Studies on Increasing the Specificity of anti-Carcinoembryonic antigen (CEA) Antisera by means of Solid Phase Immunoabsorption.

by

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ABSTRACT

Clinical studies have suggested that the anti-CEA antiserum directed against purified CEA reacts with a variety of constituents. This phenomenom may account for the lack of tumor-specificity seen in the clinical radioimmunoassays in use.

The object of the experiments presented here was to see if anti-CEA antisera could be made more specific to the tumor portion of the CEA molecule. The method of approach was by the use of affinity chromatography. Normal bowel material was coupled to glass beads and used as a matrix through which anti-CEA antisera was recycled. Both a single column with sequential passages and separate columns with successive passages were employed.

The results obtained indicate that the anti-CEA antiserum is indeed heterogeneous. It reacts primarily with normal bowel constituents and contains little, if any, tumor-specific reactivity.

RESUME

Des études cliniques ont démontré que l'immunsérum anti-CEA obtenu avec du CEA pure réagit avec plusieurs substances antigéniques. Ce phenomène peut être la cause du manque de spécificité tumorale vu par la methode radio-immunologique clinique.

Le but des expériences présentées ici était d'améliorer la spécificité de l'immunsérum contre la portion tumorale du CEA. La méthode employée consistait de chromatographie par affinité. Le matériel extrait de l'intestin normal fut fixé à des billes de verre qui servirent des matrices par lesquelles l'immunsérum anti-CEA fut recyclé. Une seule colonne avec une séquence de passages ainsi que plusieures colonnes avec des passages successifs furent employés.

Les résultats obtenus ont indiqué que l'immunsérum anti-CEA est vraiment hétérogène. Ce sérum réagit principalement avec les substances de l'intestin normal et contient peu de spécificité tumorale.

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CHAPTER 1

1) Introduction

A major objective of tumor biology studies is to specify differences between normal and neoplastic cells so that methods can be developed to selectively kill all cancer cells without endangering the viability of normal cells. Theoretically, the ultimate goal is the protection of the population from the development of primary tumor by vaccination, or protection against micrometastatic nodules by boosting the host's own antitumor response. Attaining this end is the main thrust and impetus of the study of cancer biology.

2). Experimental Approaches

Most of what we know about tumor immunology today is the result of work done on syngeneic (genetically identical) animal systems. In 1953, Foley produced the first clear demonstration of tumor-specific antigenicity in a class of experimental animal tumors using inbred strains of mice (61). It was reasoned that host rejection of a tumor of syngeneic donor origin must, then, be due to the development of new tumor specific transplantation antigens (TSTA) within the tumor tissue. A vast amount of work has since been done in syngeneic animal systems to demonstrate the existence of these TSTA's using tumors induced either by oncogenic viruses or by chemical carcinogens.

The search for similar antigens in humans is precluded by the virtual lack of syngeneic donor-host combinations (other than the rare instance of cancer in an identical twin), and the obvious moral and ethical considerations. Most of the evidence has, therefore, been obtained by indirect methods, adopting the hypothesis that what is observed in animal systems may well extend to the human situation.

The development of humoral antitumor antibodies and/or cell-mediated responses to a host's own tumor cells and to similar cells from other individuals is indicative of tumor-specific antigenicity. Furthermore, this demonstrates that tumors of a given type and of similar tissue origin possess common antigenic determinants, and leads to the assumption that tumor-associated antigens are probably present on all tumor cells.

Another approach which gives much insight into the nature of these tumor-specific antigens is the immunization of animals with a small amount of human tumor tissue. However, this method has two inherent drawbacks which are constant problems. All human tissue extracts contain large quantities of normal tissue "contaminants" from both the cellular and subcellular level. Hence, heteroimmunization may result in a predominantly antinormal response. Also, it must be remembered that the normal control tissue used for comparative studies is usually taken from noncancerous patients. This has given rise to problems of alloantigenic differences between the donors of the tumor and nontumor tissues. It's thus difficult to determine whether some antigens considered unique to a particular cancer are, in fact, tumor-

specific or simply individual-specific components.

3) Tumor Antigens

The study of tumor immunology is based on the assumption that tumor cells express antigens not seen on normal cells (114, 152, 153, 187). These are often referred to as "neoantigens". However, tumor cells often manifest several antigens, detectable by various techniques, and expressed on different tissue types as well as in different stages of the development of the organism. Hence, the concept of "neoantigen expression" should be qualified accordingly. Also, even though these tumor antigens are able to elicit an immune response on the part of the host, it is doubtful that this is the primary function of these antigens. More likely, they play important roles in maintaining the integrity of the cancer cell.

One other observation that underlies the study of tumor immunology is the ability of the host to recognize these neoantigens and mount an immunologic reaction against them (6, 7, 99). In man, however, these antigens have not, as yet, been isolated and chemically characterized. Nevertheless, the host's capability to demonstrate an anti-tumor response has been employed in establishing in vivo and in vitro assays, some of which are used in the diagnosis and management of cancer patients.

There are three basic types of tumor antigens:

A) Tumor-Specific Antigens (TSA)

Tumor-specific antigens (TSA) are detectable only on tumor cells differ-

ing qualitatively from antigens expressed on normal cells. However, the techniques for detection of the antigens may not be sensitive enough to detect minute amounts of similar specificity present on non-neoplastic tissue. Also, the type of cell used as a control to compare with a given tumor cell type is important. Absolute determination of specificity is virtually impossible given the vast products of mammalian genes, many of which are only transiently active during development. This applies even when the normal cell is of the same histological type as the tumor one.

B) Tumor-Associated Antigens (TAA)

Tumor-associated antigens (TAA) are antigens that appear to be tumor-specific but for which the appropriate specificity controls are inconclusive. These include the phase-specific or embryonic antigens, which are present during certain stages of embryonic development but are virtually undetectable, or present in only trace amounts, in adulthood (2), and antigens induced by oncornaviruses which are present in the morphologically normal cell before it becomes transformed, but do not appear in noninfected animals.

C) Tumor-Associated Transplantation Antigens (TATA)

Tumor-associated transplantation antigens (TATA) are capable of inducing resistance to tumor transplantation in the autochthonous host or in syngeneic recipients. These are also known as tumor rejection antigens. Both definitions are operational, based solely on in vivo observations. These antigens used to be called tumor-specific transplantation antigens (TSTA); however, due to the insensitivity of in vitro techniques, the more general term

TATA, is used to refer, collectively, to those antigens which fall into this category. TATA's are situated on the plasma membrane where, like histocompatibility antigens, they induce immune recognition and serve as targets for subsequent immune attacks (143).

4) Tumor-Inducing Agents

A) Antigens of Chemically-Induced Tumors

Due to the increasing evidence linking environmental factors to many types of human cancers, a considerable body of work has been carried out with chemically-induced tumors in animals. Two types of neoantigens have been identified on these tumors. One is the TSTA, which is unique to each tumor produced by a chemical carcinogen, even if the same agent induces a tumor at another site in the animal (114, 187). This type of antigen has been found on sarcomas and bladder carcinomas induced by methylcholanthrene and on hepatomas induced by aminoazo dyes in rats and mice, or by nitrosamines in guinea pigs. These antigens are also found on chemically transformed cells in vitro, and are thought to result from interaction of the carcinogen with the genome of a single cell or a clone and are stable products of these transformed cells.

The other type of neoantigen produced is the tumor-associated fetal antigen. This antigen is easily differentiated from the TSTA's because it is a common component of different chemically-induced tumors (6, 7). It is uncertain if this type of antigen plays any role in tumor rejection.

B) Antigens of Tumors Induced by Viruses

Although viruses have often been implicated in some human cancers, a direct cause and effect relationship has yet to be established. In vitro studies have shown that induction of tumor cells can be achieved by infecting normal cells with the virus, using morphological changes and abnormal growth as sign of transformation. In vivo, transformation can be brought about by injecting the virus either locally or systemically.

DNA viruses in their natural hosts are not oncogenic. However, when they infect the cells of other animal species, they will transform these cells. Three groups of DNA viruses are oncogenic in animals: the papovaviruses (papilloma, polyoma and vacuolating virus, the adenoviruses, and the herpes viruses, which includes the Epstein-Barr virus responsible for infectious mononucleosis and associated with Burkitt's Lymphoma and nasopharyngeal carcinoma in humans. Also included is the Herpes simplextypes 1 & 2. Tumors produced by the same DNA virus usually have common TSTA's (125, 19), but individually specific TSTA's may be seen (6, 125). These include the T antigen, located in the nucleus, and the S antigen, found on the cell surface of SV40 transformed cells (166).

RNA viruses, on the other hand, have been shown to be oncogenic in their natural hosts. Tumors induced by an RNA virus manifest common TSTA's and virion antigens common to cells transformed by the same virus (125). The C-type viruses, best illustrated by the murine leukemia viruses, contain an RNA core which specifies common internal virion group-specific antigens.

These are specific for the species of virus. There are also viral envelop antigens and virally-induced cell surface antigens which are not antigenically related to any part of the virus particle. Many of these RNA viruses, also known as oncornaviruses, contain an RNA-dependent DNA polymerase (reverse transcriptase) which can transcribe viral RNA onto DNA. This explains the vertical transmission of RNA viruses with the genome of the transformed cell.

5) Specific Antitumor Immune Reactions

Cell-mediated immunity is generally considered to be the primary cause of tumor rejection in experimental tumor systems, since resistance to tumor growth can be transferred to normal histocompatible hosts by means of lymphocytes from tumor-immune or tumor-bearing hosts. Recently, evidence has shown that antibody-mediated tumor cell killing can occur through either complement-dependent or antibody-dependent cellular cytotoxic mechanisms. Much work has been done to try and evaluate the role of cell-mediated antitumor immunity by using *in vitro* assays that can measure or monitor tumor cell rejection (See Table 1). However, there is an immediate need for such assays if they can reliably diagnose cancer and monitor the patient's status.

A) Delayed Hypersensitivity Skin Reactions to Tumor Antigens

In the 1960's, experiments were performed to determine the result of subcutaneous injections of viable autologous tumor cells (27, 75, 189). The studies showed that large doses were necessary to produce a nodule. Small doses were ineffective. This indicated that patients were resistant to tumor

TABLE 1

Assays for Cell-Mediated Immune Responses to Tumor Antigens

1. Skin tests for delayed hypersensitivity using extracts of tumor cells
2. Cytotoxicity assays against tumor cells or tumor-derived cells in cultures
3. Leukocyte migration inhibition by tumor antigens
4. Leukocyte adherence inhibition
5. Proliferation response to tumor antigens
6. Macrophage electrophoretic mobility test

cell growth. To determine the immunologic basis for this penonmenon, neutralization studies were performed whereby leukocytes from either healthy individuals or tumor patients were injected subcutaneously into tumor patients (27). The normal leukocytes, when injected with the tumor cells, were not able to interfere with nodule formation. However, autologous leukocytes inhibited in half the cases. These results are suggestive of cell-mediated immunity against the autologous tumor.

Tumor-associated antigens of bowel cancer have been detected by skin testing; analogous preparations of normal tissues gave negative reactions (107, 108). However, in patients with malignant melanoma, positive reactions were seen using both autologous tumor extracts as well as control extracts from autologous normal skin (22, 59).

To date, two distinct skin-reactive antigens have been purified from malignant melanoma tissue, using physiochemical methods (105, 106). One of these appears to be specific for melanoma tissue, while the other is less specific and produced positive reactions in patients with other types of cancer.

Although there is limited correlation with clinical status for the acute leukemias (33) and Burkitt's lymphoma (23, 60), the significance of skin testing is still unclear (110, 192). It is shown that tumor rejection responses are due to antigens expressed at the plasma membrane, yet intact tumor cells, viable (81) or attenuated (175), are not capable of eliciting the strong responses that are seen in cell-free assays. Delayed hypersensitiyity reactions are used mostly to monitor the purification of tumor-associated antigens.

B) Cytotoxicity Assays Against Tumor Cells

Cytotoxicity manifests the effector stage of several immune responses. Cytotoxicity assays are based on the theory that lymphocytes are lytic for tumor target cells to which they have become sensitized in vivo.

The forerunner of many assays in use today, despite some of its shortcomings, is the colony inhibition test of the Hellstroms (97). Using this test, it was observed that lymphocytes from patients with various cancers are able to inhibit colony formation or become cytotoxic against tissue culture cells from tumors of the same origin and histological type (93, 96).

The microcytotoxicity assay, which has supplanted the colony inhibition test, involves visual counting of target cells or the use of radioisotope-labelled tumor cells. It was found that blood leukocytes were cytotoxic for tumor cells obtained from the patient's tumor but not for normal cultured cells. Leukocytes from control patients, including normal subjects and patients with non-neoplastic disease, were also not cytotoxic for normal cells.

By using these CMC assays, immunoreactivity against many types of human malignant disease has been examined (144). Until just a few years ago, there was unanimity in the belief that the peripheral blood lymphocytes of patients with a particular neoplasm showed preferential reactivity against cultured autochthonous and/or allogeneic cells derived from histologically similar neoplastic tissue. In other words, human neoplasms expressed antigens that are common to tumors originating in the same tissue (98). This conclusion

differs from the type of antigenicity observed in experimental tumors evoking rejection in vivo where the tumor antigen expressed is unique to the tumor-inducing agent, regardless of the species or tissue origin of the neoplasm. Hence, early human cytotoxicity data tended to lean towards the idea that organ-specific tumor-associated antigens, discovered by the use of colony inhibition or CMC assays, were virus-related (95) or due to products of derepression, i.e. fetal antigens (62, 94).

However, more recently, with increased CMC testing, it has become apparent that the cytotoxic potential of effector cells from cancer patients, as well as being directed against both related and unrelated tumor targets, can also exhibit non-disease related cytotoxicity (21, 102, 154, 195, 196, 203). Those favoring the concept of tumor-type specificity have tried to explain non-disease related cytotoxicity by virtue of disparity in lymphocyte preparation techniques. However, while it is quite probable that variation in the preparation methods may result in quantitative differences in the CMC (46), many investigators have not been able to find tumor-type specificity regardless of the procedure used for lymphocyte isolation (21, 154). These differences between assays and the possible reasons for disparate results have been discussed at length (5, 101). Nevertheless, under well-defined conditions, the test may yield meaningful information, but the need for standardization and base lines for normal reactivity preclude its use as a human diagnostic tool.

C) Influence of Serum Factors

Early studies showed that the cytotoxicity of patients' lymphocytes was

relatively unaffected by the stage of the disease except in very advanced cases (10, 92). Since *in vitro* manifestations of lymphocyte killing was thought to be indicative of *in vivo* events, it was postulated that the progression of the disease was aided by the presence of factors in the serum which interfered with CMC expression (98). Support for this theory came from observations that melanoma patients with progressive disease demonstrated blocking factors more often than patients with localized tumors (90). Initially, it was thought that since tumor cells could absorb the blocking activity, the factor involved must be immunoglobulin, in the form of tumor-specific antibody. However, after surgical excision of the tumor, the serum blocking factor was very rapidly depleted. This was incompatible with the idea of a blocking tumor-specific antibody.

Experiments by Sjögren in human tumor systems (181, 182) and Baldwin *et al.* in experimental systems (11) have shown that blocking is due to antigen-antibody complexes rather than antibody alone. The method of action is not yet clearly understood. It is theoretically possible that the immune complexes of tumor antigens and antibodies mask the target cell itself, or, more likely, interact with receptors on the sensitized lymphocytes and therefore block the effector lymphocyte's antitumor response.

Another factor implicated in CMC abrogation is the tumor antigen. First suggested by Brawn with respect to transplantation immunity (26), this type of activity is named "inhibition". The tumor antigens are shed from the tumor cell membrane into the circulation and react with its specific receptor on the surface of the sensitized lymphocytes. This process can be visualized

as "desensitization" in that as the disease stage progresses, more antigen is shed and the immune lymphocytes become coated with these antigens, rendering them ineffective to kill the tumor cells. Experimental data, wherein soluble antigen preparations have been demonstrated to inhibit CMC in both experimental (9, 159) and human tumor systems (8, 57) support and justify this idea of "inhibition". Sjögren and his associates showed that the low molecular weight fraction of the antigen-antibody complexes inhibited the cytotoxic ability of effector cells (182). Currie and Basham also showed that the inhibitory factors directed against the sensitized lymphocytes could be removed with protracted washing, resulting in a reappearance of CMC against the target cells (43).

In addition to factors that block CMC reactions, sera with the ability to "unblock" has been described by the Hellstroms (91). They showed that the sera of patients who, clinically, were disease-free, were not only unable to block per se, but could also unblock the CMC inhibitory activity of "blocking" sera from patients with disease. Experimentally, "unblocking" antisera from rats preimmunized against a certain tumor, was shown to interfere with the same tumor growing in vivo, thus acting as an immunotherapeutic agent (17). Since "blocking" serum is thought to contain antigen-antibody immune complexes, investigators have envisioned "unblocking" serum to contain free (non-complexed) tumor antibody. Thus, when the two types of sera are mixed, a state of antibody excess is obtained, saturating all the free antigenic sites within the complex, thereby minimizing the inhibitory effect of the antigens on the immune lymphocytes. Although this

work of the Hellstroms was indeed elegant and promising, its validity has since been questioned due to the irreproducibility of the phenomenon described.

Aside from the fact that blocking factors of patients' sera can, depending on the stage of the disease, block, inhibit or unblock CMC reactions at the effector and/or target cell level, there is evidence of many CMC reactions in human systems that are not related to a neoplastic condition (158). This leads one to question the significance of blocking factors in relation to tumor antigenicity, a mechanism which has subsequently been coined "epiphenomena" by some investigators. (154).

The involvement of tumor antibody in CMC reactions can be illustrated by the mechanism of antibody-dependent cellular cytotoxicity (ADCC) (133, 157). This phenomenon, observed in nontumor systems as well, is also referred to as "lymphocyte-dependent or cell-dependent antibody" or "K-cell" cytotoxicity. The antibodies are of the IgG class and are specific for their appropriate target cells. The K cells (killer cells) must possess the receptor for the Fc portion of the IgG, a requirement that possibly implicates, as K cells, several types of lymphoid cells, including activated T cells, monocytes and polymorphonuclear leukocytes (213). However, most of the investigation in this area centres around a class of K cells, probably lymphocytic, lacking both T and B cell markers, and thus known as "null" cells (76).

The specific role antibody plays in inducing ADCC is still uncertain. However, studies done in several animal tumor systems have demonstrated that certain immune sera were capable of conferring specific cytotoxicity onto

lymphocytes from nonsensitized donors in a process known as "arming" (161, 162). It has also been reported that sera could increase or "potentiate" the cytotoxic effect of sensitized effector cells. In addition, xenogeneic antibody, passively introduced into leukemic rodents, has been effective most probably due to the ADCC mechanism (103, 212).

Thus, immune complexes can be implicated in ADCC reactions, as well as the blocking of CMC reactions. The relative concentrations of the interacting components determine which mechanism will ensue (184). However, the implications for immunotherapy are antagonistic, as antibodies that are capable of inducing ADCC might also enhance tumor growth. It should be noted that the evidence for the involvement of ADCC reactions in tumor rejection in vivo and for immune complexes facilitating tumor growth in vivo is indirect.

D) Lymphokine Secretion

One of the consequences of the reaction of sensitized lymphocytes with their corresponding antigen is the production of pharmacologically active substances known as "lymphokines". Many of these soluble mediators have been identified, but only one, the migration inhibition factor (MIF), has been used extensively to demonstrate immunity to human tumors. A test, known as the macrophage migration inhibition assay (MMI), is one of the well-established in vitro correlates of delayed hypersensitivity and measures the ability of MIF, generated by exposure of sensitized lymphocytes to their specific antigen, to inhibit the migration of normal macrophages, obtained from guinea pig peritoneal exudate cells (20).

Although there is good correlation of MMI with tumor immunity in certain animal models (119, 202), and between MIF production and in vivo reactions of the delayed cutaneous hypersensitivity type, the application of the above "indirect" assay has not proved reliable (36). Hence, another approach was tried whereby the capacity of the tumor antigen (supposedly present in tumor cell extracts) to impede the migration of the leukocytes of cancer patients was measured (18, 171, 188). This was named the leukocyte migration inhibition (LMI) assay and is also mediated by a lymphokine, whose relationship to the MIF is as yet unknown.

The LMI assay was first used as an indicator of cellular immunity in breast carcinoma by Anderson (3). Crude extracts of breast cancer tissue were able to inhibit the migration of autologous leukocytes in 36% of cases tested, but were ineffective with leukocytes from control subjects. Furthermore, noncancerous breast tissue from the patients tested also failed to inhibit migration. Subsequently, reactivity to the tumor-associated antigens of malignant melanoma, bowel cancer, lung cancer, lymphoma and leukemia by this direct LMI assay has been described (25, 29, 37, 137, 138). In most cases, the responses have been directed against common antigens on tumors of the same organ and histologic type, with normal reactivity being infrequent.

Migration inhibition assays show good correlation with delayed hypersensitivity to tuberculin and other soluble protein antigens. However, their relationship to other assays of cellular immunity remains to be elucidated. It now appears unlikely that any degree of correlation exists among

delayed cutaneous hypersensitivity reactions to various tumor cell preparations, in vitro lymphocyte-mediated tumor cell cytotoxicity, as measured in different ways, in vitro lymphocyte transformation (blastogenesis) when incubated with tumor cell preparations and the search for lymphokines which have been defined.

Due to the limited success with MMI, it was suggested that the alteration of the normal properties of adherence of leukocytes to a solid surface during incubation with antigen might be an indicator of lymphocyte activity, similar to the property of antigen-induced inhibition of macrophage migration (84). This concept leads to the leukocyte adherence inhibition (LAI) assay (84), which was modified by Thomson et al. into the tube LAI for the study of tumor immunity in human breast cancer (78) and malignant melanoma (134), and which shows promise for early diagnosis of human cancer.

6) Antigenic Reversion in Man

The majority of disease-related research today is devoted to that of cancer. However, lack of knowledge about normal cell processes, genetic control and regulation and the organization of cells in tissues and organs has prevented researchers from understanding and controlling cancers. Many scientists firmly believe that until much more is known about the differentiation processes that occur during ontogeny, the answers to ways of regulating and controlling cancer will not be found.

For a long time, biologists have considered the analogies between cancer development and cell differentiation. In 1829, Lobstein and Recamier (80)

proposed that proliferating embryonic cells, which had persisted into adulthood, were responsible for cancer. Pathologists, noticing morphological similarities in cancer and embryonic tissues, have long supported this theory. Today, techniques and instruments are finer and more sensitive, yet the concept that differentiation-like changes are involved in neoplastic transformation still remains.

In the previous decade, a number of studies undertaken have reinforced the theory that malignant tumors may carry products of trophoblastic tissue, derived from either a germ or somatic cell, in which derepression may lead to aberrant cell growth (141, 142, 167, 186).

Since 1944, a variety of fetoproteins have been described in mammals, including humans. A number of these have subsequently been shown to be present in tumor tissue and fetal serum, but absent from the circulation and tissues of corresponding adult animals. Thus, these materials have been termed "oncofetal proteins". The two most widely studied fetoproteins are alpha$_1$-fetoprotein (AFP) and the carcinoembryonic antigen (CEA) (to be discussed in the next chapter). Other examples are: placental alkaline phosphatase, an isoenzyme which has been identified in the sera of patients with various malignant tumors (193); fetal sulphoglycoprotein antigen, found in gastric juice of patients with histologically verified gastric cancer (83); alpha$_2$H ferroprotein, found in children with teratomas and a variety of other cancerous diseases (28); gamma-fetoprotein, found in 75% of benign and malignant human tumor tissues and in the serum of 10% of patients with solid tumors or leukemia and is unlike the other ferroproteins in that it

does not show species-specificity, being also observed in the sera of bovine, porcine, canine and feline fetuses (51).

The ectopic synthesis of hormones by neoplastic tissues of non-endocrine origin is also regarded as another example of antigenic reversion. However, low levels of the same hormone in corresponding normal tissues suggests that the distinction may be quantitative rather than qualitative.

In 1963, it was reported that some chemically-induced mouse hepatomas synthesized an alpha-globulin. This substance did not appear in the organs of normal adult mice, but was antigenically identical to a protein found in embryonic and neonatal mouse serum (1). Two years later, human $alpha_1$-fetoprotein was detected in the sera of patients with primary hepatomas (197). Purification of AFP by Nishi (149) enabled the development of a variety of radioimmunoassay techniques (104, 174, 180) which are capable of detecting virtually all hepatomas and testicular teratoblastomas (180). Small, but significant, elevations of AFP have been observed in other malignancies, such as bronchogenic carcinoma, cancer of the stomach, Hodgkin's disease and several nonmalignant hepatobiliary disorders. The presence of low levels of AFP in normal adults has also been described (104). However, the role of the antigenicity of AFP in the autologous host is still unknown, although functions akin to albumin have been proposed due to physiochemical similarities between the two proteins (148, 173). Immunosuppressive properties have been suggested for AFP (201), but the evidence is still inconclusive (146, 156). Nonetheless, AFP is important in that it represents one of the first recognized examples of antigenic reversion in human cancers.

