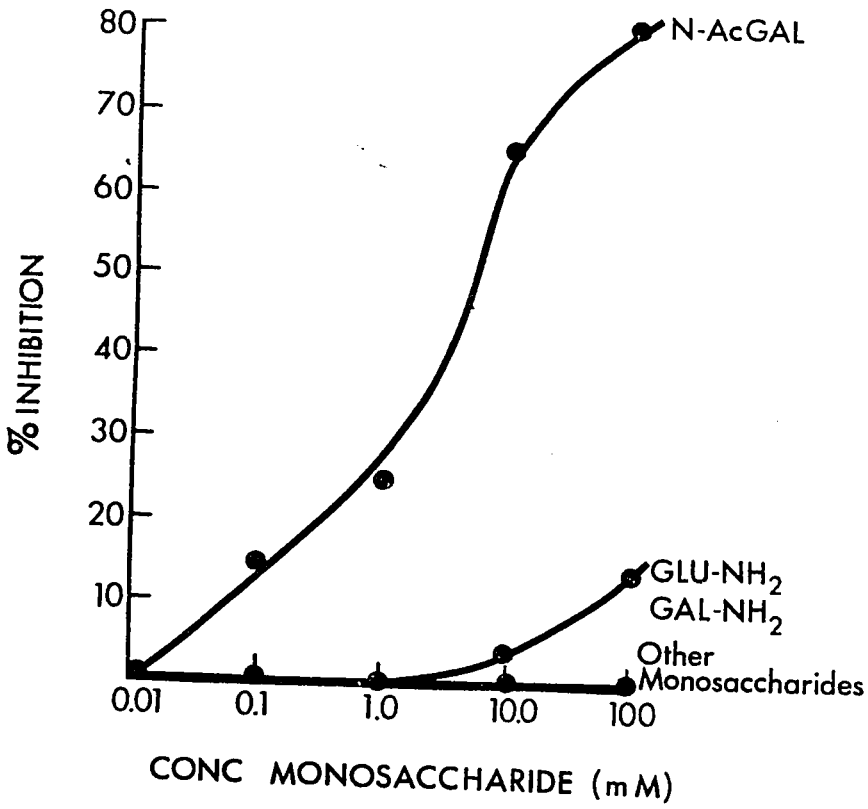

Figure 13 Binding-inhibition of ^{125}I-A to Sepharose-coupled anti-A produced by monosaccharides. These studies were performed with conditions which produced the curve in Fig. 11, at the point at which 30% of 0.5 ng ^{125}I-A was bound. If no alteration in binding was produced by a monosaccharide, then 0% inhibition is said to have occurred. N-acetyl-D-galactosamine was the only sugar demonstrating significant inhibition in the range of concentrations tested. D-glucosamine and D-galactosamine were capable of some inhibitory activity, but only at 100- to 1000 times the concentration of N-acetyl-D-galactosamine, required. The "other monosaccharides", which failed to show any inhibitory activity, are listed in Table III.

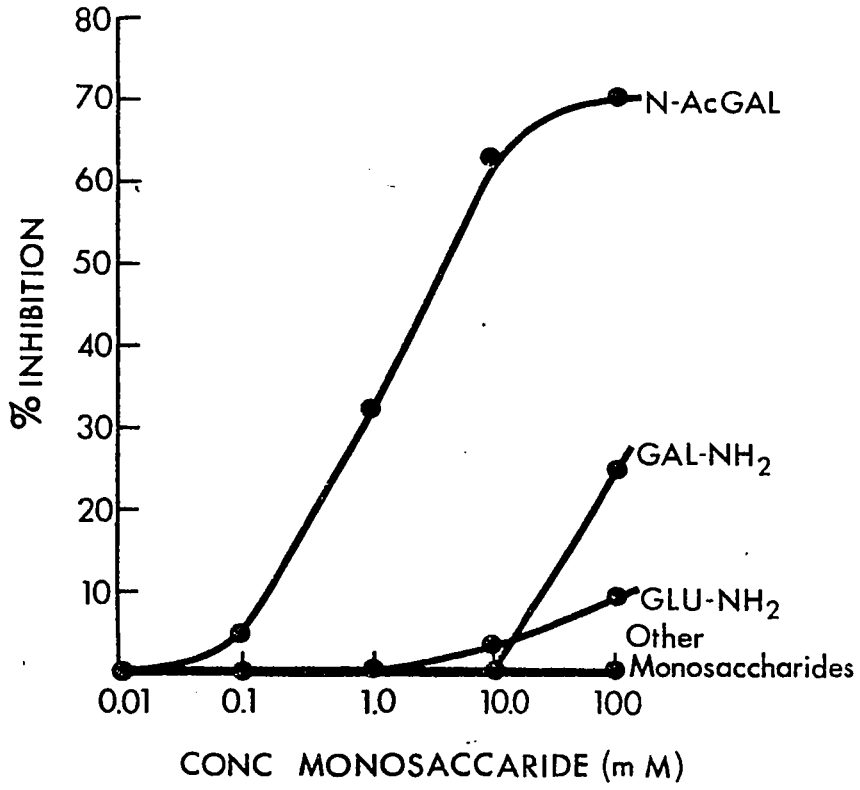

Figure 14 Binding-inhibition of Sepharose-coupled anti-A binding to ^{125}I-CEA by simple sugars. These studies were performed with the conditions which produced the curve in Fig. 12, at the point at which 6% of 0.5 ng ^{125}I-CEA was bound. If no alteration in binding was produced by a monosaccharide, then 0% inhibition is said to have occurred. N-acetyl-D-galactosamine was the only sugar demonstrating significant inhibition in the range of concentrations tested. D-glucosamine and D-galactosamine were capable of some inhibitory activity, but only at 100- to 1000 times the concentration of N-acetyl-D-galactosamine, required. The "other monosaccharides", which failed to show any inhibitory activity, are listed in Table III.

TABLE III

Sugars which Failed to Inhibit the Binding of Anti-A to ^{125}I-A or ^{125}I-CEA to any Extent in the Range of the Concentrations Employed

D-glucose, D-galactose, L-fucose, D-fucose, D-mannose, D-arabinose

N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, D-fructosamine, N-acetyl-neuraminic acid

2-Deoxy-D-glucose, 3-O-methyl-D-glucose, Methyl β-D-glucopyranoside, Methyl β-D-galactopyranoside, Methyl α-D-mannose

α-D-melibiose, β-D-gentibiose, D-raffinose

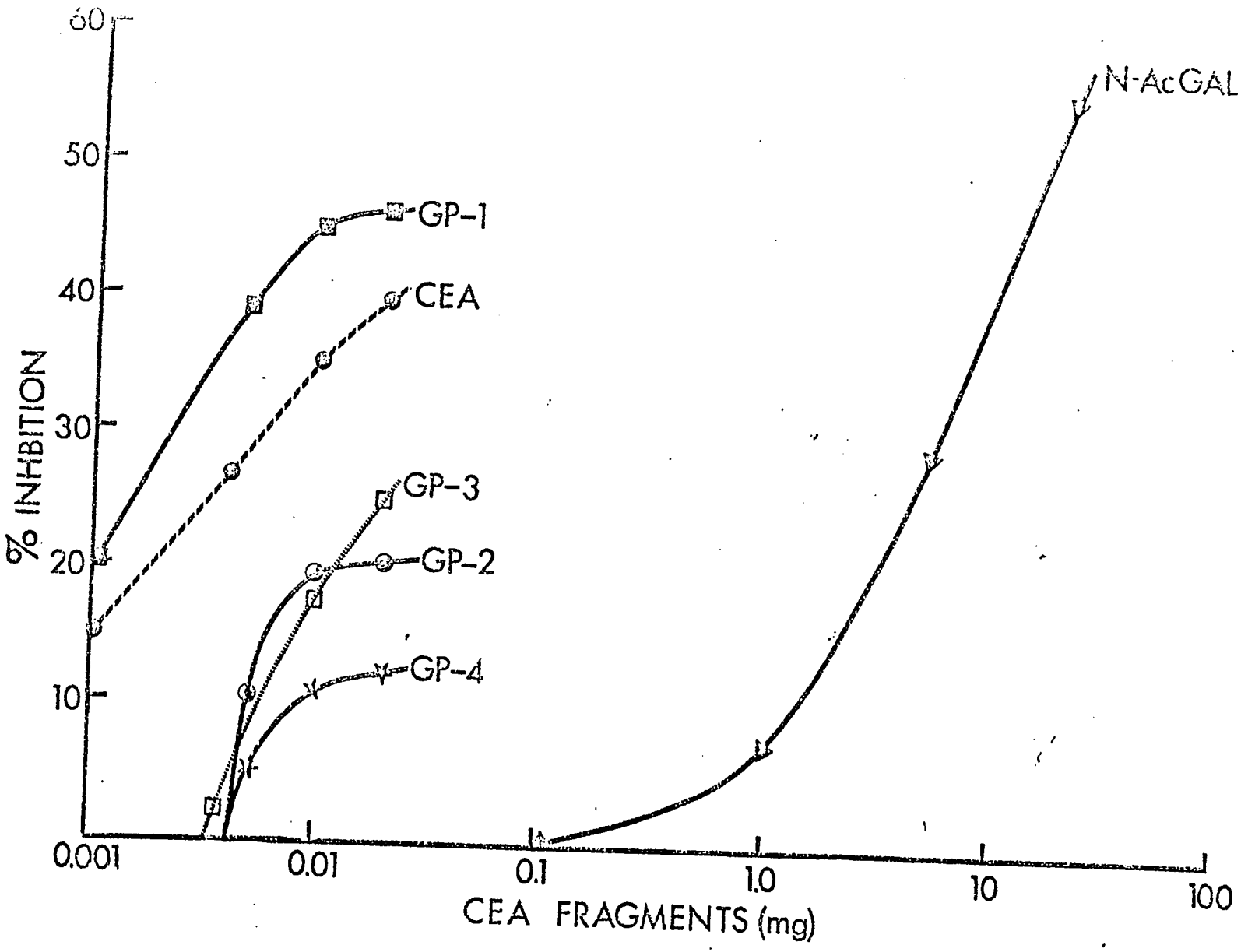
60
50
40
30
20
10
% INHBITION
GP-1
CEA
GP-3
GP-2
GP-4
N-AcGAL
0.001
0.01
0.1
1.0
10
100
CEA FRAGMENTS (mg)

Figure 15 Binding-inhibition of human anti-A antibody (coupled to Sephadex) to ^{125}I-A substance by CEA-derived glycopeptides (GP) and N-acetyl-D-galactosamine. The studies were performed under conditions at which 6% of 0.5 ng ^{125}I-A was bound. As the molecular weight of GP-1 is approximately 4000 Daltons, the graphs indicate that 10^3 to 10^4 moles less of GP-1 than N-acetyl-D-galactosamine is required to attain equivalent inhibition in the test system under consideration. However, the maximal degree of inhibition achieved with GP-1 was 46%.

although the slope of the inhibition curve of the fragment designated GP-3 was similar to those of fragment GP-1 and N-acetyl-D-galactosamine. The monosaccharides D-galactosamine, D-mannose, N-acetyl-D-glucosamine and L-fucose, which were tested, demonstrated no inhibition.

The Titration of Sephadex-Coupled Anti-CEA with ^{125}I-CEA and ^{125}I-A

The binding curves of Sephadex-coupled G-81 abs anti-CEA antibodies to ^{125}I-CEA and to ^{125}I-A are shown in Figures 16 and 17, respectively. In neither system did the antibody titration curve reach a plateau. Hence, even at the highest antibody concentrations, the maximal binding was antibody-limited. For anti-CEA binding to ^{125}I-CEA the maximal binding achieved was 65% of 0.5 ng ^{125}I-CEA. For anti-CEA binding to ^{125}I-A, the maximal binding achieved was 6% of 0.5 ng of ^{125}I-A.

Inhibition of Anti-CEA-^{125}I-CEA Binding by Materials Possessing Blood Group ABH Antigenic Activity

The studies of the inhibition of binding of anti-CEA to ^{125}I-CEA by CEA and ovarian cyst-derived A, B, H, Le^a and pneumococcal polysaccharide type XIV were performed under conditions in which 30% binding of 0.5 ng of ^{125}I-CEA occurred in the absence of any inhibitor (Figure 18). These studies demonstrated that to achieve 50% inhibition in this system, 100 to 1000 more of each of the blood group substances was required as compared to CEA. The pneumococcus-derived material did not show any inhibitory activity at the concentrations employed.

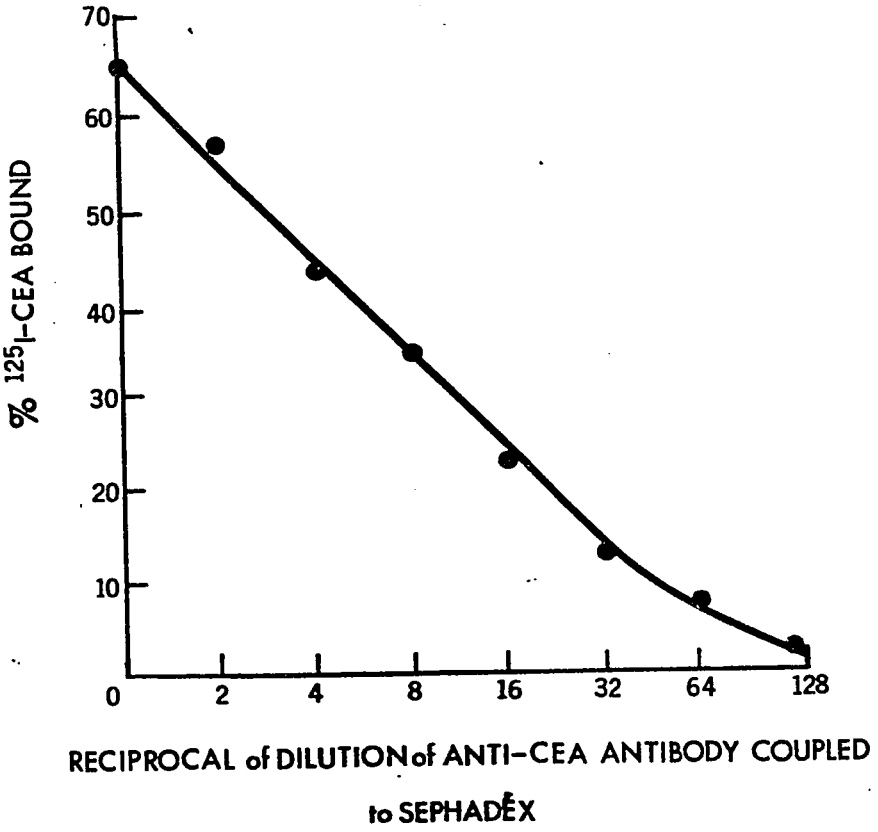

Figure 16 The titration curve of goat anti-CEA (G-81 abs coupled to Sephadex) with 0.5 ng ^{125}I-CEA. The maximal binding of 65% attained, appears to be antibody-limited.