CHAPTER 2

1) Introduction

The term 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 (71). The initial demonstration of CEA followed a series of experiments involving adenocarcinomas of the human colon (70). This lesion was specifically chosen for study because its growth pattern is such that it does not extend intramurally for more than 6 or 7 cm either distal or proximal 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. Hence the problem of distinguishing tumor-specific antigenic differences from alloantigenic variations was circumvented.

Heterologous antitumor antisera were initially prepared in rabbits and rendered tumor-specific either by absorption with an excess of corresponding normal tissue extracts or by injecting neonatal rabbits with normal tissue extracts and thus inducing a state of immunologic tolerance to this material. The neonates were then immunized with tumor material in adult life in order to induce a tumor-specific response. The antisera prepared in both procedures were tested for their content of antibodies by a number of different serologic methods.

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 tissues (70, 117, 120). Appropriate studies excluded the possibility that the antibodies responsible for these tumor-specific reactions were directed either against the bacterial flora of the bowel or the usually high concentration of fibrin often 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. The data suggested that the presence of the tumor antigen was dependent on the tissue of origin, rather than the tissue of growth, of the tumor. Hence, embryonic gut and fetal tissues were examined for its presence. It was found that embryonic and fetal gut, pancreas, and liver during the first two trimesters of gestation, contained this material. For these reasons, the material was named carcinoembryonic antigen (CEA) of the human digestive system (71).

A number of studies using the very sensitive radioimmunoassay (RIA) techniques for the detection of CEA suggest that this material may be present in very low concentrations in tissues other than those just described (73, 135, 165). Whether this material is identical to CEA or is CEA-like in that it interferes in the assay due to the use of incompletely absorbed anti-CEA antiserum will be considered more fully in subsequent chapters.

2) Cellular Location

Agglutination studies with tissue-cultured cells of colonic cancer origin suggested that the CEA was a constituent of the tumor cell surface (68). This observation was confirmed by immunofluorescence microscopy using frozen or alcohol-fixed sections of digestive system tumors and fetal intestines, as well as viable cells explanted from freshly resected colon cancers (45, 68, 74, 116). 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 (67). In this manner, it was found that at least a portion of the CEA is situated in the glycocalyx of the tumor cell immediately adjacent to the surface membrane (67). It would therefore appear that CEA is not a component of the trilaminar image usually referred to as the plasma membrane, but lies even further to the periphery of the cell in what has been termed the "greater membrane" of the cell surface. At this location it can be seen how easily CEA may be released into the surrounding body fluids.

There has been some debate as to whether or not CEA is an integral portion of the glycocalyx or simply material in transit from the cytoplasm across the cell membrane (63, 198). Recent studies have shown that antibodies specific for CEA are able to cap the CEA expressed on the surface of human intestinal cancer cells grown in tissue culture (172). Since capping occurs with components of the plasma membrane, it would seem that CEA is one of these components.

Another aspect that is still under question is if CEA is synthesized within the tumor cell or if it is made elsewhere, transported to the tumor cell and either absorbed onto the membrane or somehow interiorized. However, a number of observations clearly demonstrate that CEA is indigenous to the cancer cell. Human colon cancer cells serially passaged in unconditioned golden hamsters continue to produce and release CEA in the animal host (72), and the established cultured cell line, HT-29, derived from primary colon adenocarcinoma tissue, has been shown to synthesize and secrete CEA (55).

3) Chemistry of CEA

A) Isolation and Purification

Most procedures for the purification of CEA have utilized perchloric acid (PCA) extraction and gel filtration (63, 198). These procedures may yield pure CEA, but many other additional steps have been used to achieve final purification. These include block electrophoresis, ion exchange chromatography, isoelectricfocusing, density gradient centrifugation, lectin affinity chromatography and immunoaffinity chromatography (4, 63, 168, 198). Similar products have been obtained by these different methods, but subtle and important immunochemical differences may result from variations in the purification procedures. Treatment with PCA may cause chemical and/or conformational changes in CEA or select certain molecular subpopulations (113, 168). CEA prepared by methods which omit the PCA step should be more representative of the physiological state of the antigen.

Purified CEA shows a single diffuse band on sodium dodecyl sulfate polyacrylamide gel electrophoresis with an apparent molecular weight of 200,000

daltons. It was found to give a single symmetrical peak in the analytical ultracentrifuge with a sedimentation coefficient of 7-8S. Immunoelectrophoresis against anti-CEA antiserum reveals a single band in the β -globulin region (122). Ion exchange chromatography and isoelectric focusing studies show that CEA is an acidic molecule with considerable charge heterogeneity (63, 168, 198). This was found not to be due entirely to variation in sialic acid content since even after removal of the sialic acid with neuraminidase, some heterogeneity remained (13, 14, 41, 63, 122, 198). By electron microscopy, the molecule appears as a morphologically distinct cruller shaped or twisted rod with dimensions 9x40 nm (185), being a single chain structure with multiple intrachain disulfide bonds, at neutral pH (63). At lower pH, the particle chain length decreases.

B) Carbohydrate Portion

CEA is a glycoprotein with the carbohydrate content varying from about 80% for purified CEA of gastric origin to about 40% for CEA obtained from colon cancer tissue (14, 63, 198). In general, the most variable sugar is sialic acid which occurs to the extent of 11.0 mol/10^5 g CEA. Fucose, galactose and mannose are present in roughly equal amounts (about 60 mol/10^5gCEA) and there is almost double this amount of N-acetyl glucosamine. Little or no N-acetyl galactosamine is present in highly purified preparations. The carbohydrate portion of CEA seems to be linked to the protein via an N-glycosidic bond between N-acetylglucosamine and asparagine (53, 211), and is unusual in having a large amount of branching mannose residues, 3/4 of the

mannose being branched. The variation seen in the carbohydrate portion of the CEA from different origins, as well as variations that are seen from preparations in many labs, is partly due to the fact that the biosynthesis of the sugars is a post-ribosomal event (150, 190).

C) Protein Portion

CEA appears to be a single chain by electron microscopy and by virtue of the fact that reduction and alkylation does not greatly change the molecular weight of CEA in SDS gel electrophoresis (52, 198) or by gel filtration (86). CEA yields a single N-terminal amino acid sequence which also supports the single chain idea (199). Amino acid analyses of purified materials have revealed a fairly consistent pattern with some minor variation from preparation to preparation and suggests that the protein is relatively hydrophilic in nature (121, 122), with six intrachain disulfide bonds (208). The major amino acid is always aspartate and/or asparagine. There are low levels of basic and aromatic amino acids. The N-terminal amino acid sequences of several different CEA preparations were virtually identical for the first 15-30 residues (35, 38, 199).

D) Antigenic Determinants as Defined by Heteroantisera

Attempts have been made to localize and chemically characterize the tumor-specific antigenic site, but results so far have been inconclusive. One of the major questions asked is whether the site resides in the carbohydrate portion of the molecule or in the protein backbone. Neuraminidase treatment of CEA removes all the sialic acid residues without

any loss of antigenic activity (40). Other studies have shown that periodate oxidation, which destroys most of the sugar residues, does not affect the activity of CEA in its radioimmunoassay (38, 39, 53, 87, 210).

However, treatment of CEA with dilute alkali destroys its activity (209) as does reduction and alkylation of CEA, but to a lesser extent (86, 209). When the thiol blocking agents are removed by mercaptoethanol, more than half of the original activity is restored (208). Chemical substitution of a number of amino acids by the appropriate reagents affected immunoreactivity only in those cases where gross conformational changes were observed (129).

On the other hand, there have been reports that indicate that the carbohydrate residues in CEA are the important antigenic determinants (14, 15, 16). Heterosaccharide fragments of CEA (16), synthetic compounds containing the N-acetylglucosamine-asparagine linkage (15) and nagase fragments of CEA (14) all inhibited in the RIA. However, the specific activity of these fragments was many thousand times less than that of CEA.

Thus, the majority of evidence indicates that the peptide portion contains the antigenic determinants that are measured in the RIA. It is possible, however, that some if not all, anti-CEA antisera have some antibodies directed against the carbohydrate portion, but their affinity for the CEA molecule is not as great as those antibodies directed against the protein portion (56).

Studies using monkey, rabbit and sheep anti-CEA antisera (85) have shown that colorectal CEA contains between 10-20 determinants per molecule depending on the antiserum used. Sheep recognizes about 18, rabbit about 15

and monkey about 10. Furthermore, monkey serum does not seem to have antibodies directed against certain cross-reacting antigens, the non-specific cross-reacting antigen (NCA) and the biliary glycoprotein (BGP), and thus may be a good choice as a clinical antiserum. However, whether or not other absorptions are necessary remains to be seen.

E) Metabolism of CEA

As already indicated, it would seem that CEA is produced by the tumor cell. However, much is not known, or yet to be determined, with respect to the catabolism of the molecule. In studies using the sera of patients who have undergone curative tumor bowel resection, it was observed that CEA was rapidly catabolized, and the serum levels of CEA 2-14 days postoperatively fell to virtually undetectable levels (47, 109). Although the site of CEA breakdown in man is as yet unknown, experiments in animals indicate the liver to be the most probable site (178).

4) Host Immunity to CEA

A) 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 (108). Negative reactions were observed when comparable normal tissue fractions were used. 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

reactive antigen(s) involved and purified CEA are quite distinct from one another (107, 108). In addition, purified CEA was found to be incapable of stimulating transformation of lymphocytes taken from patients with colon cancer (128).

In vitro correlates of cell-mediated immunity have also been sought in patients with colon cancer. Using the colony inhibition technique, peripheral lymphocytes from these patients were shown to possess cytocidal properties directed against their own tumor cells as well as those from other patients (97). It was suggested that the CEA might be the common factor involved, but no studies were done to investigate this point (128). It should be noted, however, 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 (128).

B) Humoral Immunity

A specific IgM humoral anti-CEA antibody response was detected in patients' sera, using at least two different techniques (64, 65, 69). The fact that the response is IgM mediated without a conversion to IgG as yet is unexplained. However, the peculiar IgM response is found not only in humans, but has also been observed in the golden hamsters bearing the CEA-producing tumors (163).

In analyzing these results, much attention must be given to the method of antibody detection employed. Using the bis-diazotized benzidine hemagglutination technique, it was shown that the sera of patients with digestive

system cancers were positive, but only if there was no metastatic dissemination of the malignant tumor. In cases where metastases occurred, the sera were invariably unreactive (69). However, the techniques of radioimmunoelectrophoresis and radioimmunochromatography have shown the presence of anti-CEA antibodies in the sera of patients who manifest metastatic cancer (64, 65). A modified Farr RIA technique, using acid dissociation to detect antibody bound to antigen, was unable to show the presence of anti-CEA antibodies under any circumstances. Hence, it may be the technologic problem involved, as well as the type of reagent used, that account for the inability of workers to show the presence of anti-CEA antibodies in the sera of patients with digestive system cancer (118, 130). It should be noted that the specific IgM humoral response was detected in pregnant women in all trimesters of pregnancy and in the immediate post-partum period, but the function of these antibodies remains to be determined.

5) Antigens Cross-Reacting with CEA

An anti-CEA antiserum was made in a xenogeneic animal in order to obtain an antiserum specific to the CEA molecule, particularly to the tumor-specific antigenic determinants on the molecule. However, as more investigations of this molecule were performed, it became evident that CEA is heterogenous both inter- and intramolecularly and that the CEA of bowel system cancers may just be one of a family of molecules located in tissues all over the body. The antiserum, even after appropriate absorptions, was also found to contain

many different antibody populations, immunologically defined as being directed against many cross-reacting substances i.e. substances believed to be clearly distinct from CEA yet sharing common determinants.

In the last five years, twelve cross-reacting antigens have been described, all of them identified by the use of the anti-CEA antiserum (see Table 2). It has since been shown that the first six of these materials are serologically identical. Further investigation must be done to ascertain what relationship, if any, exists between the rest of the antigens to the first six. There is some preliminary data that suggests that NCA-2 and CELIA are identical to each other and to the fecal antigen described by Matsuoka (136).

A) Non-Specific Cross-Reacting Antigen

Of all these cross-reacting substances, the non-specific cross-reacting antigen is the one which has been studied the most extensively, and henceforth will refer to those first six antigens in Table 2. NCA was isolated from colonic tumors, but was also found in normal colon, spleen, lung and plasma (32, 115, 117). NCA shows most of the physical characteristics of CEA. It is soluble in PCA and is a PA-Schiff positive glycoprotein which migrates towards the cathode. At concentrations of between 0.1 and 2 mg/ml it gives a single identical precipitin line in immunodiffusion with rabbit and sheep anti-CEA and goat anti-NCA, and a line of partial identity with CEA using anti-CEA antiserum. The molecular weight of NCA is approximately 60,000 daltons. Amino acid analysis shows marked similarity to CEA. Carbohydrate comparisons show slight differences. Immunoelectrophoresis has

TABLE 2

Antigens Cross-Reacting with CEA

		NAME	ABBREVIATION	REFERENCE
I	1.	Normal Glycoprotein	NGP	J.P. Mach and G. Pusztaszeri (131)
	2.	Non-Specific Cross-Reacting Antigen	NCA	S. von Kleist et al. (115)
	3.	CEA-associated Protein	CEX	D.A. Darcy et al. (44)
	4.	Colonic CEA - 2	CCEA-2	C. Turberville et al. (200)
	5.	Colon Carcinoma Antigen III	CCA-III	E.S. Newman et al. (147)
	6.	Beta External Protein		H. Orjasaeter (155)
II	7.	Fetal Sulphoglycoprotein	FSA	I. Hakkinen (82)
III	8.	Breast Cancer Glycoprotein	BCGP	T. Kuo et al. (123)
IV	9.	Second Non-Specific Cross-Reacting Antigen	NCA-2	P. Burtin et al. (31)
V	10.	Biliary Glycoprotein	BGP-1	T. Svenberg (194)
VI	11.	Gastric CEA-like Antigen	CELIA	M. Vuento et al. (206)
VII	12.	Pancreatic Tumor Ascites Fluid Glycoprotein	PAFG	T.M. Chu et al. (34)

shown NCA to be distinct from CEA, having its own specific unshared determinants. Thus, when anti-NCA serum is absorbed with CEA, it will still react with NCA. It was found that pulmonary tissue is particularly rich in NCA and is usually the tissue of choice for its extraction.

The clinical evaluation of serum NCA markedly differs from CEA. The assay used was a double antibody assay and it was shown that CEA does not interfere in this assay (49, 126). Normal circulating values of NCA are 150 ng/ml compared with 2.5 ng/ml for CEA. Elevated NCA levels were mainly found in pulmonary tissue diseases, especially in tuberculosis. However, whereas CEA levels rise substantially in neoplastic diseases, NCA values show a moderate augmentation, with rapid levelling off. Indeed, NCA values in cancerous diseases, regardless of the tumor site, rarely exceeds 260 ng/ml. Hence, although NCA may be interesting in that it is CEA-like and is an important tool in further specifying the anti-CEA antiserum, its clinical value in neoplastic or any diseases (except perhaps for TB) has not yet been realized.

B) Breast Cancer Glycoprotein

This material was discovered by Kuo and workers (121) using the method of Rosai et al. (170) to isolate membrane-bound CEA on individual breast carcinomas metastatic to the liver. BCGP is also found in lung tissue, which may indicate a relationship to NCA. Anti-CEA antiserum can be absorbed of its anti-BCGP activity, leaving behind activity to only CEA. It has been suggested that CEA reactivity in breast cancer as seen with unabsorbed

anti-CEA serum may be due to the BCGP rather than by colonic-type CEA. The molecular weight of BCGP is around 200,000 daltons, the only cross-reacting antigen that resembles CEA in that respect.

C) Fetal Sulfoglycoprotein

This material is a fetal type of a sulfoglycoprotein and has been demonstrated by double immunodiffusion in the gastric juices of patients with histologically verified gastric cancer (83). Because secretion of FSA seemed to precede the development of morphologically distinct cancer cells, this molecule became ofinterest to workers as a possible tool in screening gastric cancers. Its relationship to CEA was also investigated and studies have ascertained its cross-reactivity with CEA, implying a shared determinant (82) but was also found to contain unique, unshared determinants.

D) Second Non-Specific Cross-Reacting Antigen

This antigen was described by Burtin et al. (31). It was identified in the PCA extracts of feces of noncancerous and cancerous patients and meconium. Recent studies have shown that NCA-2 is quite similar to CEA (30). Its molecular weight is slightly less, being 160,000 daltons, but its electrophoretic mobility is comparable as is its chemical composition. Comparisons of various anti-CEA antisera show different types of reactivity from lines of complete identity to no cross-reactivity at all. Since strong anti-NCA-2 antiserum is as yet unavailable, the degree of cross-reactivity with CEA remains to be determined.

E) Biliary Glycoprotein 1

This molecule is derived from hepatic bile and was reported by Svenberg

(194) as being a CEA-like, PCA-soluble glycoprotein. Partial identity with CEA as well as cross-reactivity with NCA has been demonstrated. BGP-1 shows α-electrophoretic mobility with a molecular weight in between that of CEA and NCA as determined by Sephadex G-200 mobility. BGP-1 did not inhibit in the enzyme-linked immunoabsorbent assay (ELISA), hence the common determinant observed on immunoelectrophoresis does not appear to be the tumor-associated determinant of CEA.

F) Gastric CEA-Like Antigen

This material is a PCA-soluble antigen described by Vuento (206) and found in gastric juice. This molecule may be identical with NCA-2 on the basis of molecular weight similarities, this same evidence establishing non-identitywith the other antigens.

This study of cross-reacting antigens is important not only in trying to eliminate non cancer-specific reactions, but in further assessing the antibody populations found in the anti-CEA antiserum and the reliability of the RIA's in use today.

6) Radioimmunoassay for CEA

The initial assays for CEA, which involved precipitation reactions in gel, were sensitive and appeared specific for the embryonic antigen. However, since then, a number of reproducible and more sensitive assays have been introduced (177), among them the ammonium sulfate Farr technique, the double antibody assay and the zirconyl phosphate gel assay. These

techniques, due to their increased sensitivity, proved to be less specific than their forerunner (176), repeatedly giving false positive and false negative results. Therefore, clinical investigators began to suggest reasons for these apparent findings. The first of these is the possibility that CEA is present in very low concentrations in tissues other than gastrointestinal cancers and fetal and embryonic digestive system organs. Similarly, data has accumulated indicating the presence of CEA or CEA-like substances in the circulation of patients with nonenteric cancers or those manifesting other forms of tissue pathology. Whether or not these materials are identical to the CEA of gastric origin, or are CEA-like substances which mimic the presence of CEA due to the use of poorly absorbed antiserum, remains to be elucidated. Lastly, the question is asked whether the interference is not simply due to large molecular weight serum proteins reacting in a nonspecific manner in the assay. In view of these persistent problems, the parameters influencing the detection of CEA in the radioimmunoassay will be briefly described.

The antigens used in the assays are usually purified from hepatic metastases of colon cancer; however, purification methods differ from laboratory to laboratory. Immunologic and chemical comparisons are available for only a few preparations of purified CEA. Thus, a universally accepted standard must be introduced. In light of recent evidence, it now seems as if CEA of the gastrointestinal tract is probably one of a family of CEA-like molecules found in pathologic tissues (63), and that there are likely a number of nontumor-specific determinants on CEA (in addition to the

tumor-specific ones) that are shared with molecules produced by various tissues in other disease states. For example, in one study done using four different "standard" CEA preparations from four different laboratories, and two different anti-CEA antisera, significant antigenic dissimilarities were observed between some of the "standard" CEA's employed. In addition, the serum CEA and the tumor CEA from the same patient appeared to be antigenically different (204, 205).

The heterogeneity of CEA has been further investigated by using additional steps beyong those generally used to purify CEA (63). Concanavalin A affinity chromatography can separate CEA into several peaks, one of which was shown to have greater antigenic activity when measured by certain antisera (169). A fraction of CEA, called CEA-S, constituting less than 5% of the "standard" CEA's in use, has been isolated by a group of investigators to be employed in diagnostic testing (160). RIA's using CEA-S show greater specificity; however, the sensitivity of the assay was decreased (50, 100).

Aside from the obvious need for homogeneous CEA populations, a standard for the absorption of the antiserum must also be adopted. Different antisera used in various assays have variable degrees of specificity and recognize both the CEA tumor site as well as other antigenic sites on the molecule (63) and, as mentioned before, describe many cross-reacting antigens. Figure 1 shows preliminary data gathered in our laboratory whereby unabsorbed anti-CEA antiserum prepared in a horse was absorbed on a Sepharose affinity column coupled with normal tissue extracts, towards which the antiserum had

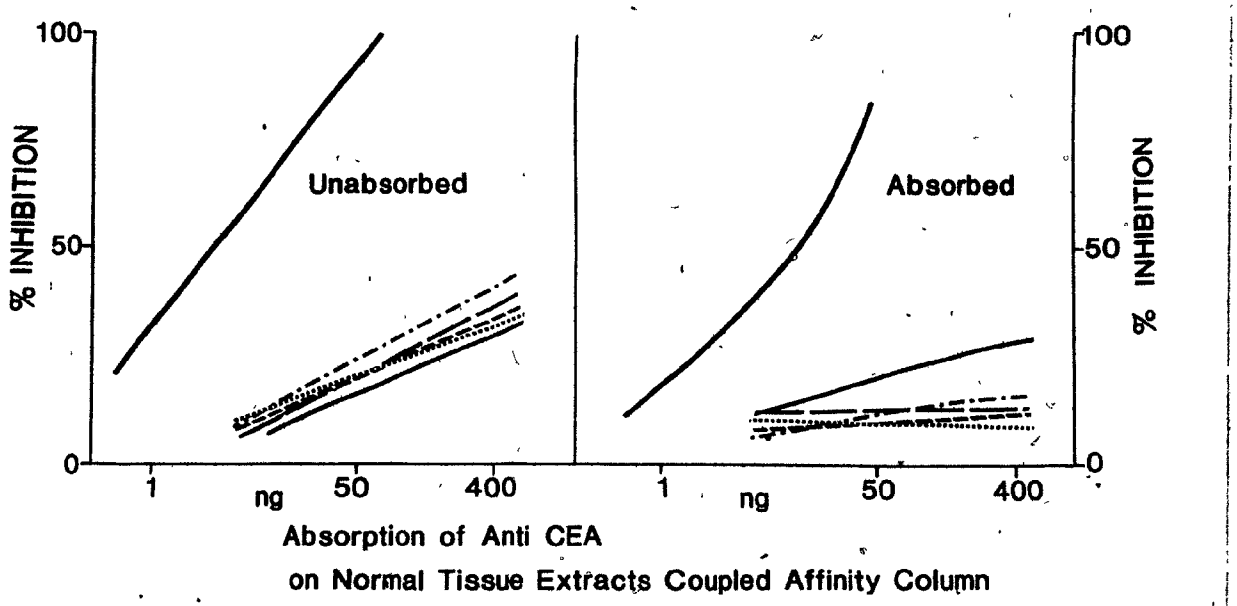


Fig. 1: Results of an RIA established between unabsorbed anti-CEA antiserum and the same antiserum absorbed on an affinity column coupled with various normal tissues. The curves compare the inhibitory activity in the assay of CEA (————), NHS (— — —), lung (·····), bowel (————), stool (— · — · —) and liver (- ——— -).

activity. After one passage of the antiserum through the column, reactivity to two of the normal materials (bowel and stool) remained and was not removed after successive recycling. Aside from those antigens already described, it is most likely that there is a number of materials not yet detected that would also interfere in the RIA. Absorption with all these materials would be needed to get an antiserum as specific as possible. However, it must be remembered that the amount of antibody produced in the xenogeneic animal to any of the CEA cross-reacting substances may not be indicative of the quantity of the immunologic determinants in the immunizing preparation. This, of course, depends on the immunogenicity of the animal. With this in mind, absorption of anti-CEA antisera becomes more intricate and conventional methods may not be adequate.