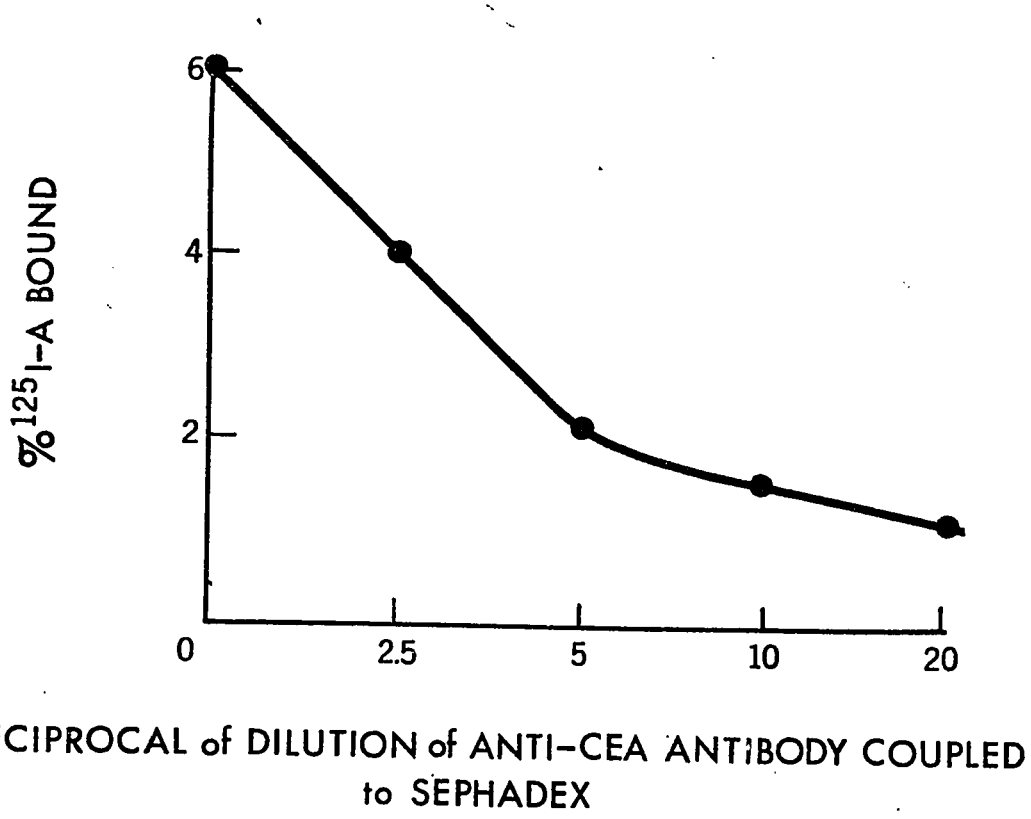

Figure 17 The titration curve of goat anti-CEA antibody (G-81 abs coupled to Sephadex) with 0.5 ng ^{125}I-A substance. The maximal binding of 6% attained appears to be antibody-limited.

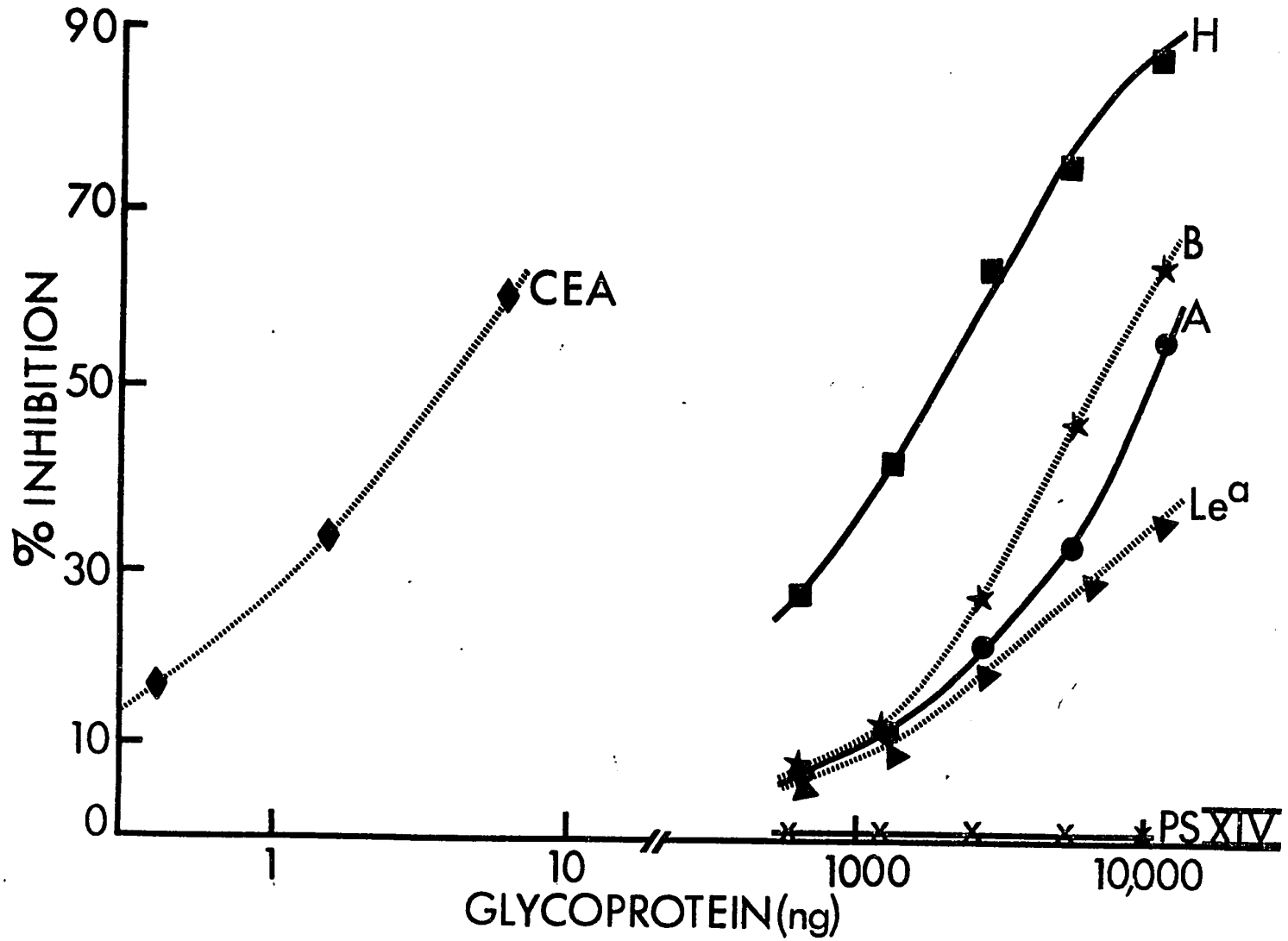
90
70
50
30
10
0
% INHIBITION
CEA
H
B
A
Le^a
PS XIV
1
10
1000
10,000
GLYCOPROTEIN (ng)

Figure 18 The inhibition of binding of goat anti-CEA (G-81 abs) to ^{125}I-CEA by CEA, ovarian cyst-derived materials with A, B, H and Le^a reactivity, and pneumococcal polysaccharide type XIV. These measurements were made by the solid phase coupled antibody radioimmunoassay, and similar data were obtainable when the Farr technique was employed. The ovarian cyst-derived materials were 100 to 1000 less potent on a weight basis than CEA in inhibiting binding in this system.

Inhibition of Anti-CEA-^{125}I-CEA and Anti-CEA-^{125}I-A Binding by Simple Sugars

Attempts to demonstrate mono- and oligosaccharide inhibition of the binding of anti-CEA to ^{125}I-CEA with all the sugars used previously (Table III), as well as the tetrasaccharide, stachyose, were unsuccessful. By contrast, 100 mM N-acetyl-D-galactosamine inhibited the binding of anti-CEA to ^{125}I-A (studies performed at 5% binding of 0.5 ng of ^{125}I-A) by 30%. The other sugars tested (Table III) were incapable of inhibiting this system.

The Effect of the Method of Purification on the A-Like Site on the CEA Molecule

A CEA-enriched preparation was obtained from a crude, aqueous tumor extract by immunoprecipitation with anti-CEA antiserum followed by chromatography of the solubilized precipitate on CMC. The tumor tissue consisted of hepatic metastases from a gastric carcinoma-bearing patient of blood group O.

The spectrophotometric elution pattern and the eluate volume containing CEA activity, as determined by Ouchterlony reactions against anti-CEA antiserum, is shown in Figure 19. When this CMC-CEA was examined with monospecific goat anti-CEA, it gave lines of complete identity with standard CEA by Ouchterlony reaction (Figure 20). Further, when assayed by the Farr technique for CEA (100), the CMC-CEA possessed 80-90% of the activity of standard CEA by weight. After radiolabeling by the usual chloramine-T technique, 90% of the ^{125}I-labeled CMC-CEA was bound by the monospecific G-81 abs

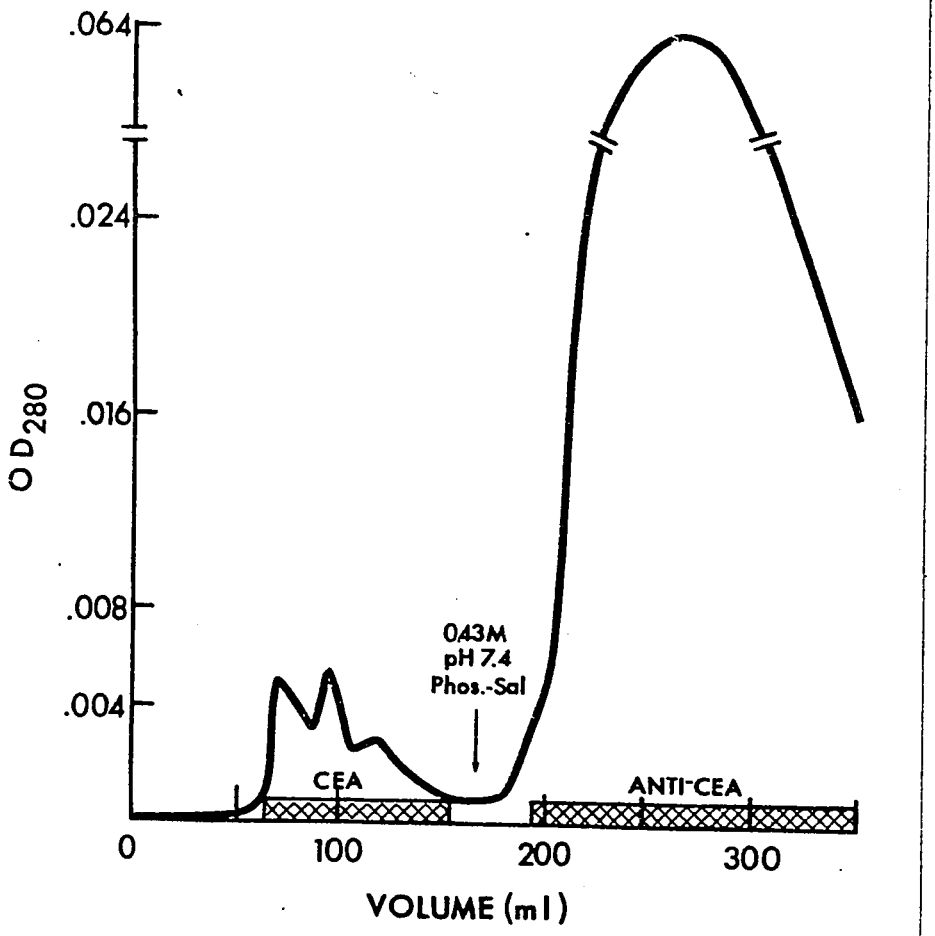

Figure 19 Absorption spectrum, at 280 nm, when solubilized immune precipitate (hepatic metastases from gastric carcinoma) was chromatographed on a column containing Carboxymethyl Cellulose at pH 3.0. The material possessing CEA activity was eluted in the first three peaks and the shoulder following the third peak.

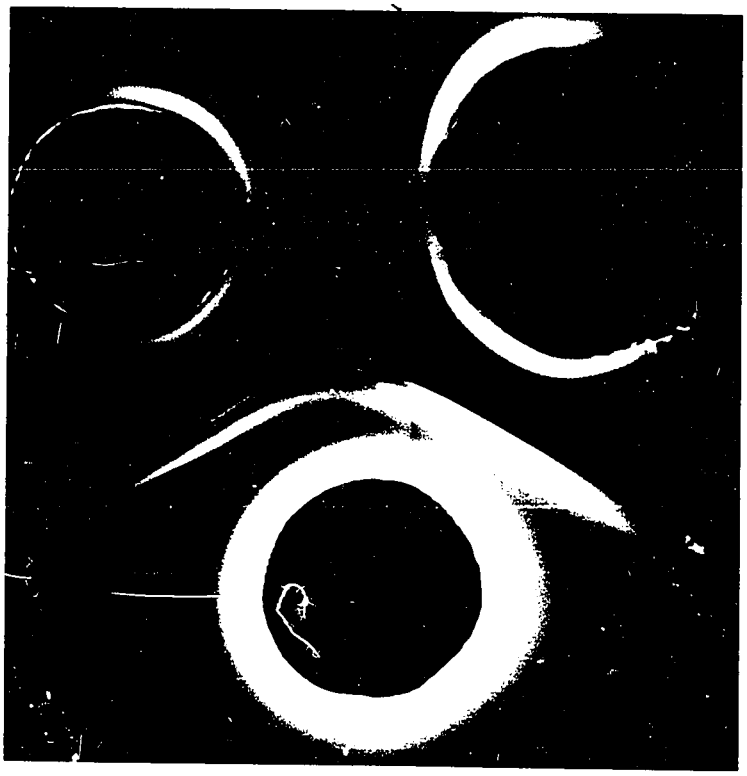

Figure 20 Ouchterlony reaction. Upper left, standard CEA (0.2 mg/ml), upper right, CMC-CEA (0.2 mg/ml), lower well, G-81 abs. Lines of complete identity were obtained between CEA and the CMC-CEA with this antibody preparation.

Figure 20 Ouchterlony reaction. Upper left, standard CEA (0.2 mg/ml), upper right, CMC-CEA (0.2 mg/ml), lower well, G-81 abs. Lines of complete identity were obtained between CEA and the CMC-CEA with this antibody preparation.

anti-CEA antibody preparation in the zone of antibody excess. When the ^{125}I-CMC-CEA was tested in the molecular sieve radioimmunoassay, the elution volumes and patterns of eluate radioactivity were identical to those obtained in Figures 1 and 2 when ^{125}I-CMC-CEA was chromatographed alone, and after incubation with an anti-A antibody preparation. This anti-A preparation, which was capable of binding 14% of standard ^{125}I-CEA, bound 11% of ^{125}I-CMC-CEA.

CHAPTER V

DISCUSSION

The experimental findings indicate that the CEA molecule manifests intramolecular heterogeneity with respect to the antigenic determinants which it contains. The various determinant groups appear to be as follows:

(1) A tumor-specific grouping(s) characteristic of cancer arising within the entodermally-derived digestive system and defined by heterologous antisera.

(2) A tumor-specific grouping(s) recognized by the sera of patients bearing digestive system tumors and pregnant women. This determinant may or may not be identical to the tumor-specific site defined by the heterologous antisera.

(3) An antigenic grouping recognized by anti-blood group A antibodies, and designated the A-like site.

The Tumor-Specific Site on the CEA Molecule

The experimental findings reported here, utilizing purified preparations of CEA and extensively absorbed, monospecific anti-CEA antiserum, demonstrated the existence and specificity of the tumor-specific antigenic determinant(s) on the CEA molecule. These studies employed the Ouchterlony technique and a solid-phase coupled antibody radioimmunoassay procedure. The findings confirm and are in keeping with data previously reported (74-77, 80, 100, 113).

Cross-Reactivity Between the Tumor-Specific Site and a Grouping Present in Preparations of Human Ovarian Cyst-Derived Blood Group Substances

The extensively absorbed, monospecific heterologous anti-CEA antiserum employed in the radioimmunoassay for CEA was capable of recognizing a site present in preparations of human ovarian cyst-derived materials possessing blood group A, B, H and Le^a reactivities*. Pneumococcal polysaccharide Type XIV, alpha acid glycoprotein, ovomucoid, and ovalbumin do not appear to possess such determinants. The extent of this cross-reactivity is of the order of 10^{-3} to 10^{-4} for all the blood group substances, as compared to CEA itself. An exact determination is difficult because of the molecular weight heterogeneity of the preparations of blood group substance employed.