The most widely used clinical assay is the "indirect" Z-gel assay method (88). This involves subjecting serum samples to extraction with perchloric acid followed by dialysis against low ionic strength buffer. This treatment tends to minimize the problem of specific and nonspecific interference factors in different plasma matrices and the sensitivity of CEA to ionic strength. However, a direct assay is still preferable. There has been some progress in this area (54, 127) whereby undiluted and untreated plasma samples are used. Good correlation between the direct and indirect assays are observed at high plasma concentrations of CEA, but, due to the presence of PCA-labile materials which cross-react with many of the anti-CEA antisera employed, discordance is seen in the 0-10 ng/ml range, an area that is highly significant in certain clinical stituations.

7) Role of the Radioimmunoassay for CEA in Clinical Medicine

The ideal immunodiagnostic assay can be visualized as being an important tool in three areas: cancer screening, which entails the assaying of large populations to determine those at risk, or the screening of asymptomatic individuals for evidence of impending tumors; cancer diagnosis, which involves the detection of malignancies in symptomatic individuals where the CEA assay is part of the diagnostic workup; cancer management, in which further CEA determinations in previously diagnosed individuals are used for the establishment of a prognosis, in detecting the occurrence of metastases or the recurrence of disease, or for monitoring the results of therapy.

The CEA assay cannot be reliably used in the area of cancer screening or diagnosis. To do so would imply that the assay is sensitive enough to allow a low or negligible incidence of false negative assays, specific enough to obviate false positives and, in addition, should show organ specificity to allow for the localization of the tumor mass (100). Present assays, although sensitive and reproducible, do not meet these requirements. Negative results may be obtained in patients with early cancerous lesions, while positive results may not always indicate malignancies. However, although many of the nonmalignant conditions which give positive results do so with only transient or low levels of CEA, rather than persistent, rising levels, and thus are distinguished from cancerous growths (63), at the present time screening of healthy individuals does not seem feasible. By itself, the CEA assay cannot be reliably used as a diagnostic tool. Recent data has suggested the use of

the assay in conjunction with other diagnostic procedures for cancer. For example, the assay has been found to be positive for pancreatic cancer and when used along with barium enema, the detection rate, in colon cancer, is greater than when each test is used alone (66).

The majority of definitive data for the reliable use of the CEA assay has been accumulated in the area of cancer management. In general, a good correlation has been observed between CEA elevation in the serum and tumor stage. High values usually indicate more advanced tumor stages, as seen in the comparison of levels for colorectal cancer Dukes A and D stages (140). Usually, high preoperative CEA values (20 ng/ml) indicate presence of metastatic lesions (63), and, in patients with bowel cancer, the higher the preoperative levels of CEA, the more rapid and frequent the rate of recurrence of the disease postoperatively (24, 132). CEA levels below 5 ng/ml or even below the normal range of 2.5 ng/ml do not rule out metastatic cancer, but suggest a resectable lesion (63).

Postoperative use of the CEA assay has proven to be reliable when interpreted at least one month post surgery. Generally, a decline in the plasma CEA levels correlates well with complete resection of the tumor (63). In those patients whose postoperative CEA level was negative or very low, the majority did not manifest any evidence of recurrence of the particular type of cancer. In those cases where the CEA level did not drop after surgery, incomplete tumor resection was responsible, and where the CEA levels increased steadily, recurrence or continuation of the tumor growth was observed. Of greater importance is the fact that patients with previous

postoperative negative CEA levels, who suddenly become positive in the assay, usually manifest tumor recurrence or continued tumor growth. This reappearance of circulating CEA frequently precedes clinical and laboratory evidence of the cancer anywhere from a few weeks to two years (24, 63) and has suggested early application of chemotherapy or radiotherapy in an attempt to arrest the disease in its early stages. "Second-look surgery" has also been suggested and a study of it is being undertaken by a number of groups (12, 191). This surgery has already been employed, based on rising serial CEA levels in symptomatic patients, and single metastatic nodules were discovered and successfully resected (139). However, consideration must be given to the possibility that postoperative rises in CEA levels may be caused by nonmalignant states, especially in cases of hepatic malfunction, and that this must be distinguished from CEA elevations in patients due to tumor recurrence (211).

The use of CEA assays to monitor chemotherapy and radiotherapy of the patient seems to correlate well with the repression or progression of the cancer.

Recently, much interest has been given to examining CEA levels in body secretions and excretions, digestive juices, ascites and pleural efflusions, and other body fluids in conjunction with serum CEA analyses to detect both primary and secondary tumors (176).

CHAPTER 3

Purpose of Study

It is apparent from the previous section dealing with the history of CEA that the overriding problem in the clinical study of this material is that of the specificity of the radioimmunoassay, or, more directly, the specificity of the antigens and antisera employed.

The problem of antigenic cross-reactivity observed in the assay resolves itself into three possibilities:

(1) CEA is present in minute quantities in normal bowel tissue;

(2) Antigenic moieties exist in normal bowel tissue which are cross-reactive with, but distinct from, CEA;

(3) There is interference in the assay due to nonspecific materials, such as high concentrations of alphaglobulins.

The difficulty encountered with the antisera arises from the inability, by heteroimmunization, to obtain monoclonal antibody populations. The multiple epitopes on the CEA molecule give rise to a variety of diverse antibody populations upon immunization of a xenogeneic animal, the specificity of which is never really certain.

Thus, this thesis focuses on antiserum specificity and how it relates to the problem of cross-reacting normal bowel antigens.

The method of making the anti-CEA antiserum more specific to the CEA molecule has, in the past, been by liquid phase absorption with a variety of

normal tissue extracts. However, this absorption method did not eliminate the problem of interference by cross-reacting substances in the radioimmunoassay.

This study attempted an alternate approach to improving the specificity of the antiserum. The method employed was solid phase affinity chromatography with normal bowel tissue extracts. This technique has previously been used to isolate specific antigen moieties. Thus, this method was adopted in order to absorb out those antibody populations not directed against the tumor portion of the CEA molecule, and to determine if, in deed, there exists a specific tumor epitope on the CEA molecule.

In the final analysis, the effectiveness of solid phase immunoabsorption versus liquid phase absorption are compared with respect to the type of anti-CEA antiserum produced.

CHAPTER 4

Materials and Methods

1) Principles of Affinity Chromatography

The primary method of approach in these studies was affinity chromatography. First introduced by Anfinsen and co-workers in 1968 (42), this technique has since been employed in the selective isolation and purification of enzymes and other biologically important macromolecules.

The technique exploits the unique biological property of macromolecules or proteins to bind ligands specifically and reversibly (42). The basic principle involves a ligand attached covalently to a water-insoluble matrix to form chromatographic material suited to absorb from a mixture just those components having an affinity for the ligand. Proteins or other molecules not exhibiting appreciable affinity for the ligand will pass unretarded through the column, whereas those which recognize the ligand will be retarded to an extent related to the affinity constant under the experimental conditions. This method thus closely parallels the use of insolubilized antigens as immunosorbents for the purification of antibodies (179).

The primary advantage of affinity chromatography over conventional separation techniques is its specificity. In addition, as a consequence of the tiny proportion of total protein absorbed from a crude mixture, a relatively small amount of efficient absorbent is required. Also, the

absorbed material is rapidly separated from proteolytic enzynes and may be stabilized by ligand binding at the "active site". In addition, the absorbent can usually be regenerated many times. In designing an affinity chromatographic system, a number of points must be considered (79):

(i) No great modification of the ligand must occur either during attachment to the support or under the experimental conditions;

(ii) The ligand must be of suitable length such that the binding determinants are accessible;

(iii) The ligand must interact specifically and reversibly with the molecule to be purified. Interactions involving dissociation constants greater than 10^{-3} mole l^{-1} are likely to be too weak;

(iv) The ligand must be suitable for coupling to a matrix with the minimum amount of modification to that part of its structure essential for binding;

(v) The matrix must be capable of mild chemical modification without undergoing gross structural changes (particularly shrinkage), be free of ionic residues which would cause nonspecific interactions with proteins, have a loose lattice structure of sufficient hydrophilic nature to permit interaction between the two phases (liquid and solid). Also, the matrix should be spherical, rigid and of uniform size to permit uniform and unimpaired entry and exit of large macromolecules, and retain good flow properties before and after coupling. Beaded agarose, polyacrylamide and glass fulfill these requirements.

It should be noted that the amount of ligand coupled to the matrix carrier represents the maximum theoretical binding capability and should not be equated with the capacity as a bioabsorbent. In practice, only a fraction of the molecules coupled may be accessible for binding, since the matrix may have nonideal porosity. Also, once a macromolecule is absorbed, it may mask adjacent ligands. Therefore, although raising the ligand concentration on a matrix improves most bioabsorbents up to a point, there is usually a limit above which the capacity no longer increases and may begin to fall (112);

(vi) It must be ensured that the ligand-carrier complex is mechanically and chemically stable to the experimental conditions of coupling and elution; otherwise, there may be leakage of ligand during the chromatography;

(vii) Often, it is necessary to insert a spacer between the ligand and its support to give greater accessibility. This is achieved by either coupling the ligand to one end of an "arm", the other end of which is subsequently attached to the carrier, or by coupling it to an arm already modifying the matrix. Due to commercial availability of matrices with spacer arms, the latter technique is preferred.

The spacer arm is usually a hydrocarbon chain of three or more carbons. The arm is especially necessary for those ligands that do not have an NH_2 group suitable for direct coupling. The coupling of ligand to matrix involves a covalent bond. It has been found that ionic bonds or physical absorption are prone to leakage or displacement of ligand from its carrier.

The greater effectiveness of ligands when attached to spacer arms is generally ascribed to their increased steric availability to the protein being absorbed. Although this is doubtless the major factor, there are others to consider. The ligands, themselves, may be more separated when on a long mobile chain so that possible masking of those adjacent by an absorbed protein molecule is minimized. Also, controls have rarely been run to ascertain whether the arm alone has any affinity for the protein, although even if there were an additional effect, it would often be desired, provided it were specific. It should be realized, however, that even a tailor-made ligand-carrier-system does not necessarily constitute a bio-specific absorbent. It has usually been tacitly assumed that spacers play little part in the chromatographic process and that ligands exhibit similar affinity characteristics in the free state as when modified to render them sterically available. Recent studies show that these assumptions are not always valid (111, 151).

Nonetheless, affinity chromatography has become an accepted part of biochemical methodology and has facilitated the isolation of many interesting macromolecules hitherto inaccessible by less sophisticated techniques. In this section, the conditions for optimal coupling of ligand to solid supporting media will be determined. This will then serve as a basis for subsequent studies in which ligand coupled to a solid support medium will be used as an immunoabsorbent to further specify anti-CEA antisera.

2) Preparation of the Carcinoembryonic Antigen

The CEA utilized in the following studies was prepared, as outlined below, by a variation of the method of Krupey et al. (67). The term CEA or standard CEA, designates preparations of the carcinoembryonic antigen of the human digestive system prepared by following this method without alterations.

A) Initial Preparations of Tumor Specimens

Whenever possible, hepatic metastases from primary adenocarcinomas of the colon or rectum were employed due to the relatively high concentrations of CEA in such lesions (12). 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 used for extraction.

In preparation for extraction, an aliquot of tumor tissue was thawed slightly at room temperature, then chopped into small sections using a stainless steel knife. The sections were added to 4 l of distilled water and homogenized in a water-cooled Virtis Mixer at 15,000 rev/min for 15 minutes. The demonstration of CEA activity in the initial homogenate, and at each stage of purification, was performed by Ouchterlony reaction against absorbed anti-CEA antiserum (to be described below). 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.

B) Perchloric Acid Extraction

500 ml of the tumor tissue homogenate was mixed with an equal volume of

cold 2.0 M perchloric acid (PCA), and then stirred for ten minutes at room temperature. The resulting suspension was centrifuged at 8000g at 4°C in an IEC Centrifuge in 250 ml aliquots for 15 minutes. The sediment was discarded and the supernantant dfalyzed either 72 hr against cold tap water or in a hollow-fiber dialyzer against cold tap water until the pH of the supernantant reached 4.0. The dialysate was then concentrated in an Amicon with a PM30 membrane to a volume of approximately 100 ml. The filtrate was then lyophilyzed.

C) Preparative Gel Filtration Chromatography

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

An aliquot of 1.5 g of the lyophilyzed powder of the PCA-extracted tumor tissue was dissolved in 50 ml of the PBS and applied to a previously equilibrated Sepharose 4B Pharmacia column (Type K100/100) with the dimensions 89 x 10 cm. Elution was performed by upward flow at a rate of 150 ml/hr. The eluate was monitored for its spectrophotometric absorption at 280 nm, and was collected in 25 ml fractions. Those fractions possessing CEA activity were dialyzed against distilled water at 4°C for 48 hr, concentrated and then lyophilyzed.

A 200 mg sample of powder derived from the Sephatose 4B column and containing the CEA activity was dissolved in 10 ml of the PBS. This solution was then applied to a 90 cm x 5 cm Pharmacia column (Type K50/100) 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 280 nm, was collected in 10 ml aliquots. Fractions containing CEA activity were then pooled, dialyzed, and lyophilyzed as described for the eluate from the Sepharose 4B column.

D) Preparative Gel Block Electrophoresis

Sephadex G-25 was washed and equilibrated with 0.05 M borate buffer pH 8.6. A thick slurry of this material was poured into a level lucite mold (61 x 7.5 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 a cotton gauze sponge until it had a firm consistency, but was not dry. The gel block was fitted with Whatmann 3 mm chromatographic paper contacts and placed in an electrophoresis apparatus where the electrode chambers contained the same borate buffer as was used in the washing of the gel.

Equilibration of the system was allowed to occur for 1 hour 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 procedures was dissolved in 0.5 ml of the borate buffer. A 1 cm strip of gel was then removed from the centre of the block and was thoroughly mixed with the solution of CEA. This slurry was then poured into the trough in the centre of the block, formed when the gel strip was removed. Ferritin (0.01 mg in 0.005 ml 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 hours. Following

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 filration 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, pooled, lyophilyzed and stored at 4°C in vacuo.

3) Preparation of Antisera

A) Preparation of Horse Unabsorbed Anti-CEA Antiserum

An adult male horse was immunized initially with 1 mg of purified CEA dissolved in 1.0 ml of sterile saline and emulsified in an equal volume of complete Freund's adjuvant. Booster doses of 500 μg were given at one to three month intervals depending on the titre of the antiserum. One week after these booster injections, the animal was bled. The bleed which gave the highest titre of CEA-reactive antibody was then employed as the antiserum for use in the radioimmunoassays and the research studies. This antiserum was thus designated Horse unabsorbed anti-CEA antiserum and abbreviated as "anti-CEA".

B) Preparation of Horse Absorbed anti-CEA Antiserum

Normal liver, lung, colon and human serum were collected and saline extracts of each were prepared. 100 mg of each normal tissue extract were added to 5 ml of anti-CEA. The slurry was stirred ovrnight at 4°C. The following morning, it was centrifuged in a Sorvall RC2B at 25,000g for 30 minutes. The supernatant was then filtered through a Millipore filter and stored in 20 ml aliquots at -20°C. This preparation of anti-CEA antiserum

was thus designated standard horse absorbed anti-CEA antiserum.

C) Preparation of the Gammaglobulin Enriched Fraction of anti-CEA

An 18% solution of sodium sulfate was prepared. To 1 ml of neat anti-CEA was added 180 mg of sodium sulfate followed by vigorous stirring. One millilitre of the 18% was then added, followed again by vigorous stirring. When the salt had dissolved the stirring was maintained for 1 hour at 25°C, followed by centrifugation at 38,000g for 5 min also at 25°C. The supernatant was discarded and the precipitate washed with 2.5 ml of the 18% solution and recentrifued. The supernatant was again discarded and the precipitate was dissolved in 1 ml of 0.1 M sodium bicarbonate pH 8.0 followed by dialysis overnight in the sodium bicarbonate at 4°C. This was then followed by dialysis against 0.2 M sodium citrate pH 6.5 for 12 hr at 4°C. A final dialysis for 4 hr was done against the bicarbonate buffer. The protein content of this gamma cut was determined by the Folin method (99).

D) Preparation of Sheep anti-Horse Gammaglobulin

An adult male sheep was initially immunized with 1 ml of a saline solution containing 1 mg of horse gammaglobulin (Pentex, fraction IV) dissolved in an equal volume of Freund's complete adjuvant. Subsequent injections of 100 µg of horse gammaglobulin were administered at two week intervals for six weeks. Booster injections of 100 µg were given every month, and the following week 300-500 ml of blood were collected. The blood was spun at 1000g in a Sorval RC2-B for 15 min. The supernatant was decanted and stored at -20°C in 10-15 ml aliquots. This preparation served as the second antibody in the double antibody radioimmunoassay for CEA.

4) Radioimmunoassay for CEA

A) Radiolabelling of CEA

CEA was shown to contain tyrosine residues (48, 164). This material was therefore radiolabelled with ^{125}I by the chloramine-T method (77) as follows:

(i) The diluent utilized in each step was phosphate buffered saline pH 7.4 0.05M (PBS);

(ii) The reaction was carried out in a 4 ml flat bottom glass vial, containing a 1/16" x 1/4" teflon-coated magnetic stirring bar. These were discarded after each labelling procedure;

(iii) Eppendorf pipets (Brinkman Instruments) of appropriate capacities were utilized to measure and dispense all reagents;

(iv) Fresh solution of chloramine-T (1.0 mg/ml, Eastman Chemicals, Rochester), sodium metabisulfite (2.0 mg/ml) and potassium iodine (10 mg/ml) in the diluent buffer were prepared before each radioiodination procedure;

(v) The pH of the ^{125}I solution was measured by placing a 1µl aliquot onto narrow range alkaline pH paper and used only if the pH of the reagent was mildly alkaline;

(vi) ^{125}I 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, Ont.);

(vii) The glycoprotein to be labelled was diluted with PBS to a final con-

centration of 1 mg/ml. The conjugation procedure was performed as follows:

To 50 µl of phosphate buffer 0.1 M, pH 7.4 was added 20 µl (20 µg) of the 1 mg/ml solution of the glycoprotein, 2 mCi of ^{125}I and 20 µl (20 µg) of Chloramine-T under constant stirring. The reaction time, at 25°C was 90 sec. 50µl (100 µg) of sodium metabisulfite was then added. The reaction solution was stirred vigorously for 20 sec. The reaction mixture was then applied to a 10 ml plastic pipet packed with Sephadex G-100 with a glass wool plug at the outlet. This column had been previously equilibrated with the diluent buffer. After the reaction mixture had soaked into the column, the reaction vessel was washed with 100 µl (1 mg) potassium iodide, and the washing was applied to the column, which was then eluted with the diluent buffer.

Fractions, which were 1.0 ml in volume, were collected into 1.0 ml of 5% BSA solution in diluent buffer, using a Gilson fraction collector which was preset at 44 drops per tube - the equivalent of 1.0 ml liquid. The radioactivity of 10 µl aliquots of the eluate was determined in a Nuclear Chicago Gamma counter calibrated for ^{125}I with a Counting Effiency of 50%. All of the radioactive measurements described in this thesis were performed in this Gamma Counter. Radioactivity in the void volume peak, as determined by prior calibration with Blue Dextran 2000 (Pharmacia), represented the labelled glycoprotein, while the radioactivity eluted in the column volume peak represented free ^{125}I.

B) Techniques of anti-CEA Antibody Demonstration

(i) The Detection of anti-CEA Antibody by the Farr Technique

To measure the binding of an anti-CEA antiserum to ^{125}I-CEA, a radioimmunoassay based upon the phenomenon of the co-precipitation of soluble immune complexes in 50% saturated ammonium sulphate (SAS) was employed, as first described by Farr (58). For this procedure, normal human serum was diluted 1:100 with 0.05 M borate buffer pH 8.6 and was subsequently used as the diluent for the anti-CEA antiserum and the ^{125}I-CEA.

A titration curve was obtained as follows: doubling dilutions of the anti-CEA antiserum in 500 µl of the diluent, starting at 1/400, was added in duplicate to polypropylene test tubes (Falcon, 12 x 75 mm). 500 µl of ^{125}I-CEA, previously diluted to yield 20,000 cpm/500 µl, was added to each tube. The tubes were vortexed, followed by incubation at 37°C for two hours. The tubes were then transferred to an ice bath where they were allowed to equilibrate for ten minutes after which 1.0 ml of cold SAS was added. Each tube was then vigorously mixed and left in the ice bath for 25 minutes, after which the tubes were centrifuged 30 minutes at 9000g at 4°C in a Sorval RC2-B. The supernatant was carefully decanted into a plastic screw-top tube (Fisher Scientific Mtl.) and the ^{125}I-CEA content was determined in the gamma spectrometer. The radioactivity of the pellet was also noted as a check to see how much, if any, ^{125}I-CEA was lost due to adhesion to the sides of the test tubes.

(ii) The Detection of anti-CEA Antibody by the Double Antibody Assay

The Farr technique was the first radioimmunoassay for CEA to be

introduced. However, since the introduction of the double antibody assay (145, 183), this type of assay was adapted for CEA in the hope of acquiring a more sensitive method of antibody detection.

In this procedure, normal horse serum diluted 1:100 with the borate buffer was employed as the diluent for the anti-CEA antiserum and the ^{125}I-CEA.

The procedure for the titration curves was as follows: doubling dilutions of the anti-CEA antiserum in 500 µl of diluent, starting at 1/2000, was added in duplicate to polypropylene tubes (Falcon 12 x 75 mm). 100 µl of the ^{125}I-CEA, previously diluted to yield 20,000 cpm/100 µl, was added to each tube and vortexed. The tubes were then incubated for two hours in a 37°C water bath, after which 150 µl of the second antibody (sheep anti-horse gammaglobulin) was added. The tubes were then incubated in the water bath an additional hour and then transferred to a 4°C cold room overnight. The next morning, the tubes were spun at 9000g in a 4°C Sorval RC2-B, the supernatant decanted and the radioactivity of both it and the pellet were determined in the gamma spectrometer.

(iii) Standard Inhibition Curves

For the preparation of a standard inhibition curve, the antiserum was diluted in 1% normal human serum (Farr assay) or 1% normal horse serum (double antibody assay) and the CEA standards were diluted in the borate buffer. A suitable dilution of antiserum was chosen from the titration curve (see above), using the 50% binding point when the Farr technique was employed and the 35% binding point for the double antibody assay. To 500 µl

of the diluted antiserum were added, in duplicate, 50 µl of the CEA standards, which ranged from 0.78 ng/50 µl to 50 ng/50 µl. The tubes were incubated in a 37°C water bath for two hours followed by the addition of 500 µl of ^{125}I-CEA for the Farr assay or 100 µl for the double antibody assay. The remainder of the procedure was similar to that previously described for the preparation of the titration curves.

(iv) Sample Inhibition Curves

The sample inhibition curves were obtained in the same manner as the standard inhibition curves with the exception that appropriate dilutions of the samples to be tested were employed instead of the CEA standards.

5) Immunodiffusion Techniques

A) Slab Gel Electrophoresis

The procedure has been previously described (124). Briefly, the gel had a 3% acrylamide stacking gel containing 0.1% sodium dodecyl sulphate (SDS) with a pH of 6.8. The running gel, containing 0.1% SDS, employed a gradient from 5% to 20% acrylamide at a pH of 8.8. The sample to be applied was dissolved in 4% SDS. Bromophenol blue was used as the indicator of mobility. The electrophoresis was carried out initially at 5 milliamps and increased to 15 ma once the sample had passed from the stacking gel into the running gel. Phosphorylase b (94,000 MW), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and α-lactalbumin (14,000) were used as molecular weight markers.

B) Ouchterlony Technique

Double diffusion in agar was performed as previously described (207).

6) Affinity Chromatographic Materials for Stability Studies

A) Preparation of CEA-Coupled Amino-Hexyl Sepharose 4B Affinity Column

(i) Preparation of the Agarose Beads

Amino-hexyl (AH) Sepharose 4B (Pharmacia Fine Chemicals) is an agarose matrix with a six-carbon spacer arm which yields 4 ml wet gel from 1 g of powder.

The gel was swollen in an excess of 0.5 M NaCl for two hours at 37°C. The swollen gel was washed in a 0.45μ Nalgene grid filter with 200 ml of 0.5 M NaCl to remove the added lactose and dextran. The gel was then washed with distilled water to remove the salt, followed by a final wash with 0.1 M sodium carbonate-bicarbonate pH 8.5 (equilibrating buffer). Three ml of wet gel was then transferred to a 20 ml reaction vessel.

(ii) Activation of the Agarose Beads

To 3 ml of wet, packed gel was added 7 ml of the bicarbonate buffer containing 1 ml of fresh 25% glutaraldehyde (Fisher Scientific). The reaction was allowed to proceed for 10 minutes at room temperature under gentle stirring. The gel was then washed with 5 x 20 ml of the bicarbonate buffer using a 0.45μ Nalgene filter. 0.5 ml of wet gel was measured and used for the coupling procedure.