Kabat and co-workers have proposed a structure for the carbohydrate chains of the blood group-active macromolecules, the so-called megalosaccharide shown in Figure 21 (165). This structure appears to be shared by the ovarian cyst-derived materials possessing blood group A, B, H, Le^a and Le^b reactivities, except at the non-reducing end where the presence of N-acetyl-D-galactosamine, D-galactose or L-fucose confers the appropriate blood group specificity upon them. The differences between the structures described for the A, B, H and Le^a antigenic determinants suggest that it is unlikely that these moieties are the determinant(s) recognized by the anti-CEA antibodies. Thus, this common structure, present

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* Obtained from Dr. W.T.J. Morgan.

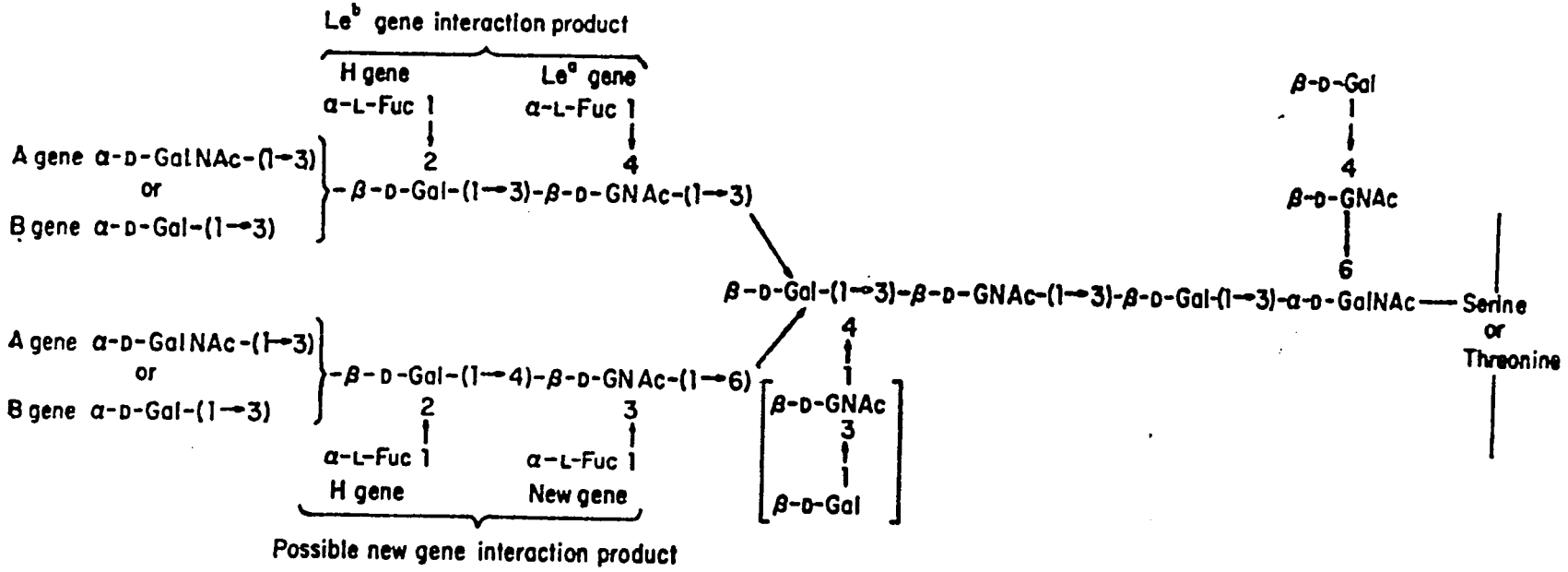

Figure 21 Proposed composite megalosaccharide structure showing the relation of the various blood group determinants, from Ten Feizi et al (165).

near the core of the human ovarian cyst-derived blood group substances might well be similar in structure to the tumor-specific site on the CEA molecule. However, the extent of cross-reactivity, mentioned above indicates that these structures are not identical.

It is also of interest, therefore, that the anti-CEA preparation was capable of binding to ^{125}I-A and that this binding could be inhibited by N-acetyl-D-galactosamine, to 30%, in spite of a lack of anti-A antibodies in the preparation as measured by hemagglutination. This finding again suggests cross-reactivity between the CEA and an ovarian cyst-derived substance. An alternate possibility is that the anti-CEA preparation contained anti-A antibodies of the IgG type which were capable of binding to the ^{125}I-A but which possessed relatively weak hemagglutinating activity (114). The suggestion by Lloyd and Kabat (94), that about half of the N-acetyl-D-galactosamine in the ovarian cyst-derived blood group substances occurs as unsubstituted residues directly attached to serine and threonine in these materials might relate to these observations.

Thus, the cross-reactivity observed between the tumor-specific CEA determinant group and a site present in preparations of ovarian cyst origin possessing blood group ABH and Le^a activity might be due to similar core structures in these molecules. Clearly, a more detailed knowledge of the structures of these entities is necessary before this relationship can be fully understood.

Monosaccharide-Hapten Inhibition Studies

It was not possible to inhibit the anti-CEA-^{125}I-CEA interaction with simple sugars as has been achieved with the ABH blood group antigens (166-169). The failure to demonstrate the binding of simple sugars to the antigen binding sites of the anti-CEA antibodies was probably not due to the radioimmunoassay system employed, as this system was capable of demonstrating monosaccharide-hapten inhibition in the anti-A-^{125}I-A system. It seems likely that the hyperimmune, IgG, anti-CEA antibodies possess larger antigen binding sites than the predominantly IgM anti-A antibodies. The studies of Moreno and Kabat, based upon the A-anti-A system indicate that a sub-population of IgG anti-A, probably those antibody molecules with the highest affinity for antigen, were not inhibitable by the monosaccharide N-acetyl-D-galactosamine at concentrations of this sugar capable of inhibiting the IgM-A-anti-A interaction by one hundred percent (170).

The interaction of these high affinity antibodies with A substance, is however easily inhibitable by the monofucosyl A-active pentasaccharide which appears to contain the complete blood group A antigenic determinant at least as it occurs in human ovarian cyst fluid material (94,170,171).

Thus it appears likely, that the tumor-specific site on the CEA molecule, because of the manner in which it is defined, does not possess a single immunodominant sugar, but may depend upon a combination of at least two monosaccharides and/or

the linkage joining them together. It is evident, therefore, that the concept of immunodominant monosaccharides in carbohydrate antigenic determinants may well require the presence of low affinity antibody molecules for its demonstration.

The Site on CEA Recognized by the Sera of Tumor Patients and Pregnant Women

The observations reported here indicate that the sera of some patients with digestive system cancers, some pregnant women and one patient with carcinoma of the urinary bladder contained IgM antibodies directed against ^{125}I-CEA. The method employed for antibody detection is obviously critical in interpreting the results of these preliminary experiments. For example, in the present study, anti-CEA antibodies could be detected by RIEP, but not by the molecular sieve radioimmunoassay or the Farr technique after acid treatment (except for 1 patient with digestive system cancer). In previous studies using the BDB hemagglutination technique 19S anti-CEA antibodies could be detected in about 70% of patients with non-metastatic digestive system cancer, but could not be detected in the sera of patients with widely disseminated digestive system cancers (99). Such antibodies are, however, detectable by the RIEP method, and do not appear to react with the A-like site discussed below. These findings, those reported by Burtin and his co-workers (172,173) and, in particular, the data recently presented by Lo Cerfo et al (174) serve to illustrate the problems in technology and antigen-

antibody binding kinetics involved in the determination of anti-CEA antibodies. It should be noted, however, that it remains uncertain as to whether or not the site on the CEA molecule recognized by the antibodies produced by patients bearing digestive system cancers is the same as that recognized by the heterologous anti-CEA antibodies.