(iii) Coupling of the Ligand to the Agarose Beads

5 mg of CEA (the protein to be coupled) was dissolved in 1.5 ml of the

bicarbonate buffer and was added to the 0.5 ml of activated gel under gentle stirring. The reaction was allowed to proceed for 30 minutes at 25°C, after which the suspension was centrifuged in a table top centrifuge, the supernatant decanted and its CEA content determined by the RIA. The pellet of gel was resuspended in 1.5 ml of the bicarbonate buffer and recentrifuged. The supernatant was again decanted and assayed in the RIA. This procedure was repeated until the supernatant showed less than 0.02 absorbance units at 280 nm. The gel was then packed into a K.9/15 column (Pharmacia Fine Chemicals), and washed with 3M potassium thiocyanate (KSCN) followed by equilibration with the bicarbonate buffer. A solution of 0.2 M glycine in the buffer, titrated to pH 8.5 with 0.1 N NaOH, was passed through the column to block reacted but uncoupled sites. All the washes from the coupling procedure were appropriately dialyzed, pooled and concentrated by ultrafiltration to a suitable volume for CEA determination in the RIA.

(iv) Assessment of Stability of Ligand-AH-Sepharose Coupling

The coupled gel was incubated with 3M KSCN for one hour at 25°C. The column was then equilibrated to and incubated with 5M guanidine-HCl (Eastman, N.Y.) for one hour also at 25°C. This was followed by equilibration with the bicarbonate buffer, utilizing a conductivity meter to ensure complete removal of the high ionic strength dissociating agents. The above procedure was repeated every second day for six days. Each time, the chaotropic elutions were collected and dialyzed for 6 hr in 12,000 MW cutoff dialysis tubing (Fisher Scientific) against 0.1 M borate buffer pH 8.5 with changes every 2 hr. The dialysate was then concentrated by ultrafiltration in an Amicon

with a PM30 membrane to a suitable volume for use in the RIA to determine its CEA content.

B) Preparation of CEA-Coupled Amino Aryl Glass Bead Affinity Column

Amino aryl glass beads (Pierce Chemicals - Distributors; Corning - Manufacturers) contain aromatic amine groups attached to the support through amide linkages. A 550 Å pore size and 120/200 mesh was employed. Such beads do not require any preparation prior to activation.

(i) Activation of the Glass Beads

1 g of beads was suspended in 10 ml of 3 N HCl and gently shaken. (No magnetic stirring rods were used due to the brittleness of the glass beads.) The beads were then cooled in an ice bath. The remainder of the activation was carried out in a brown bottle in a darkened room to avoid the photoreactivity of the next step. Solid sodium nitrite (Eastman, N.Y.) was added in small aliquots to a total of 250 mg in until the mixture turned a blue-green color. After 15 min in the ice bath, with careful shaking and degassing, the beads were filtered through a 0.45μ Nalgene filter and washed with 200 ml distilled water titrated to pH 3.0 with acetic acid. 0.5 ml packed volume of the activated beads was measured and used in the coupling procedure.

(ii) Coupling of the Ligand to the Glass Beads

5 mg of CEA was dissolved in 0.5 ml of 0.05 M Tris-phosphate buffer pH 8.0 (equilibrating buffer) and added to the 0.5 ml of activated glass beads. The mixture was incubated 1 hr in an ice bath with repeated shaking, and then poured into a K.9/15 column (Pharmacia Fine Chemicals) which was continuously washed with the Tris-phosphate buffer until the effluent was less than 0.02

absorbance units at 280 nm. To block any activated but uncoupled sites, the column was then washed with a solution of 3 mg β-naphtol/ml Tris-phosphate adjusted to pH 10.4, followed by a wash with 0.3 M glycine-HCl pH 2.8 to remove any material not covalently bound. The column was then equilibrated back to pH 8.0. All the washes from the coupling procedure were appropriately dialyzed, pooled and concentrated by Amicon ultrafiltration to a suitable volume for CEA content determination in the RIA.

(iii) Assessment of Stability of Ligand-Glass Bead Coupling

The coupled gel was incubated with 0.3 M glycine-HCl pH 2.8 for one hour at 25°C followed by continuous washing with the equilibrating buffer until the eluate showed a pH of at least 7.5. The effluent from the incubation with the dissociating agent was immediately neutralized with 1N NaOH and dialyzed for 6 hr against 0.1 M borate buffer pH 8.5 with changes every 2 hr. This was followed by concentration by ultrafiltration in an Amicon with a PM30 membrane to a suitable volume for use in the RIA to determine the CEA content. The above procedure was repeated every second day for six days.

7) Affinity Chromatographic Materials for Optimum pH Determination

A) Preparation of CEA-Coupled Longchain Alkylamine Affinity Column

Longchain alkylamine glass beads (Pierce Chemicals - Distributors; Corning - Manufacturers) contain covalently bonded extension arms, six carbons (20 Å) long, with primary amine groups at the terminal ends. A pore size of 550 Å and 120/200 mesh was employed. These beads do not require any preparation prior to activation.

(i) Activation of the Glass Beads

To 1 gm of beads was added 10 ml of 0.1 M sodium carbonate-bicarbonate buffer pH 8.5 containing 1 ml of fresh 25% glutaraldehyde. The mixture was shaken often and the reaction allowed to proceed for 30 min at 25°C. The beads were then applied to a 0.45μ Nalgene grid filter and washed with 5 x 20 ml of the bicarbonate buffer (equilibrating buffer).

(ii) Coupling of the Ligand to the Glass Beads

5 mg of purified CEA was dissolved in the bicarbonate buffer and added to 1 ml of the activated glass beads with gentle shaking The reaction was allowed to proceed for 30 min at room temperature with constant shaking, after which the coupled beads were then packed into a K.9/15 column (Pharmacia). The beads were continuously washed with the bicarbonate buffer until the effluent was less than 0.02 absorbance units at 280 nm. Activated but uncoupled sites were blocked by the application of a solution of 1 M 3-ethanolamine pH 9.0, followed by treatment with 0.3 M glycine-HCl pH 2.8 to remove any noncovalently bound protein. Equilibration to pH 8.5 was achieved with the bicarbonate buffer. All washes from the coupling procedure were dialyzed against borate buffer, pooled and concentrated by Amicon ultrafiltration with a PM30 membrane to a suitable volume for CEA content determination in the RIA.

(iii) Determination of Optimum pH for Elution of Immunoabsorbed Material.

12 ml of an enriched fraction of gammaglobulin prepared from 1 ml of horse anti-CEA antiserum was applied to the column followed by recirculation

through the immunoabsorbent overnight at 4°C utilizing the three-channel pump. The following morning, the column was removed from the cold and allowed to equilibrate to room temperature. The column was then washed with the bicarbonate buffer to elute those antibody molecules which had not bound, or were loosely bound, to the ligand (unbound faction). To remove those antibody molecules which ostensibly had specifically bound to the coupled CEA, four separate incubations, each of 15 min duration, were performed with four different pH values of 0.3 M glycine-HCl. The first incubation corresponded to pH 3.5, while the second, third and fourth incubations were done with pH values of 3.0, 2.75 and 2.5 respectively. A final wash with the bicarbonate buffer equilibrated the immunoabsorbent to pH 8.5. The four bound fractions were immediately neutralized with 0.1 N NaOH. Following this, these four fractions and the one unbound fraction were each concentrated by Amicon ultrafiltration with a PM30 membrane to volumes ranging from 13 ml to 20 ml.

8) Affinity Chromatographic Materials for Specificity of Binding Studies

A) Preparation of anti-CEA-Coupled AH-Sepharose 4B Affinity Column

The preparation and activation of the gel was as previously described for the preparation of CEA-coupled AH-Sepharose. The coupling procedure was as follows: an enriched gammaglobulin fraction was prepared by the precipitation of anti-CEA antiserum with sodium sulfate. 100 mg of this fraction, as determined by the Folin method, was concentrated to a volume of 7 ml by Amicon ultrafiltration and added to 3 ml of activated gel under gentle stirring.

The remainder of the procedure was as previously described for the preparation of CEA-coupled AH-sepharose with the exception that the washes from the coupling procedure were appropriately neutralized, followed by dialyzation and ultrafiltration for protein content determination by the Folin method.

B) Preparation of anti-CEA-Coupled Amino Aryl Glass Bead Affinity Column

Activation of the glass beads was as previously described for the preparation of CEA-coupled amino aryl glass beads. The coupling procedure was as follows: the enriched gammaglobulin fraction of antiserum containing 100 mg of protein was equilibrated with the equilibrating buffer and concentrated to 10 ml by Amicon ultrafiltration. This was then added to 3 ml of the activated glass beads. The remainder of the procedure was as previously described for the preparation of CEA-coupled amino aryl glass beads with the exception that the washes from the coupling procedure were appropriately neutralized, followed by dialyzation and ultrafiltration for protein content determination by the Folin method.

C) Preparation of anti-CEA-Coupled Longchain Alkylamine Glass Bead Affinity Column

Activation of the glass beads was as previously deacribed for the preparation of CEA-coupled longchain alkylamine glass beads. The coupling procedure was as follows: the enriched gammaglobulin fraction of anti-CEA antibodies, containing 100 mg of protein, was equilibrated with the bicarbonate buffer and concentrated to 10 ml by amicon ultrafiltration. This was added to 3 ml of activated glass beads. The remainder of the procedure was as previously described for the preparation of CEA-coupled longchain alkyl-

amine glass beads with the exception tht the washes from the coupling procedure were appropriately neutralized, followed by dialyzation and ultrafiltration for protein content determination by the Folin method.

D) Assessment of the Specificity of Binding of the Immunoabsorbent

The following applies to all three of the solid support matrices: 200 - 300 µg of either CEA or IgM, the antigen to be applied to the anti-CEA-coupled immunoabsorbents, were dissolved in the equilibrating buffer for each of the columns. Following application onto the column, the protein solution was allowed to recirculate through the column overnight at 4^0C using a peristaltic three-channel pump (Pharmacia). The following morning, the column was removed from the cold and allowed to equilibrate to room temperature. The column was then washed with its equilibrating buffer to elute those molecules that did not react or had weakly reacted with the immunoabsorbent. The protein content of the effluent (unbound fraction) was determined by the RIA in the case of CEA and by the Folin method in the case of the IgM. Subsequently, either 3M KSCN (for the agarose beads) or 0.3 M glycine-HCl pH 2.8 (for the glass beads) was applied to the column to release those molecules bound to the anti-CEA-coupled antibodies. This bound fraction was immediately neutralized and/or dialyzed in preparation for appropriate antigen determination described above. The column was then reequilibrated with buffer and returned to the cold until reutilization.

Results

1) Electrophoretic Pattern of CEA on Slab Gel

The demonstration of CEA on a 0.1% SDS slab gel is shown in Figure 2. The positions of the molecular weight markers are seen on the righthand side of the gel. They appear, from top to bottom, in decreasing order of weight. The markers used were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000) and α-lactalbumin (14,000). CEA, with a molecular weight of between 180,000 and 200,000 bands on the left, at the top of the gel.

2) The Radioimmunoassay for CEA

A) Conjugation of ^{125}I to CEA

Figure 3 shows the elution profile obtained following G-100 filtration to separate free ^{125}I from conjugated ^{125}I-CEA. Radioactivity in the void volume (peak 1), which was determined by prior calibration with Blue Dextran (Pharmacia Fine Chemicals), represented the labelled glycoprotein, while the radioactivity in Peak 11, the column volume, was due to free ^{125}I. Calculations involving the labelled glycoprotein were based on the assumption that 100% of the CEA was recovered in the void volume peak.

B) Double Diffusion in Agar of Sheep anti-Horse Gammaglobulin with Horse anti-CEA Antisera and CEA

Figure 4 shows the double diffusion reaction in agar of sheep anti-horse gammaglobulin (which was used as the second antibody in the double antibody radioimmunoassay) with CEA and both absorbed and unabsorbed horse anti-CEA

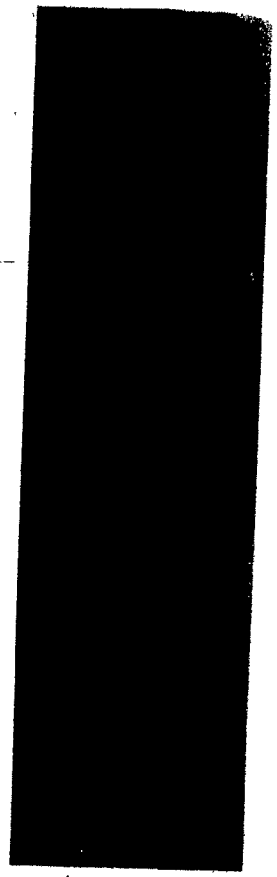


Fig. 2: Electrophoretic pattern of CEA on slab gel. Molecular weight markers, on the right, from top to bottom, are: phosphorylase b (94,000); albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,000); α-lactalbumin (14,000). CEA appears on the left at the top of the gel.

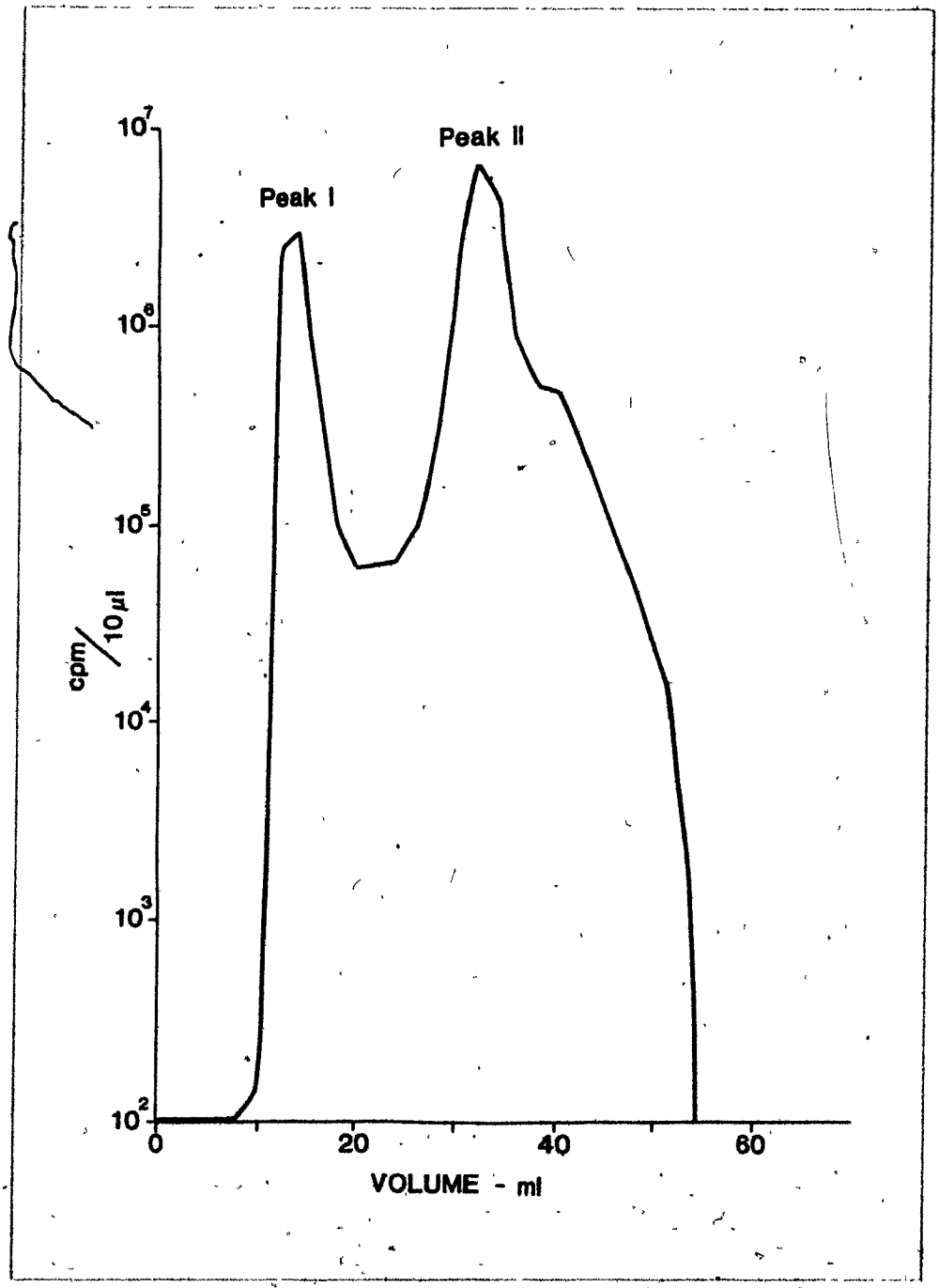
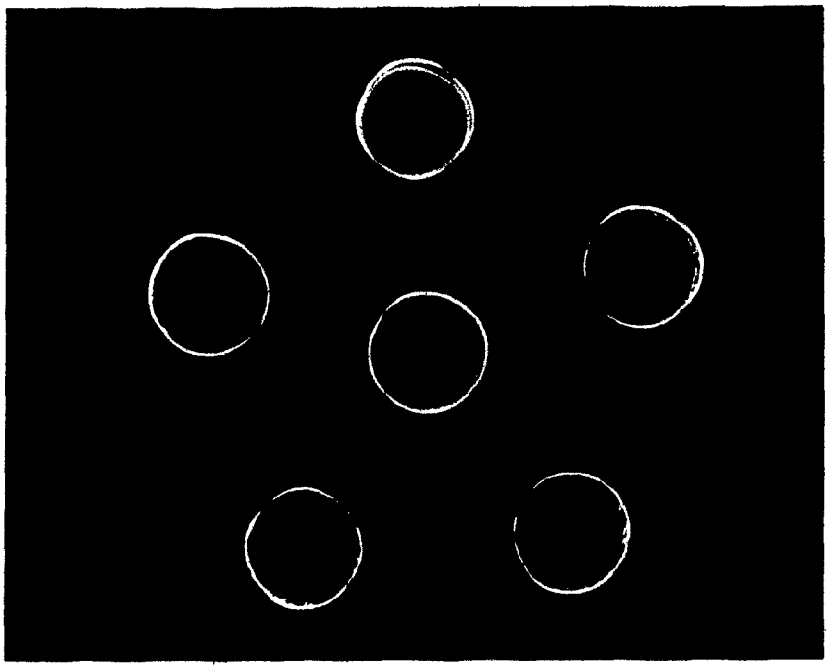


Fig. 3: Elution profile on Sephadex G-100 of an ^{125}I-labelled CEA preparation. Peak I corresponds to the radiolabelled CEA molecule. Peak II represents free unreacted ^{125}I.



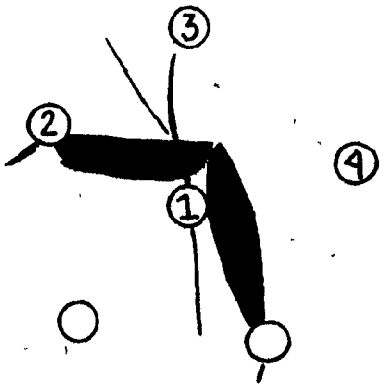


Fig. 4: Double diffusion in agar of sheep anti-horse gammaglobulin with both absorbed and unabsorbed horse anti-CEA antisera and purified CEA. Well #1 contains the sheep anti-horse gammaglobulin; well #2 contains purified CEA (1 mg/ml); well #3 contains absorbed horse serum; well #4 contains unabsorbed horse serum.

antisera. Referring to the diagram below, well #1 contained the sheep anti-horse gammaglobulin, well #2 contained purified CEA (1 mg/ml), well #3 contained absorbed horse serum and well #4 contained the unabsorbed horse antiserum. Lines of identity between the second antibody and both the anti-CEA antisera were observed. No precipitation line was seen between the second antibody and CEA.

C) Titration Curves

Both the Farr technique and the double antibody method were used to separate free from antibody-bound ^{125}I-CEA. The titration curves of both the absorbed and unabsorbed horse anti-CEA antisera, obtained with these two methods, are shown in Figure 5; those obtained with the double antibody method show typical precipitin curves, where the antigen-antibody complexes are represented by the horse antibody and the sheep anti-horse antibody respectively. At high concentrations of horse antibody, the system is in antigen (horse antibody) excess and the lattice structure is looser, causing less precipitation, thus giving lower binding values. The curves obtained with the Farr technique are typical of precipitation curves, whereby an increase in the amount of complexed ^{125}I-CEA-anti-CEA results in a concomitant increase in the amount of precipitation by the saturated ammonium sulfate.

A comparison of the four titration curves indicates that maximum binding ranged from 89% to 93%. However, with respect to the nonspecific binding, the use of the double antibody method resulted in a lower background radioactive count (10% compared with 25% for the Farr technique). When the two systems are compared with respect to their sensitivity, the Farr technique required more antibody than was needed for the double antibody

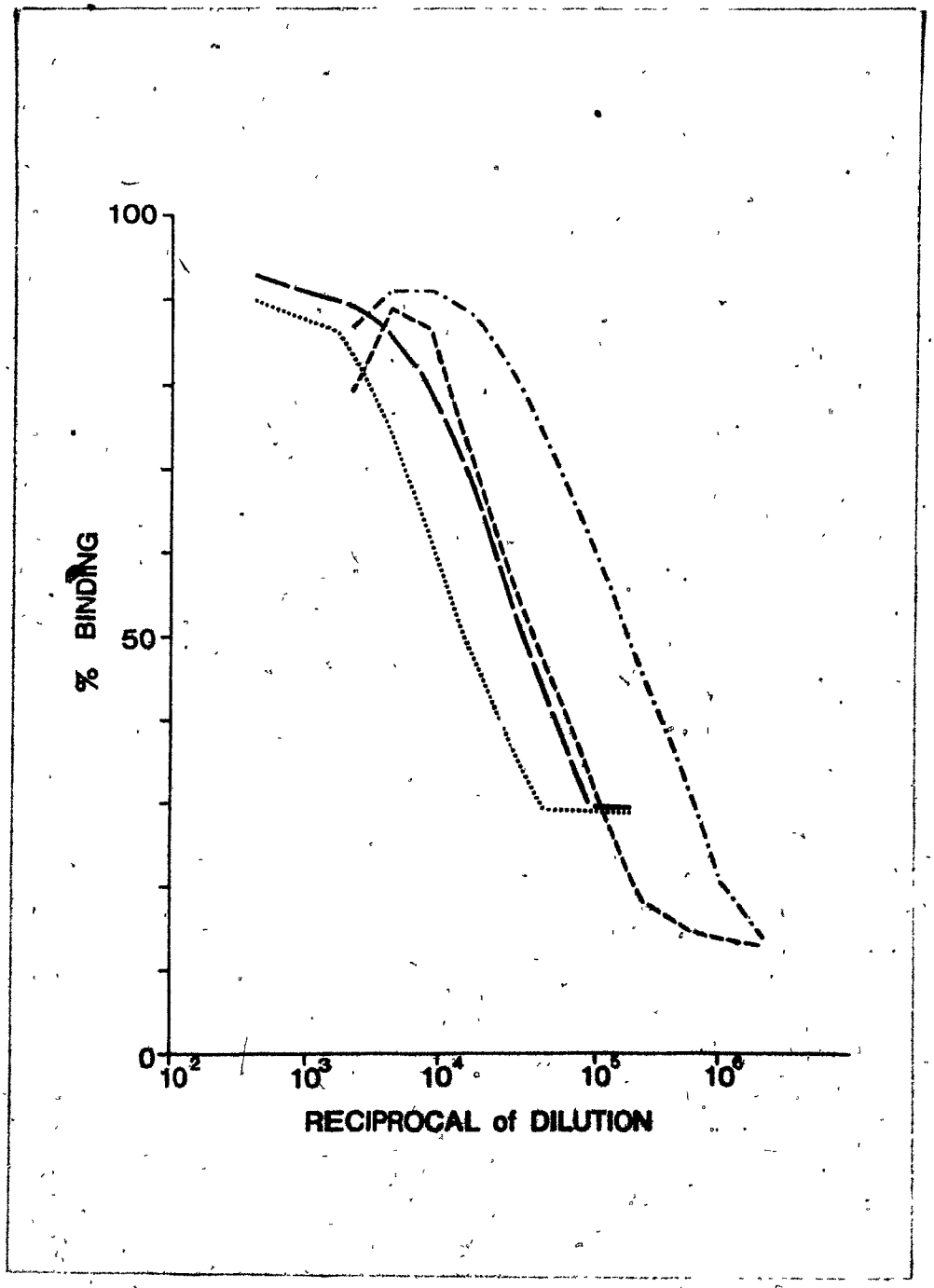


Fig. 5: Titration curves established when ^{125}I-CEA was reacted with unabsorbed and absorbed horse anti-CEA antisera using both the Farr technique and the double antibody method. Unabsorbed (— — —) and absorbed (· · · · · · · · · · · · · · ·) with the Farr technique; unabsorbed (— · — · — · —) and absorbed (— · · — · · —) with the double antibody method.

method to achieve the same degree of binding, and hence would result in a more sensitive inhibition system.