The Blood Group A-Like Site on the CEA Molecule

Serological Demonstration of the A-Like Site

The existence of a blood group A-like antigenic determinant on the CEA molecule was suggested by three studies in which the presence of anti-A antibodies in individual serum specimens correlated with the ability of those sera to bind to the ^{125}I-CEA molecule. These studies employed three techniques capable of detecting the primary binding of antigen to antibody. The procedures of radioimmunoassay by both molecular sieve chromatography and the Farr technique after acid treatment yielded quantitative data while the radioimmunoelectrophoresis technique produced qualitative determinations only.

In the study employing the molecular sieve radioimmunoassay, the quantity of ^{125}I-CEA bound by a serum indicated with a high degree of accuracy whether or not that serum specimen contained anti-A antibodies. The degree of binding clearly separated the sera studied into two groups; one group capable of binding the A-like site on the CEA molecule, and the other incapable of doing

so. The Farr technique after acid treatment produced very similar data in quantitative terms. The small amount of binding of ^{125}I-CEA by sera without anti-A antibodies observed when the Farr technique after acid treatment was employed might be attributed to the so-called "background" described with techniques which employ coprecipitation in half-saturated ammonium sulfate. This "background" has been attributed to trapping of the radioiodinated antigen in half-saturated ammonium sulfate.

Such explanations are not applicable to the molecular sieve radioimmunoassay technique which depends upon entirely different physicochemical parameters to separate the free, radiolabeled antigen from its antibody-bound form. The observed binding of ^{125}I-CEA by sera lacking anti-A antibodies could then be due to other antibodies in the sera capable of recognizing a site(s) on the ^{125}I-CEA molecule. Non-immunological binding, either through the Fc portion of IgG or by other serum proteins, might also account for these observations. At present, there is no firm evidence for any of these possibilities. However, studies employing high titer anti-blood group B antisera in both assay systems demonstrated no significant binding of this reagent to ^{125}I-CEA.

The observed increase in binding of anti-A to ^{125}I-CEA in the studies employing the Farr technique after acid treatment as compared to the Farr technique without acid treatment is not well understood. However, the observation of apparent enhancement

of antibody activity after treatment of sera at low pH is not unprecedented. Isliker reported minimal increases in activity of antibodies to PR8 Influenza A virus and antistreptolysin O titer when the antibodies in each instance were exposed to pH 3.1 for 16 hr (175). These observations, obtained by hemagglutination techniques, were unexplained and one can only speculate as to their relevance to the studies reported here. It is conceivable that some anti-A antibodies circulate in the form of soluble antigen-antibody complexes with blood group A - reactive antigens, possibly of bacterial origin (176). The acid treatment would then be expected to dissociate at least some of these complexes and ^{125}I-CEA could then be bound by the antibody moiety when the solution containing these complexes was neutralized. Clearly, more data are needed to resolve this problem.

The radioimmunoelectrophoresis technique demonstrated binding of ^{125}I-CEA by 80% of the anti-A-containing sera studied. This binding was due to IgM and, in some cases IgG antibodies as well, and correlated with the data obtained when these sera were examined by the Farr technique after acid treatment. The qualitative RIEP data confirmed results obtained with the molecular sieve radioimmunoassay employing anti-human immunoglobulin antisera. Furthermore, studies of sera containing anti-A antibodies which had demonstrated binding to ^{125}I-CEA by RIEP showed that removal of these antibodies by absorption on group A erythrocytes abrogated this binding, although the IgM precipitin lines were unchanged by this treatment. The control studies, which utilized

group O or B erythrocyte-treated sera, demonstrated no diminution in the ability of these sera to bind ^{125}I-CEA. This suggested that the binding observed was due to anti-A antibodies and not due to non-immunological binding by IgM moieties in the sera studied. The observation of inhibition of anti-A to ^{125}I-CEA binding by a preparation of human ovarian cyst origin possessing blood group A activity in the molecular sieve radioimmunoassay supported this contention.

Demonstration that the A-Like Site is on the CEA Molecule

The localization of the A-like site to the CEA molecule and not to a simple contaminant of the CEA preparation was suggested by the studies utilizing a concentrated, high titer anti-A antibody solution. The maximal binding of ^{125}I-CEA by G-81 abs in the range of antibody excess exceeded 95%. The maximal net binding of this anti-A preparation was 45% of the same ^{125}I-CEA preparation. Total binding of both antibody moieties would then approach 140% indicating that both sites were present on the same macromolecule. Furthermore, the lack of a plateau at the upper limits of the binding curve (Figure 8) indicates that the maximal binding achieved with the anti-A reagent was antibody-limited, and may well have been greater with an anti-A antiserum of higher titer. Hence, the possibility (174,177) that the interaction of anti-A antisera with purified preparations of CEA might be due to simple contamination of the final CEA preparation by blood group A substance appears highly unlikely.

Monosaccharide-Hapten Inhibition Studies of the A-Anti-A System

To define the interaction between anti-A and blood group A antigenic determinants, a radioimmunoassay technique was devised, employing Sepharose-coupled anti-A antibodies and ^{125}I-labeled human ovarian cyst-derived A substance. The binding of Sepharose-coupled anti-A antibodies to ^{125}I-A was specifically inhibited by N-acetyl-D-galactosamine and, hence, is similar to the human anti-A system described by Kabat et al (167). However, the inhibition of anti-A binding to ^{125}I-A by N-acetyl-D-galactosamine (Figure 13) differs from that obtained utilizing the quantitative precipitin analysis technique in a number of ways (167).

First, the primary binding of anti-A to ^{125}I-A was inhibited to 80% whereas the maximum inhibition which has been achieved with the precipitin method has been only 40-45%. Second, the primary binding technique required at least 10 to 100 times the concentration of N-acetyl-D-galactosamine to reach a level of 40% inhibition as compared to the precipitation method. While this variability quite possibly reflects differences in the antigen and antibody preparations utilized, a more likely explanation of these observations is that the precipitin analysis technique, by definition, measures only insolubilized antibody in the form of an antigen-antibody precipitate at equivalence (178). However, it fails to measure soluble immune complexes. The experimental situation in which a monosaccharide is utilized to inhibit an antigen-antibody system at equivalence is analogous to the addition of antigen to

the system. This results in soluble antigen-antibody complexes in the zone of antigen excess which go undetected by the precipitin method. These complexes represent antigen-antibody interactions with which the added monosaccharide has not interfered. However, as concerns the precipitin analysis, these complexes would be measured as free antibody. Consequently, the amount of inhibition measured would be greater than the actual number of antigen-antibody interactions disrupted.

When utilized for inhibition studies, primary binding systems are employed in the zone of antigen excess. Therefore, the maximum number of antigen-binding sites on the antibodies, determined by the equilibrium constant of the antigen-antibody reaction, are occupied. As the measurement performed is the amount of radiolabeled antigen bound to a constant quantity of antibody, the problems created by the soluble antigen-antibody complexes do not arise. This is true not only for the solid-phase coupled antibody primary binding technique, but for the Farr technique, which indeed is based upon the phenomenon of coprecipitation of soluble immune complexes by half-saturated ammonium sulfate.

As expected, the corresponding unlabeled A substance was capable of inhibiting the primary interaction between anti-A and ^{125}I-A to almost 100%. An inhibition of 50% binding of 0.15 ng ^{125}I-A required 125 ng of the unlabeled A substance. This apparent increase in antibody affinity for the ^{125}I-labeled A substance compared to the unlabeled A substance is unclear. However, a similar situation is observed with the affinity of anti-CEA for

^{125}I-CEA. A phenomenon, in which increasing iodination of anti-hapten antibody results in increased affinity of the antibody for the hapten is called the Pressman-Radzimski effect and has been described by a number of workers utilizing anti-hapten systems (179,180). Since the basis of this phenomenon is not understood, it is of interest only in that it bears a resemblance to the observations reported here.

Monosaccharide-Hapten Inhibition Studies of the CEA-Anti-A System

The data presented suggest that the Sepharose-coupled anti-A antibody reagent was capable of recognizing a blood group A antigenic determinant as defined by the parameters of monosaccharide inhibition (166-169). This reagent was, therefore, utilized to study the A-like site on the CEA molecule.

First, the presence of this site on the unlabeled CEA molecule was demonstrated by the ability of CEA to bind to the anti-A preparation. This was shown by the inhibition of binding of anti-A to ^{125}I-A by CEA (Figure 11). Approximately 100 times more CEA than A substance by weight was required to achieve the same level of inhibition. No definite conclusions of a quantitative nature may be drawn from these results since the concentration of the reacting antigenic determinants in each macromolecular preparation is unknown. The studies of monosaccharide inhibition of the Sepharose-coupled anti-A binding to ^{125}I-CEA were strikingly similar to those obtained in the ^{125}I-A-anti-A system. These data,

strongly suggest that it is anti-A antibodies, and not another antibody present in the anti-A preparation which recognizes the A-like site on the CEA molecule.

Although minute quantities of N-acetyl-D-galactosamine, of the order of 0-3% of the total carbohydrate content have been demonstrated in some preparations of CEA (86), there does not appear to be a correlation between the A-like activity and the presence of this amino sugar in CEA. This observation is of the utmost importance, because although almost all of the structures possessing blood group A antigenic activity which have been chemically characterized have been shown to contain a terminal non-reducing N-acetyl-D-galactosamine residue, this relationship does not appear to be absolute. Kabat has presented data in which two different human anti-A systems were studied and were inhibited by galactosido-1-4-β-N-acetyl-D-glucosamine (type 2 chain) and by D-galactosamine (167). The latter observation is of interest as the data presented here show a small but significant inhibition of anti-A binding to ^{125}I-A and ^{125}I-CEA by D-galactosamine.

Inhibition of the A-Anti-A System with CEA Fragments

The CEA fragment GP-1, approximate molecular weight 4000 daltons, inhibited anti-A to ^{125}I-A binding 1000 to 10,000 times more efficiently than N-acetyl-D-galactosamine on a molar basis although it contains no demonstrable N-acetyl-D-galactosamine. However, the inhibitory activity of GP-1 appeared to reach a plateau at 45% inhibition, suggesting that although it contains

structures similar to the A determinant, there are important differences between the A-like site on the CEA molecule and that found in ovarian cyst fluid. Although it is difficult to relate the kinetics of the precipitin-inhibition and radioimmunoassay techniques as described above, it is of interest that the potency of the A-active monofucosyl pentasaccharide MSS ARL 0.52 (170,171) in inhibiting a human anti-A system is also of the order of 1000 to 10,000 greater than N-acetyl-D-galactosamine alone. These data suggest strongly that the A-like determinant on the CEA molecule probably differs from the structures possessing A activity which have been previously described (181-185).

The observation that both anti-CEA and anti-A antibodies are capable of binding to GP-1 could be interpreted as evidence that the tumor-specific site and the A-like site share molecular constituents or are contiguous. The differences in specificity of these antibodies, described above, make the latter explanation more likely.

The Effect of the Method of Purification and Tumor Donor Blood Group Phenotype on the A-Like Site

The presence of the A-like site on the CEA molecule does not appear to be the result of the extraction in perchloric acid. This was shown by the method employing carboxymethyl cellulose ion exchange chromatography for the preparation of CEA. Furthermore, a ^{125}I-CEA preparation derived from a blood group O individual was capable of being bound by anti-A antibodies. This indicates that

the presence of the A-like site is independent of the ABH blood group phenotype of the tumor donor.

Implications of the Intramolecular Heterogeneity of CEA in the Radioimmunoassay for CEA

The finding of cross-reactivity between the tumor-specific grouping on the CEA molecule and a site present in preparations of human ovarian cyst-derived blood group substance ABH materials might have far-reaching implications in the radioimmunoassay for CEA in human sera. The observation, by Höstrup (188) that water soluble materials possessing blood group A and B activity are present in the sera of the vast majority of A, B and AB individuals in concentrations of 1-10 μg/ml, regardless of secretor status is, of great interest in this context. Since approximately 10,000 ng of ovarian cyst-derived ABH or Le^a are capable of inhibiting the radioimmunoassay for CEA by an amount equivalent to 2 ng of CEA, a demonstration of similarities between the serum blood group substances and the ovarian cyst-derived materials might provide an explanation for the difficulties reported in the measurement of CEA in whole serum and to the findings of CEA in normal individuals (108,110,186,187).

The presence of the A-like site on the CEA molecule might also affect the measurement of CEA in human serum particularly in those individuals possessing large quantities of anti-A antibodies in their sera. The binding by anti-A antibodies of part of the ^{125}I-CEA added to the radioimmunoassay system would then cause an

increase in the amount of CEA measured if an anti-globulin technique is employed to separate free ^{125}I-CEA from antibody-bound ^{125}I-CEA, as reported by Lawrence et al (186).

On the other hand, the coprecipitation of soluble immune complexes in 50% saturated ammonium sulfate would cause a diminution in the amount of CEA measured. Thus, the removal of anti-A antibodies is of utmost importance in the measurement of CEA in whole serum. Perchloric acid extraction of serum appears to accomplish this aim (100).

Thus, these findings serve to illustrate the problems in immunochemical technology involved in the determination of CEA.

Immunologic and Other Biologic Implications of the Intramolecular Heterogeneity of CEA

The demonstration of similarities between the tumor-specific site of the CEA of the human digestive system and the normally occurring blood group ABH antigens has implications concerning the relationship of the CEA-bearing tumor cell to its environment. These similarities, as well as the paucity of the anti-CEA response on the part of the digestive system cancer-bearing individual suggests that the molecular mimicry of tumor cell surface components to normal cell surface constituents might be a factor enabling tumor cells to adapt to a hostile environment. Glycoprotein antigens appear to evoke weak, if any, cell-mediated immune responses (189). They elicit relatively weak humoral antibody responses except under highly artificial situations of

intensive specific and non-specific immunological stimulation (190, 191). Thus, the glycoprotein antigens differ from the *HL-A* system of transplantation antigens, and the TSTA of animals which evoke cell-mediated as well as humoral antibody responses. It appears then, that the CEA as it has been defined may not play a significant part in the modulation of tumor growth by the interaction of humoral and cell-mediated immune responses which have been studied in a variety of *in vivo* and *in vitro* systems (51,52,54,55). This does not imply that other carcinoembryonic antigens, employing this term in a generic sense, of either species-specific or individual specificities do not exist in human digestive system cancers.

The existence of the CEA supports the view that oncogenesis involves the retrogenic activation of early fetal antigens (19). Whether this manifestation plays an important role in the process of oncogenesis or is merely a consequence of that process is presently unknown. It is conceivable that the molecular mimicry is no more than a manifestation of cellular economy, utilizing available glycocalycal "building blocks" with small modifications at various stages of cellular differentiation and tissue organization reflecting these processes.

The glycoprotein(s) demonstrated by Mach (111), Burtin (112) and Ørjasaeter (113) and designated normal glycoprotein (NGP), nonspecific cross-reacting antigen (NCA), and beta external (βE) respectively, is of great interest in this context as it might be a precursor to CEA under appropriate circumstances. The elucidation

of the structure of the carbohydrate moieties of CEA and NGP, NCA and βE as well as comparative studies of the amino acid sequences of CEA, NGP, NCA and βE might certainly be of interest in this regard. It is also possible, that small alterations in the structure of the basic glycocalycal molecules, measured as carcino-embryonic antigens, perform an important function in the growth, organization and economy of fetal, embryonic and cancerous cells. The elucidation of these relationships might provide an insight into the biologic mechanism underlying the development of cancer in the human host.