D) Standard Inhibition Curves

To establish an inhibition assay, the dilution corresponding to 50% binding for the Farr technique and 35% binding for the double antibody method was determined from their respective titration curves and used to react with the various standard amounts of CEA, ranging from 50 ng to 0.20 ng. This dilution, when the Farr technique was used, was 1/40,000 for the unabsorbed horse antiserum and 1/13,000 for the absorbed antiserum. In the case of the double antibody assay, the dilutions used for the unabsorbed and absorbed antiserum were 1/250,000 and 1/64,000 respectively.

The typical inhibition curves thus obtained are shown in Figure 6. The useful working range of the assay with the Farr technique was from 1.56 ng to 50.0 ng. In contrast, the assay employing the double antibody method was more sensitive and CEA inhibition levels of 0.40 ng to 25.0 ng could be reliably measured. This sensitivity is shown by the I^{50}, which denotes the amount of material needed to inhibit to 50% the antiserum being tested. The I^{50} for the unabsorbed horse antiserum with the Farr technique was 7.8 ng, compared with 2.0 ng when the double antibody method was used, an approximate four fold greater sensitivity with the use of the second antibody method. The sensitivity of inhibition with absorbed antiserum, similarly, showed a six-fold increase with a second antibody when compared with the use of SAS, the I^{50} values being 1.0 ng and 6.0 ng respectively.

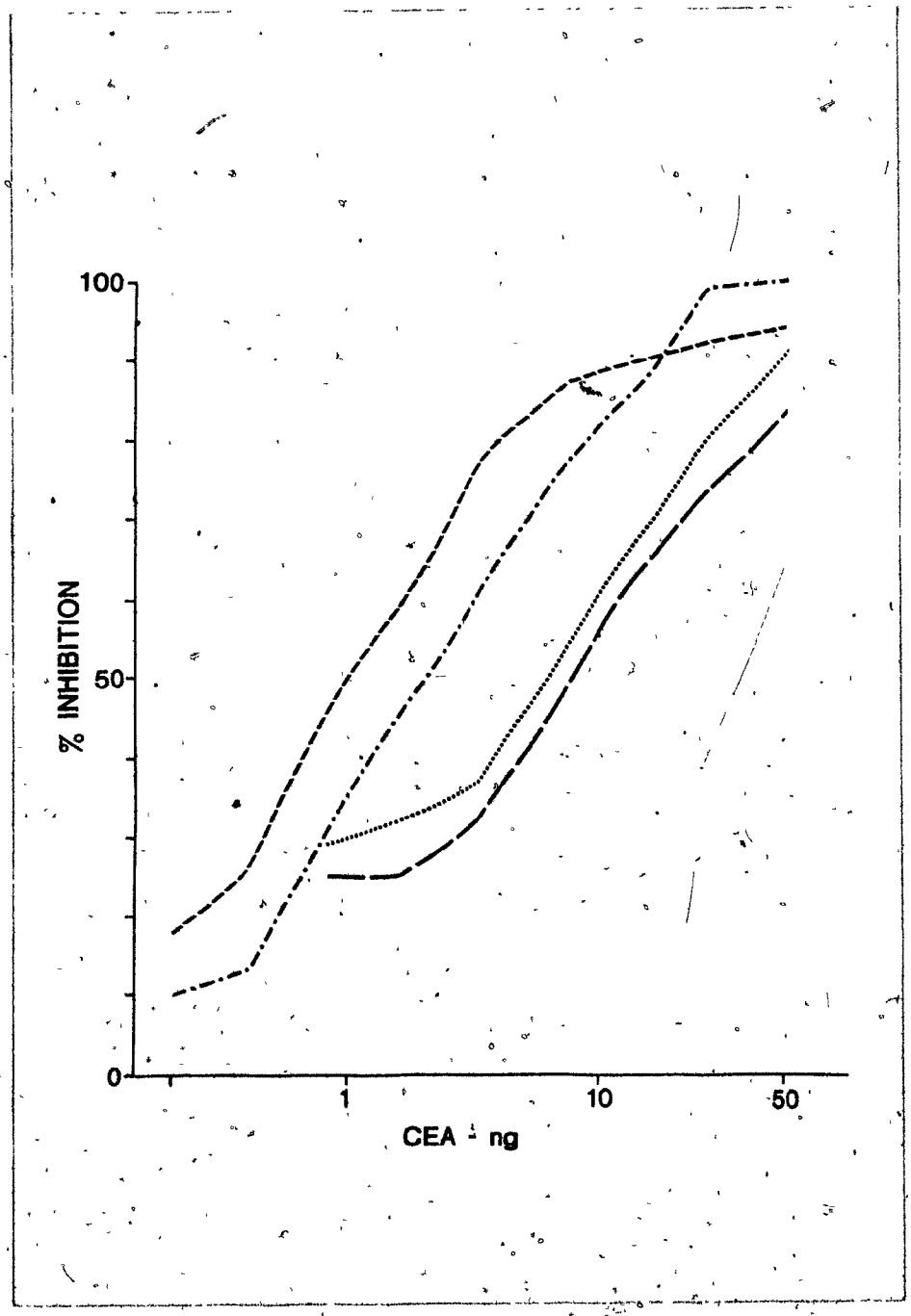


Fig. 6: Inhibition curves established between CEA, ^{125}I-CEA and both the absorbed and unabsorbed horse anti-CEA antisera using the Farr technique and the double antibody method. Unabsorbed (— — —) and absorbed (.....) with the Farr technique; unabsorbed (— · — · —) and absorbed (— — —) with the double antibody method.

3) Studies with CEA-Coupled Affinity Columns

A) Efficiency of Coupling Ligand to the Matrix

5000 g of CEA was reacted with both activated glass beads and sepharose. Table 3 shows that with respect to AH-sepharose, as well as the two types of glass beads, coupling was essentially quantitative in that almost all the CEA (90% to 96%) reacted with the carrier.

B) Stability of Ligand-Coupled Affinity Columns

The stability of a ligand (CEA) chemically coupled to a carrier arm by the use of glutaraldehyde, as demonstrated by AH-sepharose, or by diazotization, in the case of amino aryl glass beads, was assessed by measuring the ability of strong dissociating agents, 3M KSCN and 0.3 M glycine-HCl pH 2.8, respectively, to destroy this linkage. Table 4 gives the CEA content, as determined by the inhibition RIA, of the eluates obtained with each dissociation treatment (bleeding).

Essentially, both types of linkages were quite stable to the harsh treatments, resulting in less than 1% loss of coupled CEA with each bleed. The three bleeds of the AH-sepharose column yielded losses of 0.77%, 0.98% and 0.96% of CEA. The exposure of the amino aryl column to its dissociating agent resulted in CEA losses of 0.53%, 0.44% and 0.24% for its three bleedings.

C) Determination of the Optimum pH Strength for the 0.3M Glycine-HCl Elution of Immunoabsorbed Material

To determine the optimum pH value of 0.3M glycine-HCl to use for the elution of the bound antibody fractions from the immunoabsorbent, four

TABLE 3

Efficiency of Coupling CEA to Carrier

	CARRIERS		
	AH-Sepharose	Amino Aryl Glass Beads	Longchain Alkylamine Glass Beads
Amount of CEA used for coupling (µg)	5000	5000	5000
Amount of CEA coupled to the carrier (µg)	4511	4800	4650
Amount of CEA not coupled (µg)	489	200	350
Coupling Efficiency	90.2%	96.0%	93.0%

TABLE 4

Stability of Coupling Ligand to Carrier

Type of carrier	# chaotropic treatments	Am't of CEA coupled (µg)	Am't of CEA dissociated (µg)	% Dissociation
AH Sepharose	1	5000	34.76	0.69
	2	4965.24	43.66	0.88
	3	4921.58	42.44	0.86
Aryl Amine Glass Beads	1	5000	25.44	0.51
	2	4974.56	21.43	0.43
	3	4953.13	11.35	0.23

different pH strengths, over a range of pH 2.5 to pH 3.5 were successively utilized. The CEA-reactive antibodies in each of the four fractions were calculated from the titration curves each fraction elicited. The titres at 50% binding are given in Table 5, including those of the unbound fraction and that of the IgG fraction of the antiserum which was originally used for the absorption.

The calculation of the CEA-reactive antibodies in each fraction assumed 100% recovery of the labelled glycoprotein in the radioiodination procedure (see Figure 3). It was thus determined that 500 µl (20,000 cpm) contained 0.34 ng of radioactive CEA (*CEA), which was then reacted with, in the RIA, an equal volume of the various diluted antiserum fractions. Assuming, for simplicity's sake, a one-to-one antigen-antibody reaction, and taking into account the various sample volumes, the multiplication of the above variables (*CEA, antiserum dilution and sample volume) yields a measure of the antibodies in each fraction which reacted with CEA in the RIA.

The total amount applied to the immunoabsorbent was calculated to be 60 µg of CEA-reactive antibodies. The amount of these antibodies which constituted the effluent was determined to be 22.6% of the total amount applied, thus giving, as 77.3%, those CEA-reactive antibody moleculea remaining on the column. Of this 77.3%, the amount eluted with the various pH strengths of the glycine-HCl was 60.7% of the total applied, indicating a loss of about 17.0% on the column.

The effectiveness of each elution with the different pH values was represented by the per cent of the total antibody molecules applied which was

TABLE 5

Elution of Immunoabsorbed Material

with Various pH Strengths of Glycine-HCl

Sample	Dilution at 50% Binding	Sample Volume (ml)	CEA-Reactive Antibody (µg)	% Recovery
Globulin Fraction	1/14,720	12	60.0	-
Unbound Fraction	1/2000	20	13.6	22.6
Bound Fraction				60.7
pH 3.5	1/640	14.5	3.2	5.3
3.0	1/3360	18.0	20.6	34.3
2.75	1/2720	13.0	12.0	20.0
2.5	1/100	20.0	0.34	1.1

released by ech treatment with the chaotropic agent. Referring to Table 5, it is observed that pH 3.0 and pH 2.75 eluted the majority of the CEA-reactive antibodies immunologically bound to the ligand, giving values of 34.3% and 20.0% respectively. The pH values of 3.5 and 2.5 succeeded in eluting only 5.3% and 1.1% respectively.

4) Studies With anti-CEA-Coupled Affinity Columns

A) Binding Activity With CEA

Absorbent binding activity of the affinity columns was measured by the determination of the amount of CEA which was reacted with an enriched fraction of anti-CEA antibodies coupled to three types of solid support systems. See Table 6.

Between 78 mg - 80 mg of the anti-CEA antibodies were chemically coupled to the various carriers. To test for biological activity of the coupled ligand, the binding of CEA to the solid phase bound antibodies was determined. With respect to the alkylamine glass bead column, 50 µg of the 300 µg originally applied bound to the antibodies. This compares with 48 µg on the amino aryl column and 10 µg on the AH-sepharose column.

B) Binding Activity With IgM

As another measure of specificity of reaction on the columns, and non-specific absorption onto the matrix, an unrelated protein (IgM) was applied to the columns. The data in Table 7 shows that non-specific absorption was between 10 µg and 15 µg for the glass beads and 60 µg for the agarose beads.

TABLE 6

Efficiency of Affinity Chromatography of an IgG Preparation of anti-CEA Coupled to Different Carriers

Anti-CEA coupled to:	Am't CEA Applied (µg)	Am't CEA Unbound (µg)	Am't CEA Absorbed (µg)	% Absorption
Aryl Amine Glass Beads	300	252	48	16
Longchain Alkylamine Glass Beads	300	250	50	16
AH-Sepharose	300	290	10	3.3

TABLE 7

Non-specific Binding of an IgG Preparation of anti-CEA Coupled to Different Carriers

Anti-CEA Coupled to:	amount of IgM Applied (µg)	Amount of IgM Unbound (µg)	Amount of IgM Absorbed (µg)	% IgM Absorption
Amino Aryl Glass Beads	200	190	10	5.0
Longchain Alkylamine Glass Beads	200	180	15	7.5
AH-Sepharose	200	140	60	30.0

Discussion

In designing an immunoabsorbent system, careful consideration of the matrix or solid support to be used, as well as the conditions of absorption and desorption, is as important as the choice of the antigen-antibody system.

Three types of matrices, one of agarose and two of glass beads, were tested for their ability to form efficient immunoabsorbents. Two different types of ligand-carrier linkages were also investigated for their efficiency and stability of coupling - one resulting in the formation of a Schiff base using glutaraldehyde and the other involving diazotization.

From Table 3, it can be seen that both types of linkages resulted in greater than 90% efficiency of coupling, while the stability of the linkage between the ligand and the carrier (Table 4) was quite resistant to harsh dissociation treatment, allowing less than 1% leakage of CEA with each bleed. The stability of the diazotized CEA was significantly greater than the glutaraldehyde-coupled CEA; however, this is most probably due to the milder effect of the glycine-HCl over the 3M KSCN. Nonetheless, both coupling systems are quite efficient.

In order to determine which matrix would be better suited to form an immunoabsorbent, two parameters were examined: the efficiency of the ligand-coupled matrix to absorb a specific protein and the extent to which the matrix, itself, would absorb proteins, as measured by choosing one which would not react immunologically with the ligand, in this case IgM. The type of affinity column used for these studies was one where the ligand was an

enriched gammaglobulin fraction of the anti-CEA unabsorbed antiserum. The efficiency of this column was tested by measuring, in the RIA inhibition assay, the amount of CEA which would absorb to the coupled antibodies. Table 6 shows that both glass bead columns had reacted with 48 µg - 50 µg of CEA applied compared with only 10 µg for the sepharose column.

The amount of nonspecific absorption by the matrix itself was measured by applying IgM to each column. As shown in Table 7, the amount of IgM each matrix retained indicated that the glass beads, which absorbed 10 µg and 15 µg for the amino aryl and longchain alkylamine beads respectively, are chemically more inert than the sepharose, which absorbed 60 µg.

The method of dissociation of those molecules immunologically bound to the ligand was chosen to be 0.3 M glycine-HCl. This chaotropic agent is less harsh than 3M KSCN with the same eluting ability, as seen above. To determine which pH strength was to be used for the elution procedure, anti-CEA antibodies were successively dissociated from the coupled ligand (CEA) with four different pH values of the 0.3 M glycine-HCl, starting with pH 3.5, followed by pH 3.0, then pH 2.75 and finally, pH 2.5.

Following the calculation of the CEA-reactive antibodies in each fraction, it was seen (Table 5) that the majority of these antibodies were eluted with pH 2.75 and pH 3.0, while incubation with pH 3.5 and pH 2.5 resulted in only minor dissociation. Hence, one elution with pH 2.8 was determined to be sufficient.

This study also gave a measure of the nonspecific absorption onto the matrix. Using the CEA-reactive antibody determination as being representa-

tive of the total amount of protein in the gammaglobulin fraction originally applied, the amount of unrecovered antiserum was 17% (Table 5). This loss could be due to nonspecific absorption onto the matrix as well as a measure of antibody molecules with an extremely high affinity for the coupled CEA, which were unable to be eluted under the conditions employed. Separation of such high affinity complexes would probably only occur with strong reagents that may possibly destroy the individual components. Indeed, part of this 17.0% loss could have been recovered in the pH 2.5 elution as inactivated antibody molecules due to the acidic environment.

Thus, the glass beads were seen to be a superior matrix over the agarose with respect to its efficiency as an immunoabsorbent. Both amino aryl and longchain alkylamine beads, utilizing diazotization and activation by glutaraldehyde, respectively, to couple ligand, and employing 0.3 M glycine-HCl pH 2.8 to elute the immunoabsorbed fractions, were used to form the affinity columns in the subsequent studies.

CHAPTER 5

Introduction

The problems relating to the clinical use of the CEA radioimmunoassay, as described in Chapter 1, are due to the multi-epitope nature of the CEA molecule which, when injected into a xenogeneic animal, elicits a population of antibody molecules which not only react with CEA, but with a number of normal components. The unabsorbed anti-CEA antiserum will react with aqueous extracts of normal human serum, normal lung, bowel liver and stool in an inhibition assay. After a single passage through an affinity column containing various covalently coupled normal tissues, the reactivity of the antiserum to normal serum, liver and lung tissues decreases. However, normal bowel, and to a lesser extent, normal stool still significantly inhibit in the assay (page 37, Fig. 1)

In the previous chapter, two types of matrices and two different kinds of ligand-coupling systems were examined. It was seen that either method of coupling ligand to a carrier on the matrix was reliable when used with a glass bead solid support rather than an agarose one.

The aim of this study was to see if the anti-CEA antiserum could be made less reactive to normal bowel tissue, thereby improving the specificity of the RIA towards CEA and reducing the ambiguity of detection when diseases other than malignancy are involved in tissue pathology.

In this present study, normal stool and normal bowel from mucosal linings of the intestines were the two antigens used as ligands and employed to immunoabsorb anti-CEA antisera in an attempt to modify the specificity detected by the unabsorbed anti-CEA antisera.

Materials and Methods

1) Preparation of Antigens

A) Preparation of CEA

The preparation of CEA was as previously described in Chapter 4.

B) Preparation of Normal Bowel Antigen (NBA)

At all times the normal bowels used were obtained immediately after autopsy, from individuals free of any type of cancer or gastrointestinal disorders, and stored at −20°C until used for extraction.

In preparation for extraction, the tissue was dissected free of surrounding fat as cleanly as possible. The mucosal lining was then dissected away from the tissue and subsequently used for the extraction.

(i) Initial Preparation of Normal Bowel Tissue

A 500 ml volume of distilled water was added to 500 g of tissue. This was homogenized in a water-cooled Virtis mixer at 15,000 rev. for 15 min. The resulting homogenate was spun at 7000g in a Sorvall RC2-B for 20 min at 4°C. Three layers resulted - a fatty layer on the top, a middle liquid layer and then a precipitate at the bottom of the tube. The fatty layer was discarded. The supernatant was decanted, recentrifuged and redecanted. The precipitate, in each case, was also discarded. The supernatant was then filtered through a Whatman #4 filter paper. The filtrate, a volume of approximately 800 ml, was then divided into three parts. One part was kept on ice in preparation for the next step in the isolation procedure. The other two parts were lyophilyzed and stored at −20°C until further utilization.

(ii) Preparative Ion Exchange Chromatography

Both the carboxy-methyl cellulose (CM 52, Whatman) and the DEAE cellulose (DE 52, Whatman) were obtained preswollen. The two matrices were each, suspended in distilled water to yield a volume of 100 ml, followed by exhaustive washing with distilled water. Each gel was then separately packed with distilled water in a K25/60 column (Pharmacia Fine Chemicals) to a height of 20 cm for the DEAE cellulose and 18 cm for the CM cellulose, and were allowed to run, by gravity, for 36 hr with distilled water. The two columns were then set up in tandem, with the outlet of the CM cellulose column joined by plastic tubing to the inlet of the DEAE cellulose column.

The filtrate from the extraction procedure, which had been kept on ice, was then applied to the CM cellulose column using a Mariotte bottle. The effluent from the DEAE cellulose column was retained and tested for NBA cross-reactivity in the RIA for CEA. The two columns were washed with distilled water until the effluent from the DEAE cellulose column showed less than 0.02 absorbance units at 280 nm. The CM cellulose column was discarded and the material in the DEAE cellulose column was eluted, stepwise, with increasing ionic strength of a Tris-HCl buffer pH 7.8, starting at 0.05M Tris-HCl, followed by 0.1M Tris-HCl and finally with 0.2M Tris-HCl. Each tube, which contained about 6 ml, was tested for its ability to inhibit in the RIA for CEA. The active fractions of each elution were pooled, lyophilyzed and retested in the assay to obtain a specific activity of the preparation.

(iii) Preparative Ultragel Chromatography

When further purification of the NBA was required, filtration through an Ultragel (LKB Labs) column was employed. The Ultragel was washed exhaustively with distilled water and then packed in a K15/100 column (Pharmacia Fine Chemicals) by gravity. The column was equilibrated with 0.05M Tris-HCl buffer pH 7.8, which was also used as the eluting buffer.

A 50 mg sample from the 0.1M Tris-HCl eluted fraction of the DEAE cellulose isolation step was dissolved in 5 ml of the 0.05M Tris-HCl buffer and applied to the Ultragel column. The elution was carried out by upward flow at a rate of 20 ml/hr at room temperature. The eluate was monitored for its spectrophotometric activity at 280 nm and was collected in 5 ml fractions. Each fraction was assayed in the RIA for CEA, and those containing NBA inhibitory activity were pooled, lyophilyzed and stored at -20°C until further use.

C) Preparation of Stool Extract

Normal stool material was collected from patients with malabsorption syndrome. This source was employed since the stools from such patients were routinely collected in the hospital to monitor their clinical status.

To 500 gm of stools was added an equal volume of distilled water and the resulting mixture was homogenized in a water-cooled Virtis mixer at 15,000 rev., followed by centrifugation in a 4°C Sorval RC2-B at 2500g for 10 min. The supernatant was collected, lyophilyzed and stored at -20°C until further use.

2) Preparation of Antisera

A) Preparation of Horse Unabsorbed anti-CEA Antiserum

Horse unabsorbed anti-CEA antiserum was prepared as previously described in Chapter 4.

B) Preparation of Horse Absorbed anti-CEA Antiserum

Horse absorbed anti-CEA antiserum was prepared as previously described in Chapter 4.

C) Preparation of Sheep anti-Horse Gammaglobulin

Sheep anti-horse gammaglobulin for use as the second antibody in the double antibody RIA for CEA was prepared as previously described in Chapter 4.

D) Preparation of Guinea Pig anti-CEA Antiserum

A guinea pig was immunized once with 500 µg of purified CEA emulsified in Freund's complete adjuvant. The animal was given a test bleed 10 days later which gave a line in Ouchterlony against purified CEA. The animal was sacrificed and the blood, obtained by cardiac exsanguination, was divided into four 5 ml aliquots and stored at −20°C until further use. This antiserum was thus termed guinea pig anti-CEA.

3) Radioimmunoassay for CEA

The radioimmunassay for CEA was performed as previously described in Chapter 4.

4) Immunodiffusion Techniques

Double diffusion in agar was performed as previously described in Chapter 4.

5) Preparation of Affinity Chromatographic Materials for Absorption Studies

A) Preparation of Amino Aryl Glass Bead Immunoabsorbent Columns

The activation of the glass beads was as previously described in Chapter 4. The coupling of the ligand to the matrix was as follows: 80 mg of NBA material was dissolved in 10 ml of 0.05M Tris-phosphate buffer pH 8.0 and added to 3 ml of activated glass beads. The mixture was allowed to incubate one hour in an ice bath with repeated shaking. The remainder of the ligand-coupled amino aryl glass beads.

B) Preparation of Longchain Alkylamine Glass Bead Immunoabsorbent Columns

The activation of the glass beads was as previously described in Chapter 4. The coupling of the ligand to the matrix was as follows: 80 mg of either NBA or stool material was dissolved in 10 ml of 0.1 M sodium carbonate-bicarbonate buffer pH 8.5 and added to 3 ml of activated glass beads. The mixture was allowed to incubate one hour in an ice bath with repeated shaking. The remainder of the procedure was as previously described in Chapter 4 for the preparation of ligand-coupled longchain alkylamine glass beads.

C) Use of the Immunoabsorbent Columns

500 µl of either the horse or guinea pig unabsorbed anti-CEA antiserum

was applied initially to the column and allowed to recirculate overnight at 4°C with the use of the peristaltic three-channel pump at medium speed. The next morning, the column was allowed to equilibrate to room temperature followed by extensive washing with the equilibrating buffer to remove those molecules not bound or weakly bound to the ligand. This was followed by incubation with 0.3 M glycine-HCl pH 2.8 for one hour to remove those antibody molecules which had ostensibly specifically reacted with the coupled normal material. The column was then equilibrated to neutral pH and returned to the cold until further utilization. The eluate from the treatment with glycine-HCl was immediately neutralized with 0.1N NaOH to a pH of between 7.0 and 8.0.

The two fractions of antibody populations, that eluted with the equilibrating buffers (unbound fraction) and that eluted with the glycine-HCl (bound fraction) were concentrated by Amicon ultrafiltration to a volume of 2.0 ml. 0.1 ml of each fraction was used to make an initial dilution of 1/10, subsequently used in the establishment of the RIA's.