CHAPTER VI

SUMMARY

The initial objectives of the present series of experiments were:

(1) To determine if antibodies to the carcinoembryonic antigen (CEA) in the sera of pregnant women or patients bearing digestive system cancers belong to all of the three major immunoglobulin classes.

(2) To determine if the CEA and its corresponding antibodies, when present in human serum, exist as free molecular moieties or as antigen-antibody complexes.

A: For these purposes, three radioimmunoassays were developed, each employing different physicochemical parameters for the separation of CEA from anti-CEA antibodies. The principles underlying the assays were:

(a) Radioimmunoelectrophoresis

(b) Molecular sieve chromatography

(c) Coprecipitation of soluble immune complexes in half-saturated ammonium sulfate after acidification and neutralization of the serum preparations.

By the use of these procedures the following observations were made:

(1) The three radioimmunoassay techniques employed demonstrated that the great majority of human sera which contained

anti-blood group A antibodies were capable of binding to ^{125}I-CEA, and that this binding was due predominately to IgM moieties in the sera.

(2) Utilizing a high-titer, purified anti-A antibody preparation, a maximal net binding of 45% of a ^{125}I-CEA preparation was demonstrated under conditions which were still antibody limited and where goat anti-CEA was capable of binding 95% of the same ^{125}I-CEA preparation.

(3) Inhibition studies utilizing the various radioimmunoassays demonstrated that the IgM anti-A antibodies were capable of binding to site(s) present on A erythrocytes, human ovarian cyst-derived material possessing A activity and to purified CEA.

(4) The radioimmunoelectrophoresis technique revealed that in addition to anti-A antibodies, the sera of some digestive system cancer patients, some pregnant women, and one patient with metastatic carcinoma of the urinary bladder contained IgM antibodies which were capable of binding to ^{125}I-CEA, but not to A erythrocytes.

On the basis of these findings it was concluded that:

(1) There exists a blood group A-like site on the CEA molecule capable of being recognized by anti-blood group A antibodies.

(2) The demonstration of human anti-CEA antibodies, restricted to the IgM class, confirms the finding previously made with the BDB-hemagglutination technique. The restriction of the apparently tumor-specific antibodies to the IgM class may well be due to the probable carbohydrate composition of the antigenic

determinants stimulating this antibody production.

B: Employing a solid phase-coupled antibody radioimmunoassay technique, an assay for blood group A antigenic determinants was devised. This system was defined by means of monosaccharide inhibition studies and was utilized to study the A-like site of CEA. The following observations were made:

(1) The monosaccharide, N-acetyl-D-galactosamine was capable of inhibiting the interaction between ^{125}I-CEA and a preparation possessing anti-A antibodies.

(2) A glycopeptide obtained by the enzymatic degradation of CEA and possessing tumor-specific antigenic activity was capable of binding to anti-A antibody at 10^{-4} to 10^{-3} the molar concentration required to achieve equivalent binding by N-acetyl-D-galactosamine. The glycopeptide under consideration has a molecular weight of about 4000 daltons and contains N-acetyl-D-glucosamine, D-mannose, D-galactose and L-fucose, but no detectable N-acetyl-D-galactosamine.

C: Employing the solid phase-coupled antibody radioimmunoassay technique to study the interaction between the tumor-specific goat anti-CEA and ^{125}I-CEA, it was observed that there exists a molecular site or sites, in preparations of human pseudomucinous ovarian cyst origin, possessing blood group A, B, H, and Le^a activity, which is capable of binding to the tumor-specific anti-CEA antibodies.

On the basis of these findings it was concluded that:

(1) The structure of the A-like determinant on the CEA molecule differs from other immunochemically defined structures possessing blood group A reactivity at least in so far as it lacks a terminal, non-reducing N-acetyl-D-galactosamine residue.

(2) The tumor-specific antigenic determinant on the CEA molecule is structurally similar to a site(s), present in the blood group substances examined, possibly in the "core" region of the blood group megalosaccharide described by Kabat (165).

(3) The relationship of the A-like site to the tumor-specific determinant is unclear, however it is entirely possible that both sites reside in the same "megalosaccharide" chains on the CEA molecule.

D: It is proposed that the carcinoembryonic antigen is derived from available glycocalycal "building blocks" and plays a role in the maintenance of the viability of the incompletely differentiated embryonic, fetal and cancerous cells.

CHAPTER VII

CLAIMS TO ORIGINAL RESEARCH

This thesis has been concerned with the study of human anti-CEA antibodies with particular emphasis on the structure of a site on the CEA molecule recognized by human antibodies directed against the blood group A antigenic determinant. The original contributions made during this investigation may now be considered.

A: In order to demonstrate the existence of anti-CEA antibodies, present either as free constituents or as immune complexes with CEA in the serum of patients bearing digestive system cancers, the following innovations were employed:

(1) Three primary binding radioimmunoassay techniques were devised to study the interaction between anti-CEA and CEA. This served to eliminate the problems inherent in the detection of antibody by the application of immunologic techniques dependent on secondary manifestations of antigen-antibody interactions.

(2) The quality and quantity of anti-CEA antibodies was determined by means of a molecular sieve radioimmunoassay technique, and the resulting data were compared to measurements of these moieties obtained by the simultaneous application of qualitative (RIEP) and quantitative (Farr) techniques.

(3) A modification of the Farr technique, employing a method of acid treatment of the serum preparations was devised to attempt to measure CEA and anti-CEA present as constituents of immune complexes.

The use of these procedures led to the following original observations:

(1) The observation that IgM moieties present in the great majority of human sera containing anti-blood group A antibodies are capable of binding to a site on the CEA molecule. This binding is demonstrable with all of the primary binding techniques employed.

(2) The RIEP technique is capable of detecting a tumor-specific interaction between ^{125}I-CEA and an IgM antibody moiety in the sera of some patients bearing disseminated digestive system cancers, some pregnant women, and one patient bearing metastatic cancer of the urinary bladder. This finding was not dependent upon the blood group of the patient studied and was not directed against the site bound by sera containing anti-A antibodies.

B: A radioimmunoassay for blood group A antigenic determinants was devised to define the interaction between sera containing anti-A antibodies and CEA by means of monosaccharide inhibition studies.

The use of this procedure led to the following original observations:

(1) The monosaccharide, N-acetyl-D-galactosamine, specifically inhibited the binding of a preparation containing anti-A antibodies to ^{125}I-CEA. These studies confirmed the classical studies of the blood group A antigenic determinant and demonstrated that anti-A antibodies so defined were capable of binding to a site present on the CEA macromolecule which, nevertheless, lacks N-acetyl-D-galactosamine.

(2) The A-like site of the CEA molecule appears to be present on the smallest fragment of CEA containing tumor-specific activity (molecular weight of approximately 4000 daltons) studied to date.

C: Studies of the goat anti-CEA antisera demonstrated that this antibody preparation, which is capable of binding to the tumor-specific CEA antigenic determinant can also bind to a site, or sites, present in preparations of human pseudomucinous ovarian cyst fluid-derived materials possessing blood group A, B, H, or Le^a activity.

The similarities described for CEA and the A, B, H blood group antigens represent the first demonstration of a relationship between the CEA and other, partially defined antigenic structures.

Further studies will be directed along the following lines:

(1) Further studies of CEA active fragments will attempt to determine the relationship of the A-like site to the tumor-specific CEA antigenic determinant.

(2) An attempt to isolate and characterize the antigenic determinants present in ovarian cyst fluid materials as well as those substances present in normal human serum, stool and urine which are capable of binding to the heterologous anti-CEA antibodies.

(3) An attempt will be made to devise a primary binding radioimmunoassay technique to quantitate the anti-CEA antibodies

present in the sera of patients bearing primary and metastatic digestive system cancers and pregnant women. These antibodies will then be studied by the technique of hapten inhibition utilizing monosaccharides and CEA fragments to determine if the human antibodies in these groups of patients are directed against the same antigenic determinant as has been defined by the heterologous anti-CEA antibodies.

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