With respect to the unbound fraction, the remaining 1.9 ml was reapplied, 48 hours later, to either the same immunoabsorbent or to a different one and allowed to recirculate overnight at 4°C. The recycling of this unbound fraction, and subsequent recyclings, were performed as described above for the initial passage of the unabsorbed antiserum.

Results

1) NBA Isolation

Figure 7 shows the DEAE cellulose profile of an aqueous extract of normal bowel material. NBA material, reactive in the CEA RIA, was represented by three major peaks, 1, 11, and 111, which corresponded to the three distinct molarities of Tris-HCl pH 7.8 used for the elution. Fraction 1 eluted with 0.05M Tris-HCl, Fraction 11 with 0.1M Tris-HCl and Fraction 111 with 0.2M Tris-HCl. These three fractions were each dialyzed against distilled water followed by lyophilyzation. The amount of recovered material and relative inhibitory activity in the CEA assay of each fraction were determined and are summarized in Table 8. The amount of NBA material required to achieve 50% inhibition of the RIA for CEA (the I^{50} value) using the Farr technique, was employed as a means of comparison of the degree of immunological reactivity obtained with each purification step. The fraction which gave the greatest degree of immunologic reactivity was Fraction 11, having an I^{50} of 10,000 ng as compared with Fraction 1 and Fraction 11 whose I^{50} values were 37,500 ng and 20,000 ng respectively.

The second step in the isolation of NBA employed, as starting material, 50 mg of Fraction 11 from the DEAE cellulose column, which was then applied to an Ultragel column. Figure 8 shows the Ultragel elution profile. Following chromatography, two peaks were observed, a major one, Peak 1, at 200 ml of elution and a minor one, Peak 11, at 290 ml of elution. Each peak was dialyzed against distilled water and lyophilyzed. Table 9 shows the

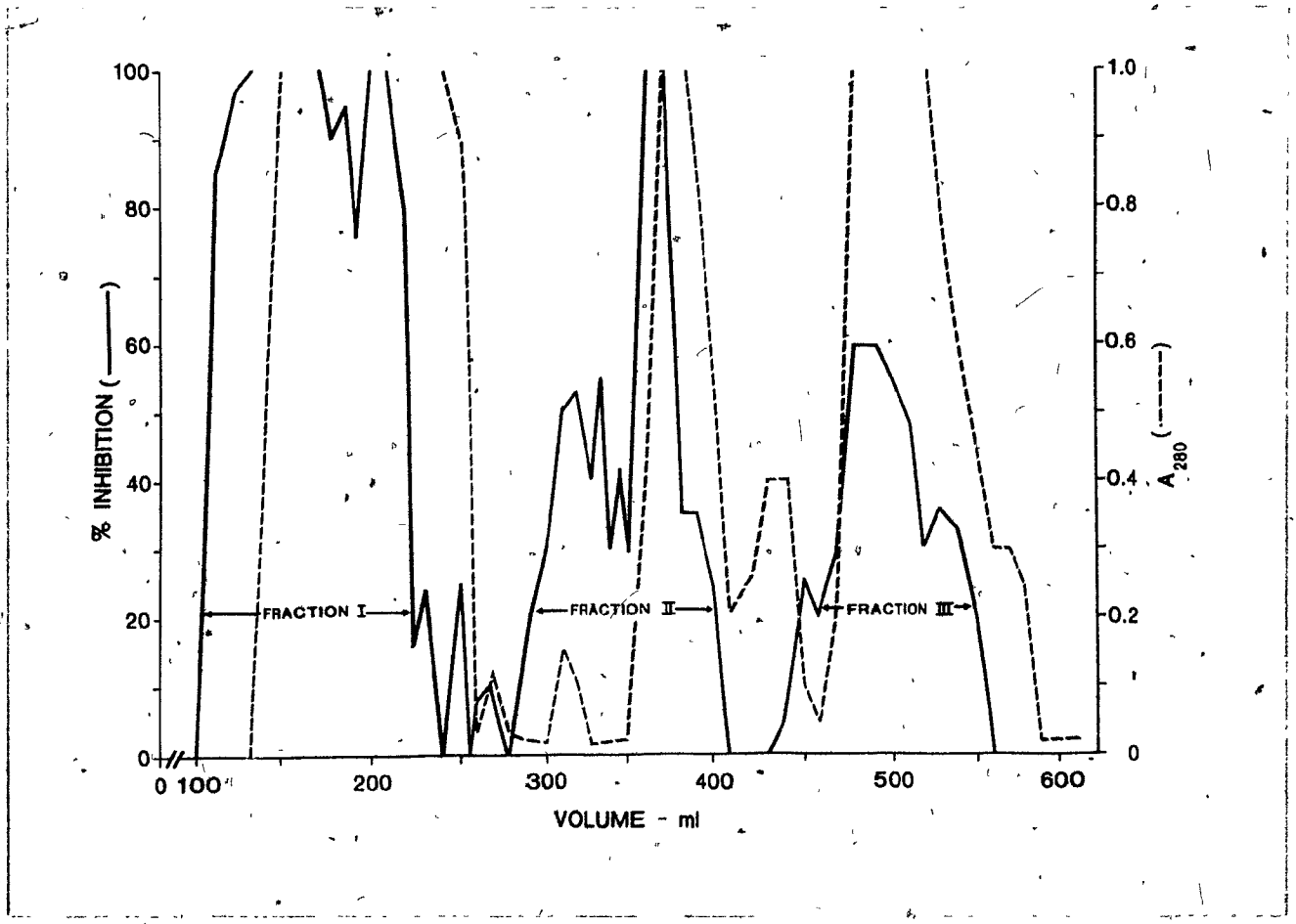


Fig. 7: Elution profile of an aqueous extract of normal bowel tissue on DEAE cellulose. A column size of 25mm x 20cm was employed with stepwise elutions using Tris-HCl buffers of pH 7.8 with molarities of: 0.05M for Fraction 1; 0.1M for Fraction 11; 0.2M for Fraction 111. The flow rates were 50-60 ml/hr. O.D. at A_{280} (– – –); inhibitory activity in the RIA (——).

TABLE 8

Preparation of NBA Material

by DEAE Cellulose Ion Exchange Chromatography

Fraction No.	Stepwise Elution with Tris-HCl at:	Recovered NBA Material (mg dry weight)	I^{50} of Recovered NBA (ng)
1	0.05M	233	37,500
2	0.1M	85	10,000
3	0.2M	125	20,000

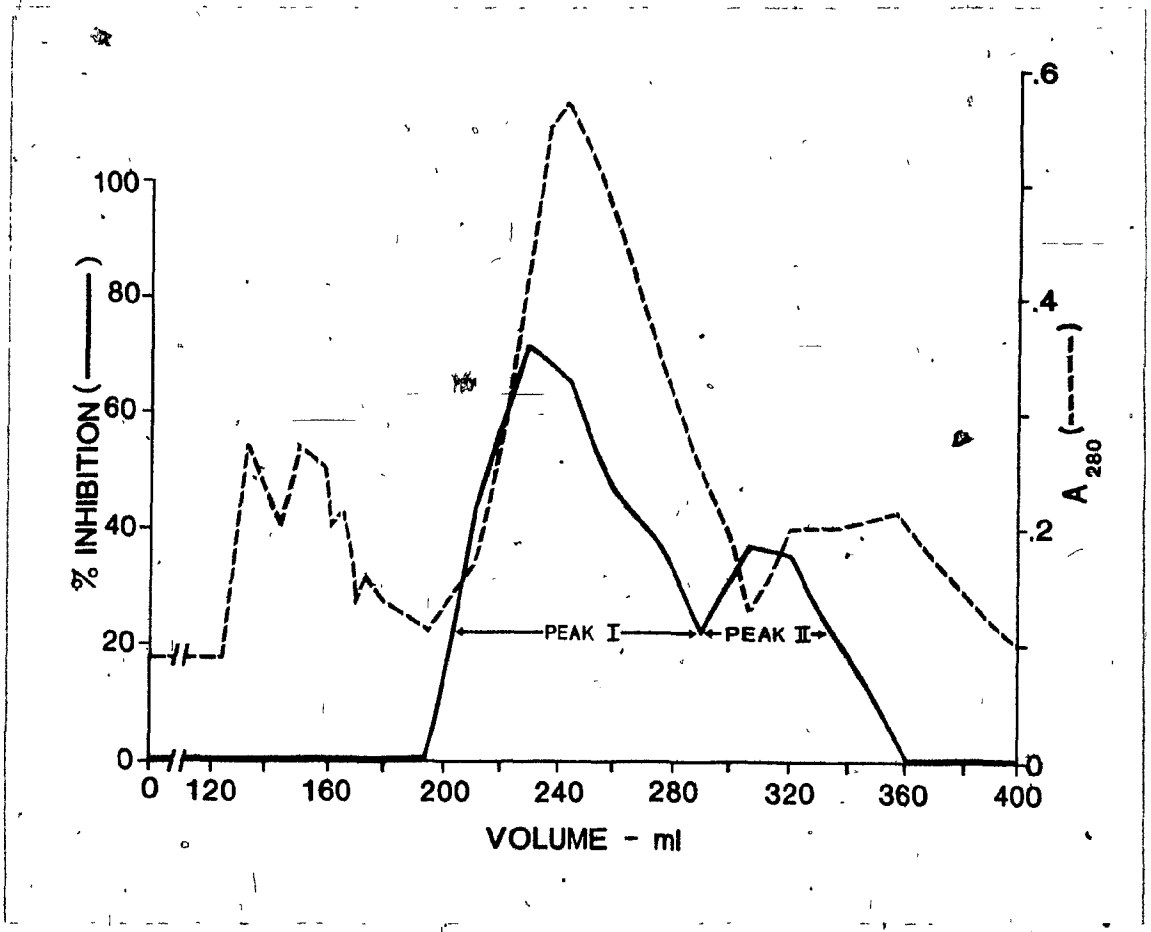


Fig. 8: Elution profile obtained by Ultragel filtration of a 50 mg sample of the material from Fraction 11 of the DEAE cellulose chromatography. A column size of 15mm x 100 cm was employed with flow rates of 40 ml/hr, using 0.1M Tris-HCl pH 7.8 as the eluting buffer. Two peaks were determined by the inhibitory activity - Peak 1 and Peak 11. O.D. at A_{280} (- - - -); inhibitory activity in the RIA (——).

TABLE 9

Preparation of NBA Material

by Ultragel Filtration

Peak No.	Recovered NBA Material (ng dry weight)	I^{50} of Recovered NBA	
		Farr Technique (ng NBA)	Double Antibody (ng NBA)
1	26	5500	2000
2	8	32,000	20,000

amount of material recovered and the quantity required for 50% inhibition in the RIA. Employing the Farr technique, the material in Peak 1 gave an I^{50} of 5500 ng compared with the material in Peak 11 which needed 20,000 ng to achieve the same degree of inhibition.

By comparing the I^{50} values of the most active peaks of both isolation procedures, it can be seen that Ultragel chromatography, with an I^{50} of 5500 ng for Peak 1, achieved only a two-fold increase in purification and specific reactivity over the DEAE cellulose isolation which gave an I^{50} value of 10,000 ng for Fraction 11.

2) Immunoreactivity of Horse anti-CEA Antisera with NBA

Figure 9 shows the inhibition curves established when both the absorbed and unabsorbed horse anti-CEA antisera were reacted with NBA material and ^{125}I-CEA in the RIA. The curves obtained by the use of the Farr technique employed, as NBA material, Fraction 11 of the DEAE cellulose isolation step; those obtained using the double antibody method used the NBA material from Peak 1 of the Ultragel filtration step.

The useful working range of the curves was between 3000 ng and 50,000 ng when the Farr technique was used, and from 1500 ng to 20,000 ng with the double antibody method. Using the I^{50} values as a means of comparison, the assay using the double antibody method was twice as sensitive than that using the Farr technique, yielding I^{50} values of 2500 ng and 5500 ng with the unabsorbed antiserum and 6000 ng and 11,000 ng with the absorbed serum for the two assays respectively.

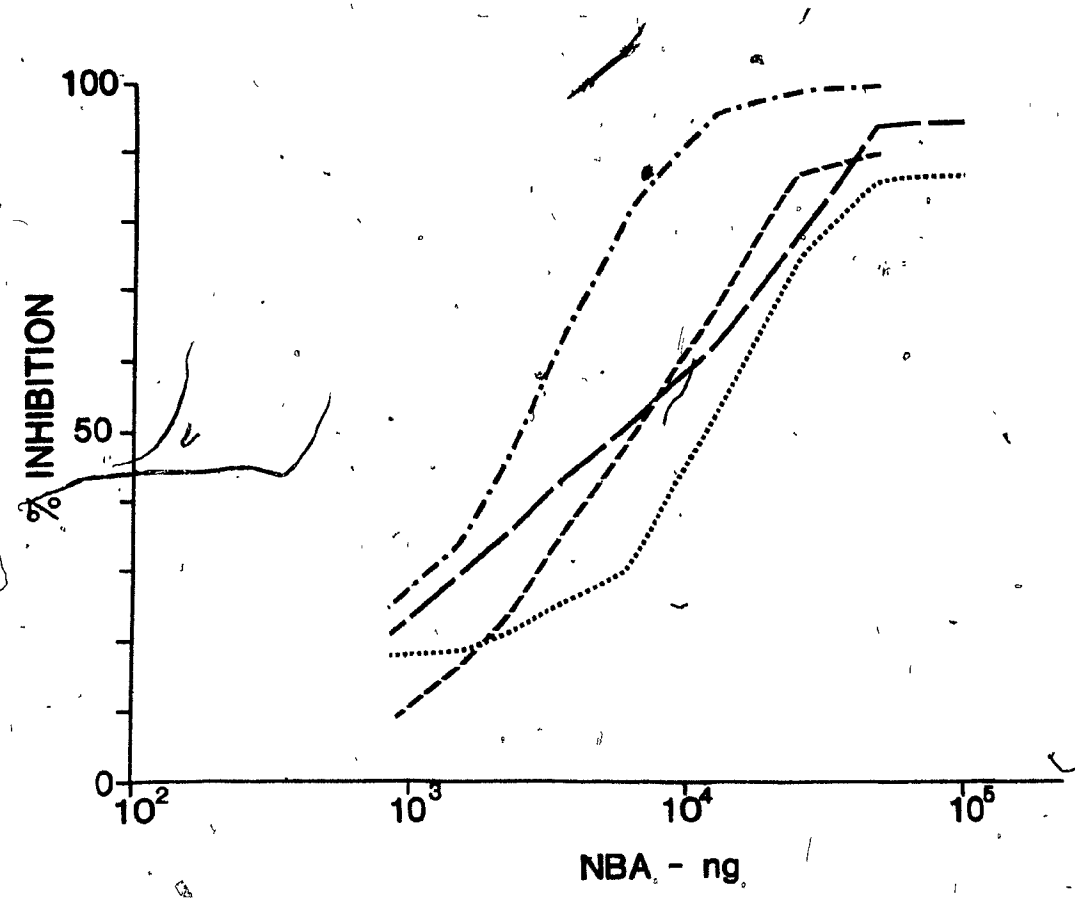


Fig. 9: Inhibition curves established between NBA (Fraction 11), ^{125}I-CEA and both the absorbed and unabsorbed horse anti-CEA antisera using the Farr technique and the double antibody method. Unabsorbed (— — —) and absorbed (·····) with the Farr technique; unabsorbed (— · — · —) and absorbed (— — — —) with the double antibody method.

3) Preparation of Covalently Coupled NBA or Stool Affinity Columns

Eight affinity columns were prepared and used as immunoabsorbents for this study. The longchain alkylamine glass beads were the matrix used for the coupling of stool material and NBA material from Fraction 11 of the DEAE cellulose isolation step. The amino aryl glass beads were the matrix used to prepare four separate immunoabsorbents coupled with NBA material from Peak 1 of the Ultragel chromatographic procedure. In each case, 80 mg of the normal materials were used for coupling. The efficiency of coupling for all eight columns ranged from 85% to 92%.

4) Double Diffusion in Agar of the anti-CEA Antisera with CEA

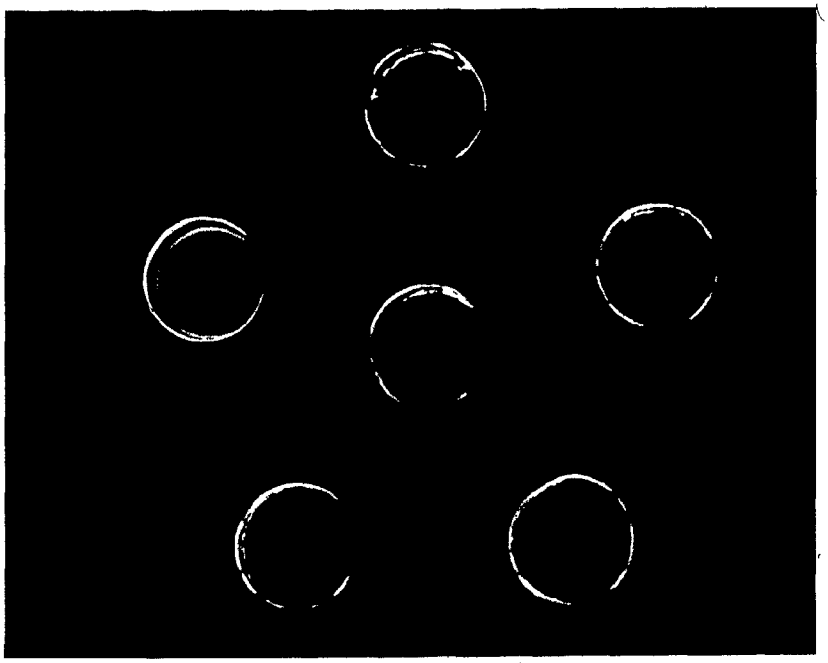
Two species of anti-CEA antisera were utilized in this study - one from a guinea pig and one from a horse.

Figure 10 shows the double diffusion reaction in agar of CEA with unabsorbed guinea pig antiserum and both absorbed and unabsorbed horse antisera. Referring to the diagram below, well #1 contained purified CEA (1mg/ml) and wells #2, #3 and #4 contained absorbed horse, unabsorbed horse and guinea pig antisera respectively. Single precipitation lines were observed between the antigen with each of the anti-CEA antisera.

5) Immunoabsorbent Chromatography of Unabsorbed anti-CEA Antisera

A) Immunoabsorption of Guinea Pig anti-CEA Antiserum

Two immunoabsorptions of guinea pig anti-CEA antiserum were performed.



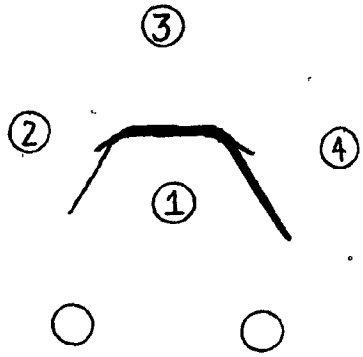


Fig. 10: Double diffusion in agar of CEA with unabsorbed and absorbed horse anti-CEA antisera and guinea pig anti-CEA antiserum. Well #1 - purified CEA (1mg/ml); well #2 - absorbed horse antiserum; well #3 - unabsorbed horse antiserum; well #4 - guinea pig antiserum.

The first one entailed the use of a stool-coupled affinity column followed by two more passages through an NBA-coupled column. A second immunoabsorption was attempted by recycling the unabsorbed antiserum three times through a single NBA-coupled column. The matrix used, in both experiments, was the longchain alkylamine glass beads. The NBA material used for coupling was from Fraction 11 of the DEAE cellulose isolation procedure.

(i) Immunoabsorption on Stool and NBA-Coupled Affinity Columns

Unabsorbed guinea pig anti-CEA antiserum was initially passed through a stool-coupled affinity column followed by two additional passages through an NBA-coupled column. The guinea pig unabsorbed antiserum and the effluents from each passage through the immunoabsorbents were titrated in the RIA, employing the Farr technique. Figure 11 shows the titration curves thus obtained. Maximum binding remained at about 80% in each case. However, the titres of the various recycled antisera decreased after each absorption step. As a means of comparison, the titre at 50% binding on the curves was chosen. Table 10 summarizes these values. The titre of the guinea pig unabsorbed antiserum decreased by 80% from 1/33,000 to 1/6400 after the initial passage through the stool immunoabsorbent. The succeeding two passages through the NBA immunoabsorbent further decreased the titre by 17% from 1/6400 to 1/5280 for the second passage and by 60% to 1/2000 for the last passage.

To determine the degree of antiserum modification produced by each absorption step, the different recycled antisera were tested in the inhibition RIA with CEA and NBA. The 50% binding point on the titration curves was chosen to give the dilution of each recycled antiserum to be used in the

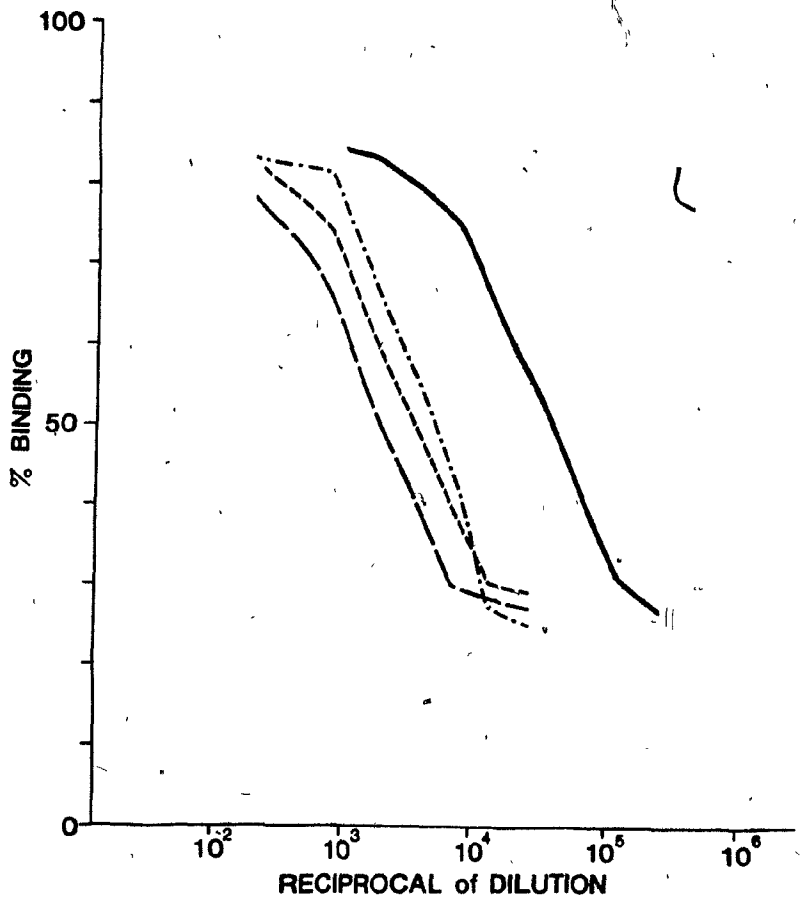


Fig. 11: Titration curves established when ^{125}I-CEA was reacted with unabsorbed guinea pig anti-CEA antiserum (——————) and the same antiserum immunoabsorbed on an initial stool affinity column (— · — · — · —), followed by a second cycle (— — — —) and a third cycle (— — — —) on an NBA (Fraction 11) affinity column. The curves were obtained using the Farr technique.

TABLE 10

Reactivity of Guinea Pig anti-CEA Antiserum Immunoabsorbed on a Stool-Coupled and an NBA-Coupled Affinity Column

Degree of Serum Immunoabsorption	Dilution of Antiserum at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	$I^{50'}$ Inhibitory Activity (ng - NBA)
Unabsorbed Guinea Pig Antiserum	1/33,000	7.80	3740
1st Cycle	1/6400	6.25	7000
2nd Cycle	1/5280	11.70	3120
3rd Cycle	1/2000	15.00	8750

establishment of an RIA. Figure 12 shows the inhibition curves subsequently obtained. The working range of the curves was between 3 ng and 25 ng for inhibition by CEA and 1500 ng and 25,000 ng for inhibition by NBA. Table 10 shows the amounts of CEA and NBA required to achieve the I^{50} value for each absorbed antiserum. Absorption through the stool immunoabsorbent resulted in a reduction in sensitivity towards NBA from 3740 ng for the guinea pig unabsorbed serum to 7000 ng after the first passage. Reactivity to CEA showed a very slight increase. The value of the I^{50} changed from 7.8 ng for the unabsorbed guinea pig antiserum to 6.25 ng following this first cycle of immunoabsorption. However, the recycling of this antiserum through the NBA affinity columns decreased the inhibition with CEA to 11.7 ng and 15.0 ng for the second and third absorptions respectively, while the sensitivity to NBA fluctuated from 3120 ng for the second passage to 8750 for the last one.

The antibody populations which were eluted with 0.3M glycine-HCl from the immunoabsorbents were similarly titrated in the RIA and tested in the CEA and NBA inhibition assays. Figure 13 shows the titration curves obtained using the bound fractions from this first attempt to modify the guinea pig anti-CEA antiserum. Maximum binding ranged between 63% and 82%, with a background radiation level of about 20%. The dilutions needed to achieve 50% binding are shown in Table 11. The first passage through the stool column resulted in dissociation of only 2.7% of the originally applied guinea pig serum, giving a dilution of 1/900. The second passage, through the NBA immunoabsorbent, resulted in elution of 12.2% of the recycled antiserum (from the unbound fraction of the first passage) for a dilution of 1/780 at 50%

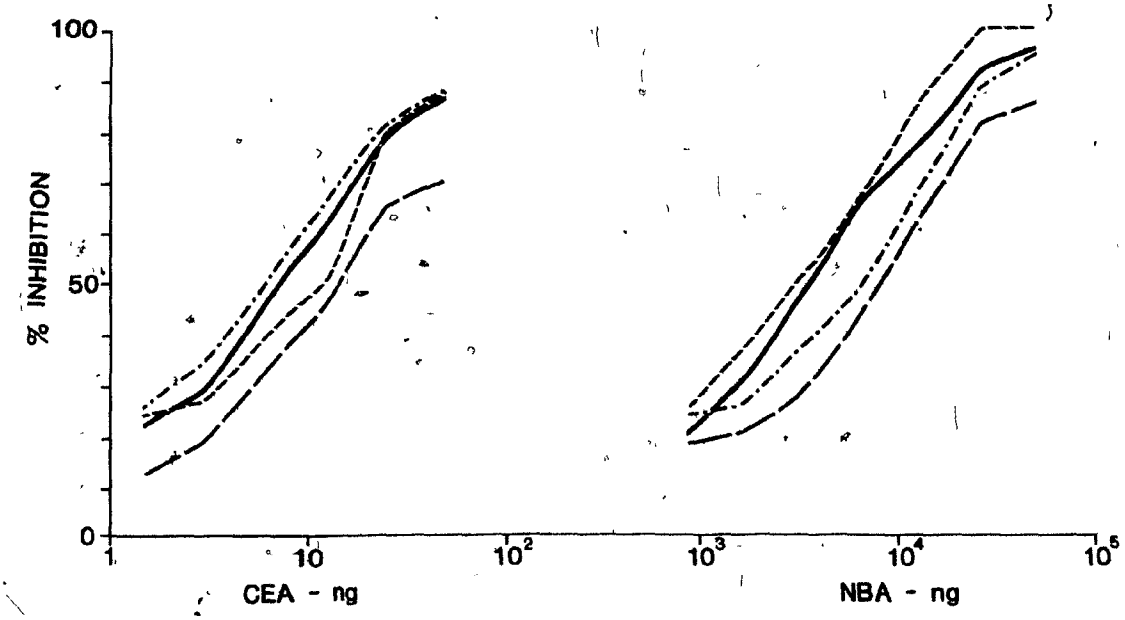


Fig. 12: Inhibition curves established between ^{125}I-CEA and either CEA or NBA (Fraction 11) and guinea pig anti-CEA antisera: unabsorbed (————); and immunoabsorbed following: 1 cycle on a stool affinity column (·—·—··); a 2nd cycle (-·—·—·-) and a 3rd cycle (-·———·-) on an NBA (Fraction 11) affinity column. These curves were established using the Farr technique.

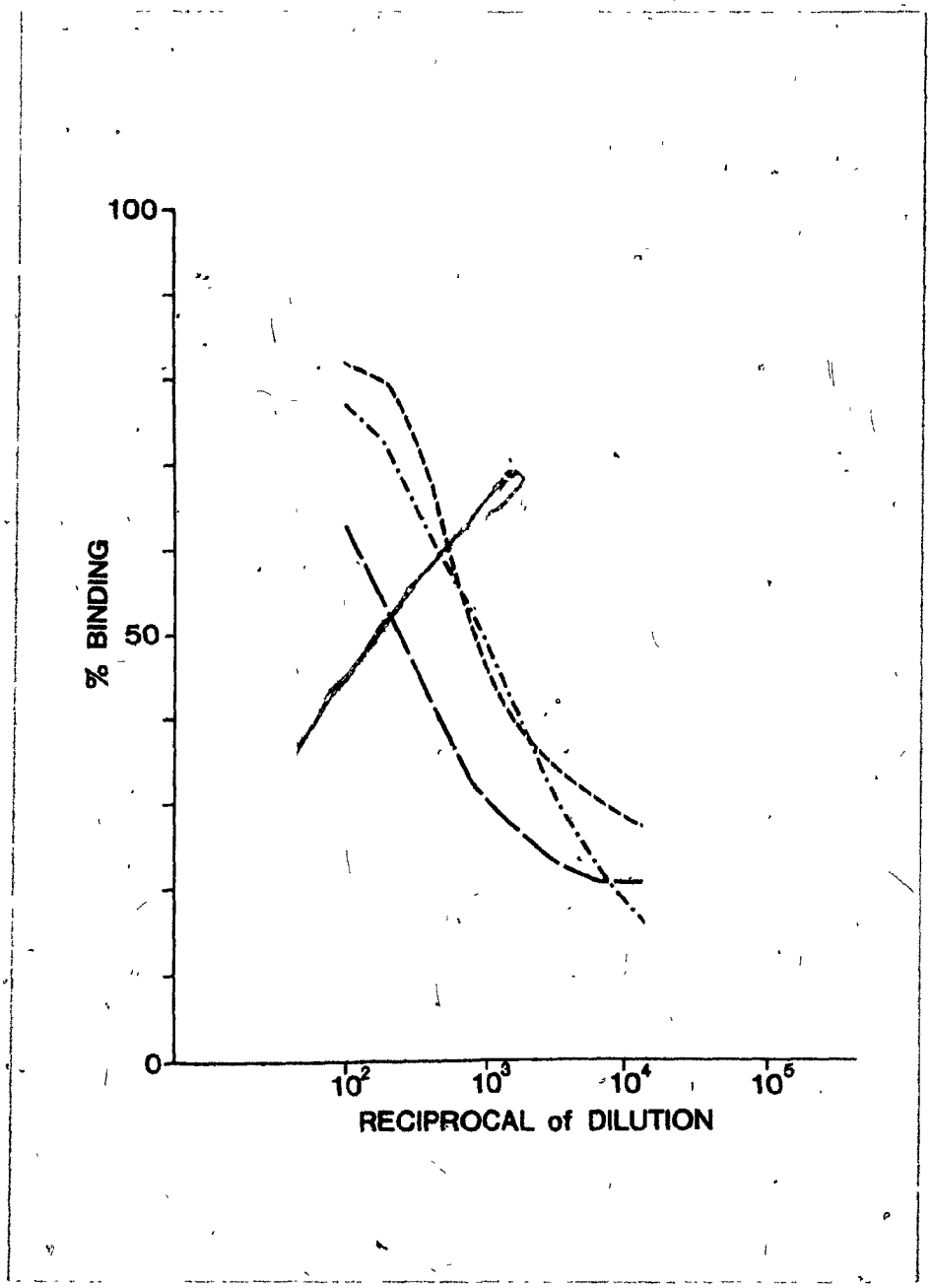


Fig. 13: Titration curves established when ^{125}I-CEA was reacted with immunoabsorbed guinea pig anti-CEA antiserum eluted with 0.3M glycine-HCl pH 2.8 following an initial cycle on a stool affinity column (-·—·—·—) and a second (-———) and third (-———) cycle on an NBA (Fraction 11) affinity column. The curves were obtained using the Farr technique.

TABLE 11

Reactivity of Bound Guinea Pig Antibody Fractions

Eluted with 0.3M Glycine-HCl

	Dilution of Eluted Fraction at 50% Binding	I^{50} Inhibotory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng - NBA)
1st Cycle (Stool Column)	1/900	12.50	4000
2nd Cycle (NBA Column	1/780	10.00	1900
3rd Cycle (NBA Column	1/250	11.00	4520

binding. The final passage, through the same NBA column, gave a dilution of 1/250, which represented the dissociation of 4.7% of the recycled antiserum from the second passage which had reacted with the ligand.

Figure 14 shows the inhibition curves obtained when each of the eluted, bound antibody fractions were reacted with both CEA and NBA in their respective inhibition assays. The I^{50} values subsequently determined are given in Table 11. Generally, these antibody fractions all showed similar sensitivities to CEA, with I^{50} values ranging from 10.0 ng to 12.5 ng, a decrease from that seen with the unabsorbed guinea pig serum (I^{50} of 7.8 ng). The sensitivity to the NBA remained mostly unchanged from that of the original guinea pig serum (I^{50} of 3740 ng), as seen by an I^{50} of 4000 ng with the first elution, increasing to an I^{50} of 1900 ng with the second elution and returning to an I^{50} value of 4500 ng with the last elution from the NBA immunoabsorbent.

(ii) Immunoabsorption on an NBA-Coupled Affinity Column

A second absorption of the unabsorbed guinea pig antiserum was attempted similar to the one described above. However, the stool immunoabsorbent was omitted and three successive passages through only an NBA immunoabsorbent, prepared from the same material as was previously used, were performed. The titration curves obtained, with the Farr technique, are shown in Figure 15. Maximum binding ranged from 70% to 85%. The titres at 50% binding are shown in Table 12. The first passage produced an 80% decrease in the titre of the unabsorbed guinea pig antiserum from 1/33,000 to 1/6400. The second passage only slightly reduced the titre to 1/5900 for an 8% decrease while the last

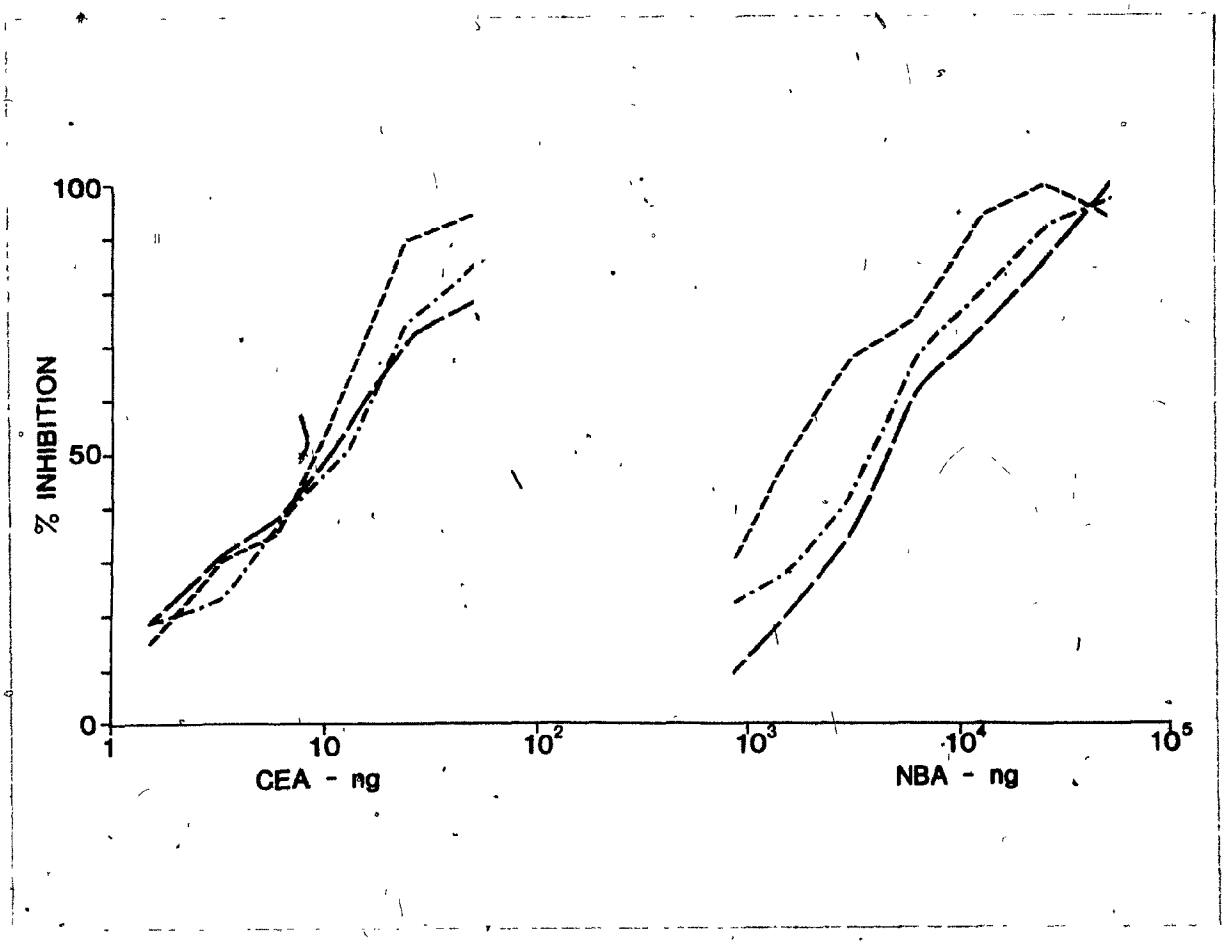


Fig. 14: Inhibition curves established between either CEA or NBA (Fraction II) with ^{125}I-CEA and the immunoabsorbed guinea pig anti-CEA antiserum eluted with 0.3 M Glycine-HCl pH 2.8 following a 1st cycle on a stool column (-·—·—); and a 2nd (-———-) and a 3rd (-———-) cycle on an NBA (Fraction II) affinity column. The curves were obtained using the Farr technique.

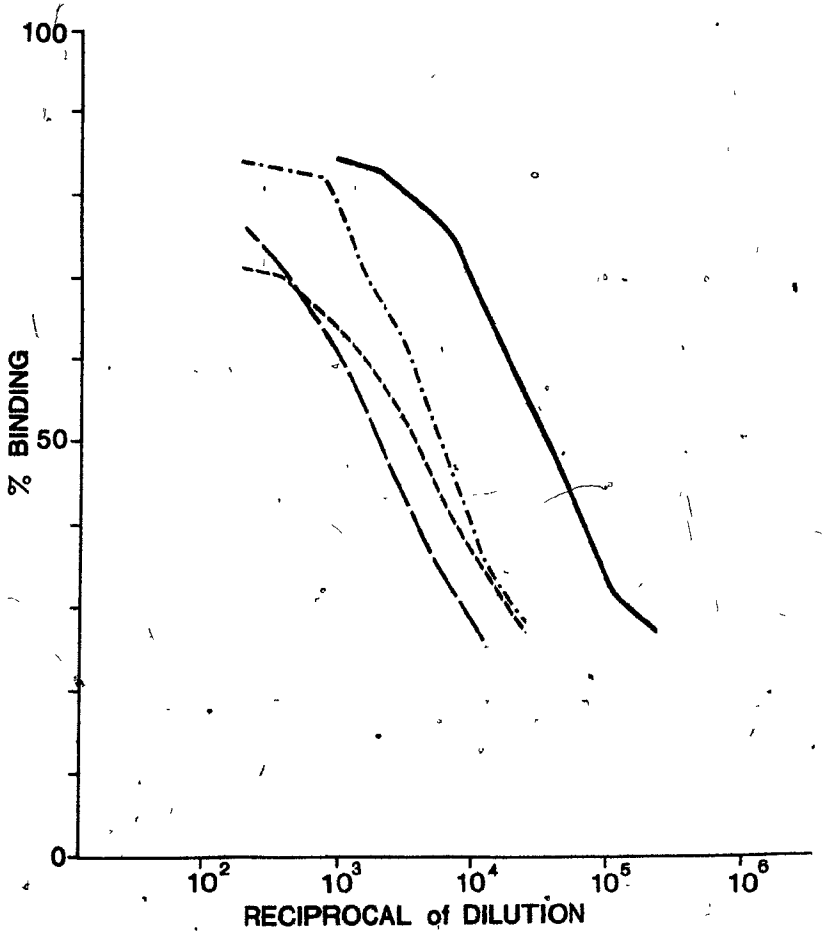


Fig. 15: Titration curves established when ^{125}I-CEA was reacted with unabsorbed guinea pig anti-CEA antiserum (——————) and the same antiserum immunoabsorbed on an NBA (Fraction 11) affinity column following a first (— · — · —); a second (— — — —) and a third (— — — —) cycle. These curves were obtained using the Farr technique.

TABLE 12

Reactivity of Guinea Pig anti-CEA Antiserum

Immunoabsorbed on an NBA-Coupled Affinity Column

Degree of Serum Immunoabsorption	Dilution of Antiserum at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng -NBA)
Unabsorbed Guinea Pig Antiserum	1/33,000	7.80	3740
1st Cycle	1/6400	8.50	7000
2nd Cycle	1/5900	15.00	4000
3rd Cycle	1/2200	15.60	8750

absorption gave a 62% reduction for a final titre of 1/2200.

Figure 16 illustrates the inhibition curves obtained when each antiserum was tested against NBA and CEA in the RIA, using the dilution given by the 50% binding point on the titration curve to construct the inhibition assay. The I^{50} values subsequently determined are summarized in Table 12. The recycling of the antisera caused a reduction, each time, in their sensitivity to CEA going from 7.8 ng for the unaborbed guinea pig antiserum to 8.5 ng, then 15.0 ng and finally 15.6 ng for the first, second and third absorption respectively. Reactivity to NBA was decreased from 3740 ng for the unabsorbed serum to 7000 ng after the first passage. The second passage resulted in an elevation of reactivity, giving an I^{50} value of 4000 ng which was again reduced to 8750 ng following the last absorption.

The antibody populations eluted from the NBA immunoabsorbents in this second attempt to modify the guinea pig antiserum were similarly titrated followed by reaction in the inhibition assays. The titration curves are shown in Figure 17. Maximum binding ranged between 69% and 82%, with a background level of 20%. The inhibition curves obtained when these eluted fraction were reacted with NBA and CEA are shown in Figure 18. Table 13 gives the dilutions at 50% binding and the I^{50} values determined by inhibition with the two antigens. The first passage through the NBA immunoabsorbent resulted in elution of 2.7% of the guinea pig serum for a dilution of 1/900. The second and third passages yielded bound fractions comprising 12.8% (1/820 dilution) and 5.5% (1/325 dilution), respectively, of the recycled antiserum preparations.

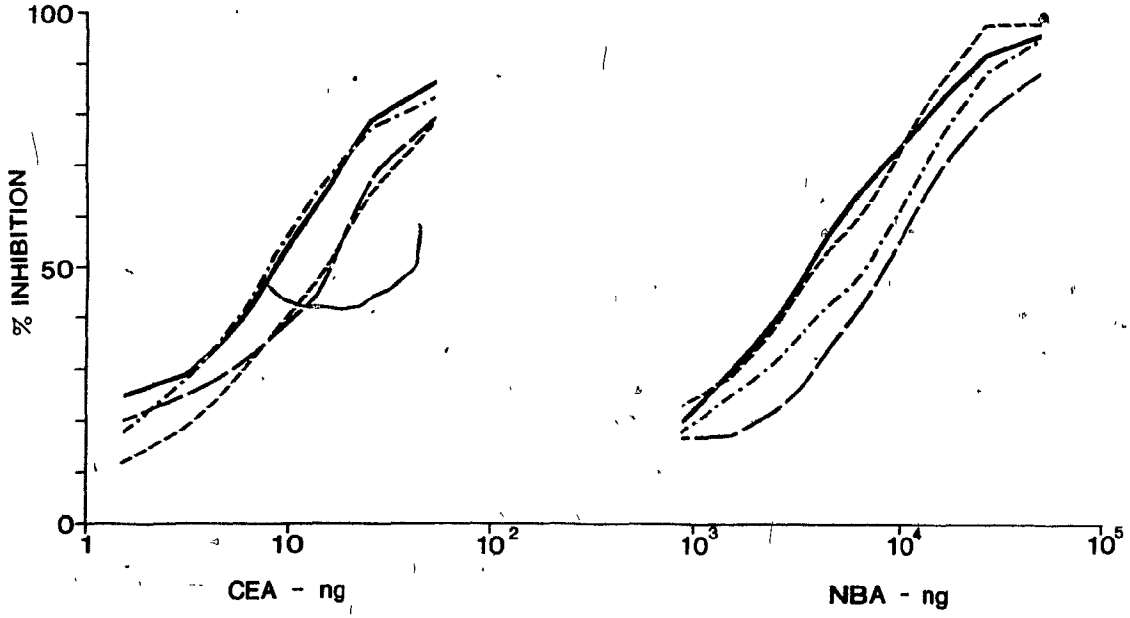


Fig. 16: Inhibition curves established between either CEA or NBA (Fraction 11) with ^{125}I-CEA and guinea pig anti-CEA antiserum: unabsorbed (————); and immunoabsorbed on an NBA (Fraction 11) affinity column following 1 cycle (·—·—·); 2 cycles (–—–—) and 3 cycles (– — –). The curves were obtained using the Farr technique.

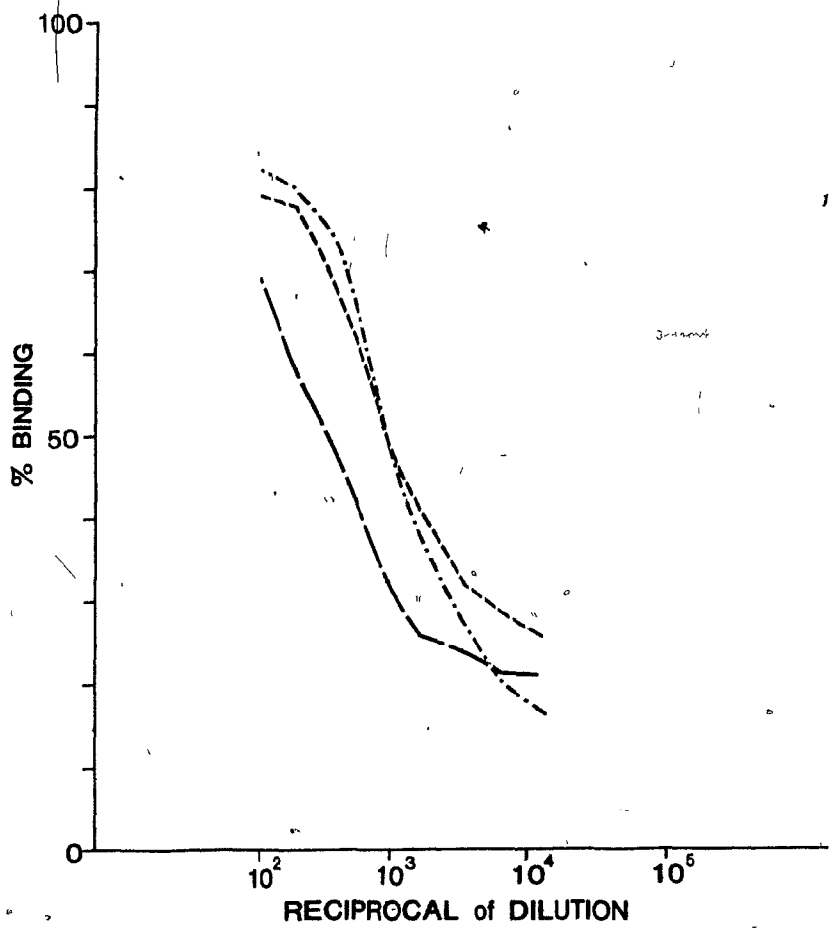


Fig. 17: Titration curves established when ^{125}I-CEA was reacted with immunoabsorbed guinea pig anti-CEA antisera eluted with 0.3M glycine-HCl pH 2.8 following 1 cycle (-·—·-—) 2 cycles (————); and 3 cycles (— ——— —) on an NBA (Fraction 11) affinity column. The curves were obtained using the Farr technique.

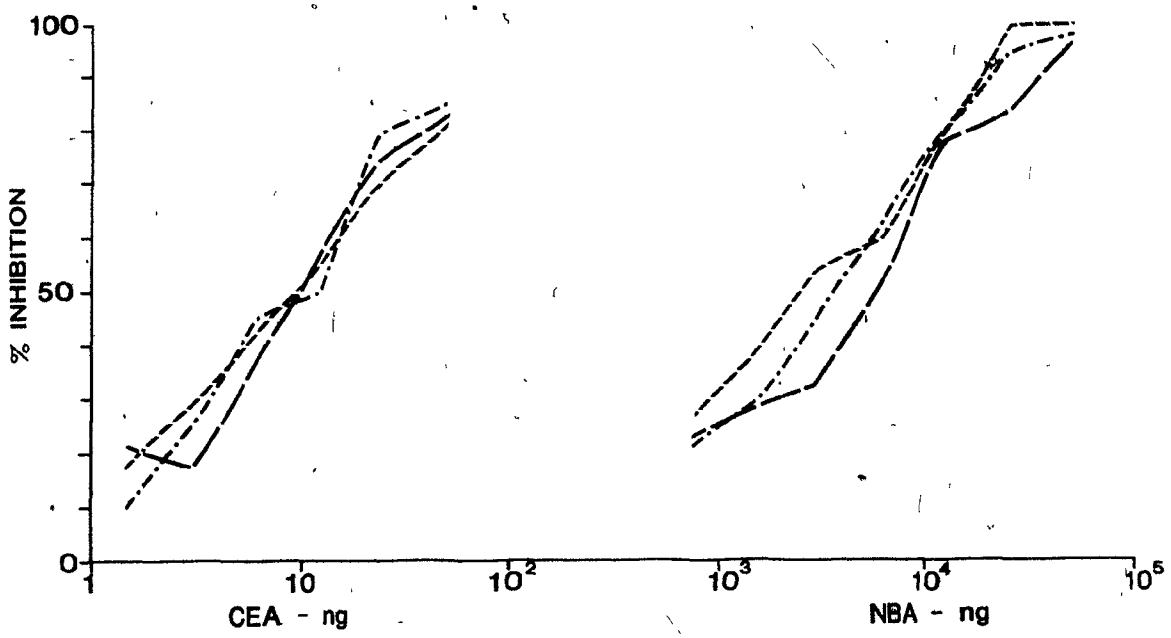


Fig. 18: Inhibition curves established between either CEA or NBA (Fraction 11) with ^{125}I-CEA and the immunoabsorbed guinea pig anti-CEA antisera eluted with 0.3M glycine-HCl, pH 2.8 following a 1st cycle (·—·—·—), a 2nd cycle (·————) and a 3rd cycle (—·——·) on an NBA (Fraction 11) affinity column. The curves were obtained using the Farr technique.

TABLE 13

Reactivity ofBound Guinea Pig Antibody Fractions

Eluted with 0.3M Glycine-HCl

	Dilution of Eluted Fraction at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng - NBA)
1st Cycle	1/900	12.50	3900
2nd Cycle	1/820	10.00	2500
3rd Cycle	1/325	10.15	5600

The three bound antibody fractions showed a decreased sensitivity to CEA with I^{50} values ranging from 10.0 ng to 12.5 ng as compared with the unabsorbed serum's I^{50} value of 7.8 ng. I^{50} values obtained when these three fractions were reacted with NBA were 3900 ng with the first elution, 2500 ng with the second, and 5600 ng with the third, showing slight variation from that obtained with the guinea pig unabsorbed serum (3740 ng).

B) Immunoabsorption of Horse anti-CEA Antiserum

Two immunoabsorptions of horse anti-CEA antiserum were performed. One series of experiments consisted of five successive passages through an NBA immunoabsorbent prepared with the longchain alkylamine glass beads coupled to material from Fraction 11 of the DEAE cellulose chromatography in the isolation procedure of normal bowel. The second absorption involved serial passages through four different NBA immunoabsorbents, each one prepared in the same manner, using the amino aryl glass beads coupled with material obtained from Peak 1 of the Ultragel chromatographic step in the NBA isolation procedure.

(i) Immunoabsorption on an NBA-Coupled Affinity Column

Figure 19 shows the titration curves, using the Farr technique, of the unabsorbed horse anti-CEA antiserum and the antisera obtained following recycling through the NBA immunoabsorbent. Maximum binding ranged between 50% and 92%. The first passage resulted in a reduction of 85% in titre, going from 1/38,000 for the unabsorbed horse serum to 1/5600 for the antiserum after the first absorption. Successive passages resulted in decreases in titre of 60% to 1/2200, 72% to 1/600, 75% to 1/150 and 66% to

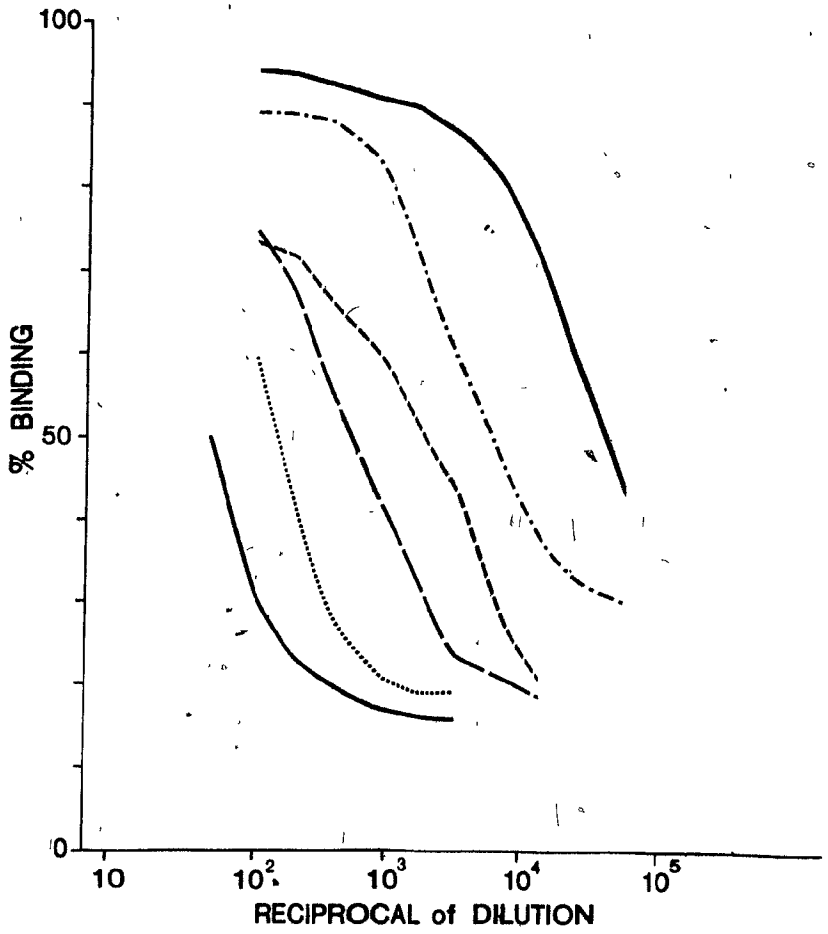


Fig. 19: Titration curves established when ^{125}I-CEA was reacted with unabsorbed horse anti-CEA antiserum (——————) and the same antiserum immunoabsorbed on an NBA (Fraction 11) affinity column after a 1st cycle (—·—·—); a 2nd cycle (—— —— ——); a 3rd cycle (— — — —); a 4th cycle ( ) and a 5th cycle (——————). The curves were obtained using the Farr technique.

1/50 for the second, third, fourth and fifth passages respectively.

Figure 20 shows the inhibition curves obtained when the titre at 50% binding on the titration curve was used as the dilution for testing each of the different recycled antisera in the inhibition assays with CEA and NBA. The working range of the curves was between 25 ng and 6.25 ng for CEA and between 50,000 ng and 6250 ng for the NBA inhibition curves. The I^{50} values thus determined are shown in Table 14. The reactivity of the unabsorbed horse serum to CEA decreased from 7.0 ng to 9.5 ng after the first passage. The second passage did not change this value; however, the third one resulted in a further decrease to 15 ng, which remained the same for the fourth passage, but decreased to 50 ng following the final absorption. With respect to the reactivity to NBA, the first passage gave an initial four-fold decrease in sensitivity, from 5500 ng for the unabsorbed horse serum to 22,000 ng, followed by an increase to 16,000 ng for the second absorption. The third, fourth and fifth passages yielded antisera in which the reactivity to NBA was again decreased to 23,000 ng, 23,000 ng and 25,000 ng respectively.

(ii) Immunoabsorption on Multiple NBA-Coupled Affinity Columns

Figure 21 shows the titration curves obtained for the unabsorbed horse serum and those antisera absorbed on the four separate affinity columns. For this absorption, the double antibody method for the RIA was employed. Maximum binding ranged between 45% and 90%. The 35% binding point gave the titre used for comparison purposes. The first passage resulted in an 84% decrease in titre of the original unabsorbed horse serum from 1/250,000 to

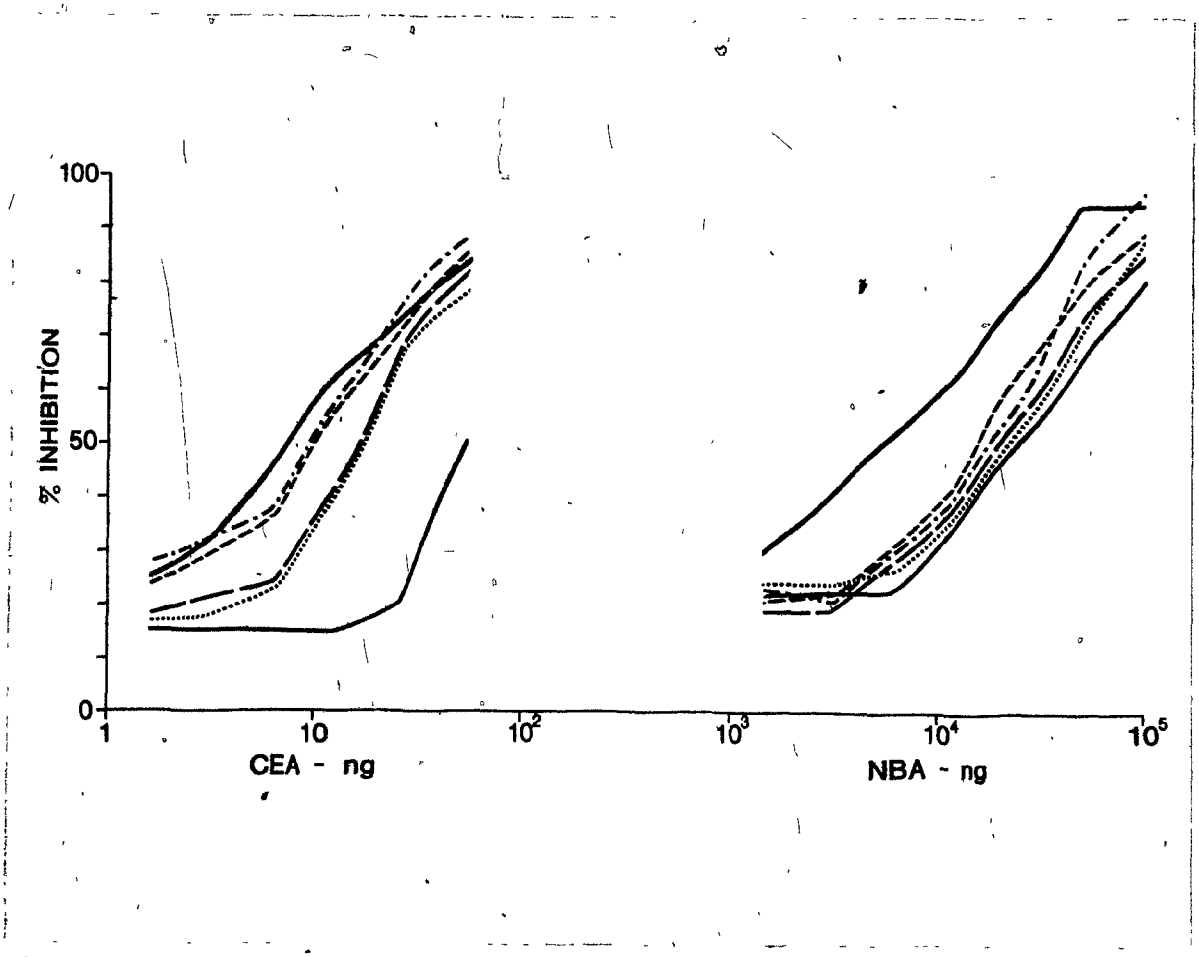


Fig. 20: Inhibition curves established between either CEA or NBA (Fraction 11) with ^{125}I-CEA and horse anti-CEA antiserum: unabsorbed (——————); and immunoabsorbed on an NBA (Fraction 11) affinity column following 1 cycle (— · — · —) 2 cycles (— — — — —), 3 cycles (— — — — —), 4 cycles (·····) and 5 cycles (——————). The curves were obtained using the Farr technique.

TABLE 14

Reactivity of Horse anti-CEA Antiserum

Immunoabsorbed on an NBA-Coupled Affinity Column

Degree of Serum Immunoabsorption	Dilution of Antiserum at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng - NBA)
Unabsorbed Horse Antiserum	1/38,000	7.0	5500
1st Cycle	1/5600	9.5	22,000
2nd Cycle	1/2200	9.5	16,000
3rd Cycle	1/600	15.0	23,000
4th Cycle	1/150	15.0	23,000
5th Cycle	1/50	50.0	25,000

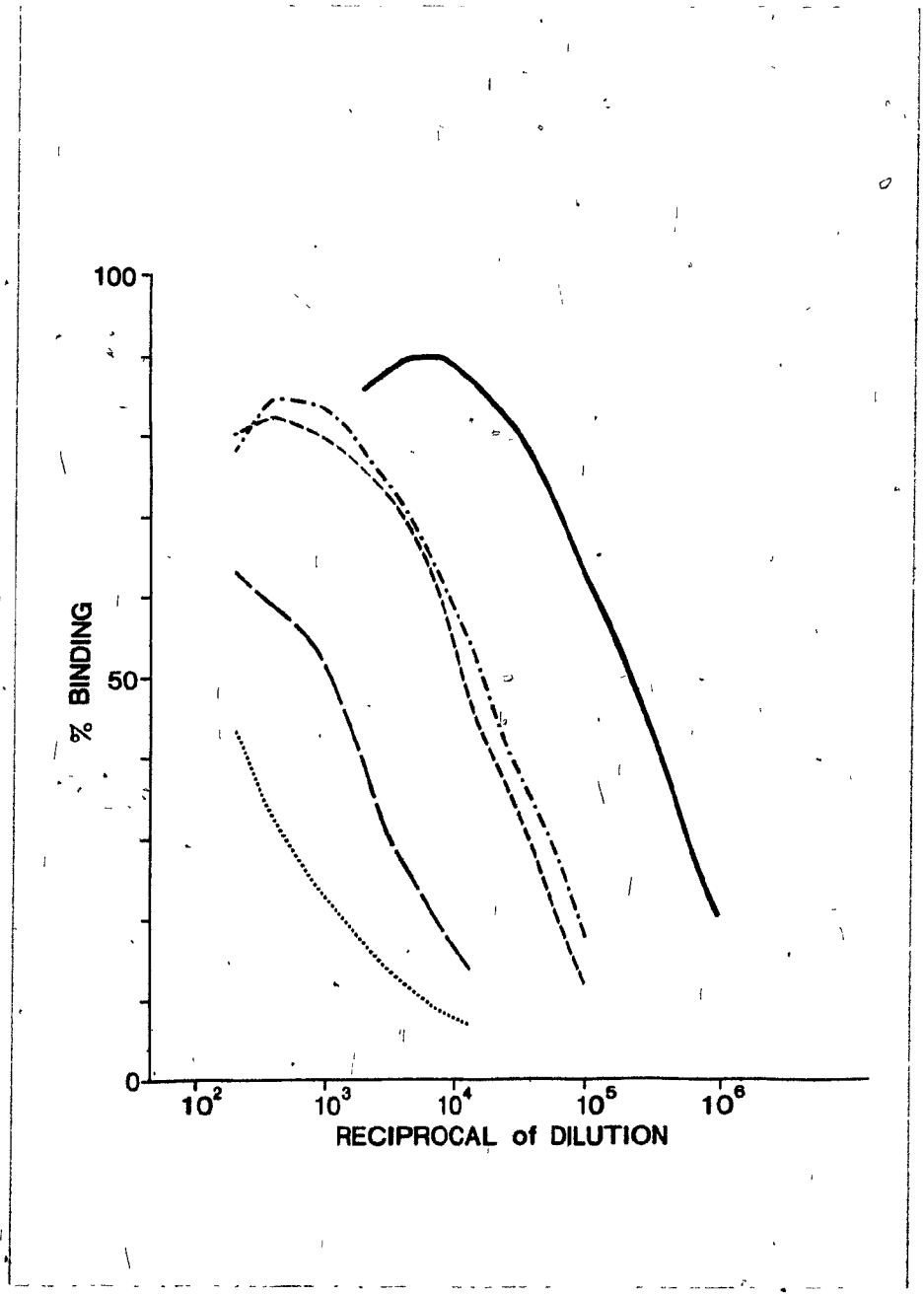


Fig. 21: Titration curves established when ^{125}I-CEA was reacted with unabsorbed horse anti-CEA antiserum (——) and the same antiserum immunoabsorbed on four separate NBA (Peak 1) affinity columns. 1st cycle (- - — - - —); 2nd cycle (— — —); 3rd cycle (— — —); and 4th cycle (.....). These curves were obtained using the double antibody method.

1/40,000. The second, third and fourth passages yielded decreases of 35% to 1/26,000, 90% to 1/2600 and 85% to 1/400 respectively.

Figure 22 illustrates the curves obtained from the inhibition with CEA and NBA of the antisera from each immunoabsorption. The dilution of antiserum used was determined by the titre needed to achieve 35% binding in its titration curve. The working range of these curves was from 1.5 ng to 25 ng for inhibition with CEA and from 1500 ng to 25,000 ng for the inhibition with NBA. The I^{50} values thus obtained are shown in Table 15. The first three passages yielded small decreases in the sensitivity of the antisera to CEA, changing from 2.0 ng for the unabsorbed serum to 3.0 ng after the first passage, then to 3.5 ng after the second and to 4.7 ng after the third absorption. The last passage resulted in a two-fold decrease in sensitivity, yielding an antiserum with an I^{50} of 10.0 ng. The reactivity to NBA decreased by more than two-fold after the first passage, the I^{50} increasing from 2500 ng for the unabsorbed serum to 6700 ng. The second immunoabsorption resulted in an antiserum more sensitive to the NBA, having an I^{50} of 2500 ng. However, after the third and fourth passages, this sensitivity decreased, shown by I^{50} values of 5000 ng and 10,000 ng respectively.

The antibody populations eluted with 0.3M glycine-HCl, from the four NBA immunoabsorbents, were similarly titrated in the RIA followed by reaction with NBA and CEA in the inhibition RIA. The titration curves, shown in Figure 23, had maximum bindings ranging from 61% to 88%, with a background level of about 7%. The dilutions at 35% binding were used to construct the inhibition curves with the two antigens, illustrated in Figure 24. The

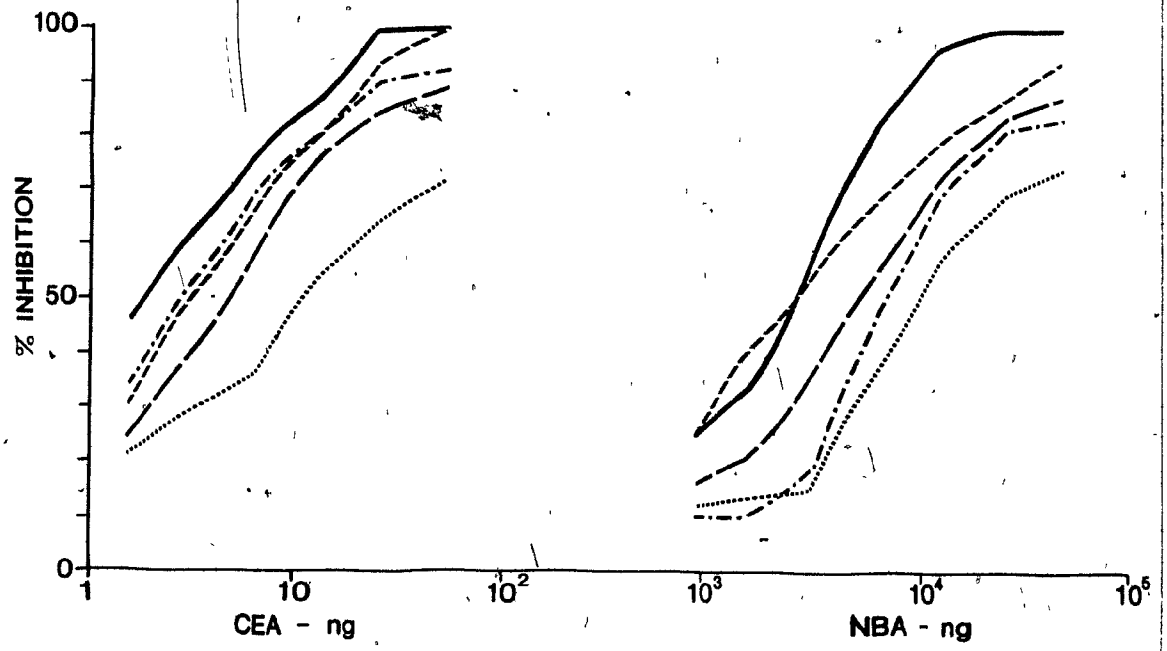


Fig. 22: Inhibition curves established between either CEA or NBA (Peak 1) with ^{125}I-CEA and horse anti-CEA antisera: unabsorbed (————); and immunoabsorbed on four separate NBA (Peak 1) affinity columns following 1 cycle (– · — · —) 2 cycles (– — – —); 3 cycles (— — — —); and four cycles (·····). The curves were obtained using the double antibody method.

TABLE 15

Reactivity of Horse anti-CEA Antiserum

Immunoabsorbed on Multiple NBA-Coupled Affinity Columns

Degree of Serum Immunoabsorption	Dilution of Antiserum at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng - NBA)
Unabsorbed Horse Antiserum	1/250,000	2.0	2500
1st Cycle	1/40,000	3.0	6700
2nd Cycle	1/26,000	3.6	2500
3rd Cycle	1/2600	4.7	5000
4th Cycle	1/400	10.9	10,000

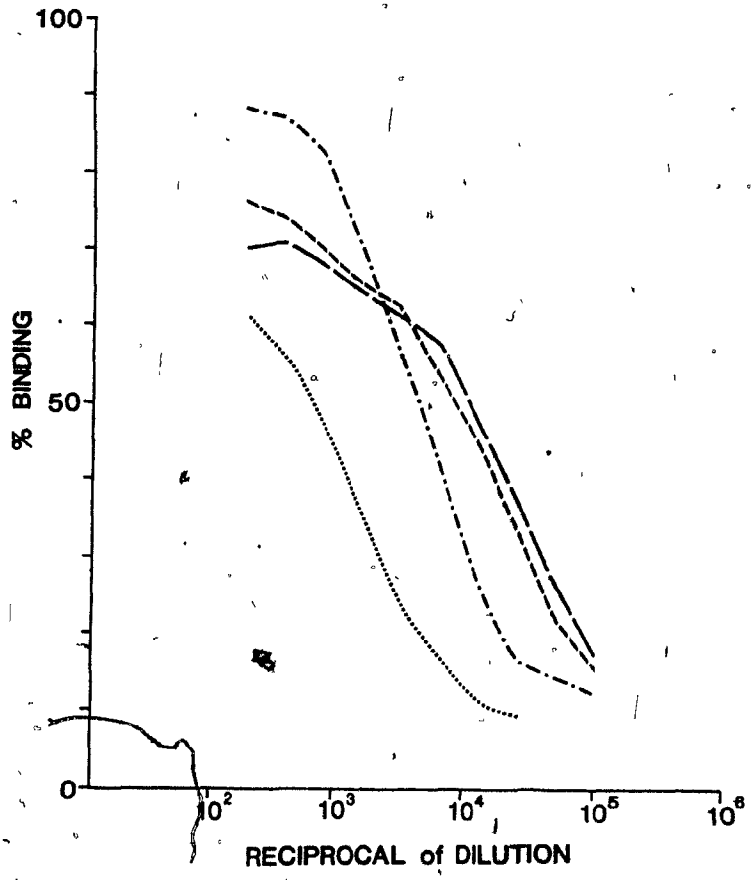


Fig. 23: Titration curves established when ^{125}I-CEA was reacted with immunoabsorbed horse anti-CEA antisera eluted with 0.3M glycine-HCl pH 2.8 following 1 cycle (·—·—·—), 2 cycles (— — —), 3 cycles (— ——— —), and 4 cycles (·····) on four separate NBA (Peak 1) affinity columns. The curves were obtained using the double antibody method.

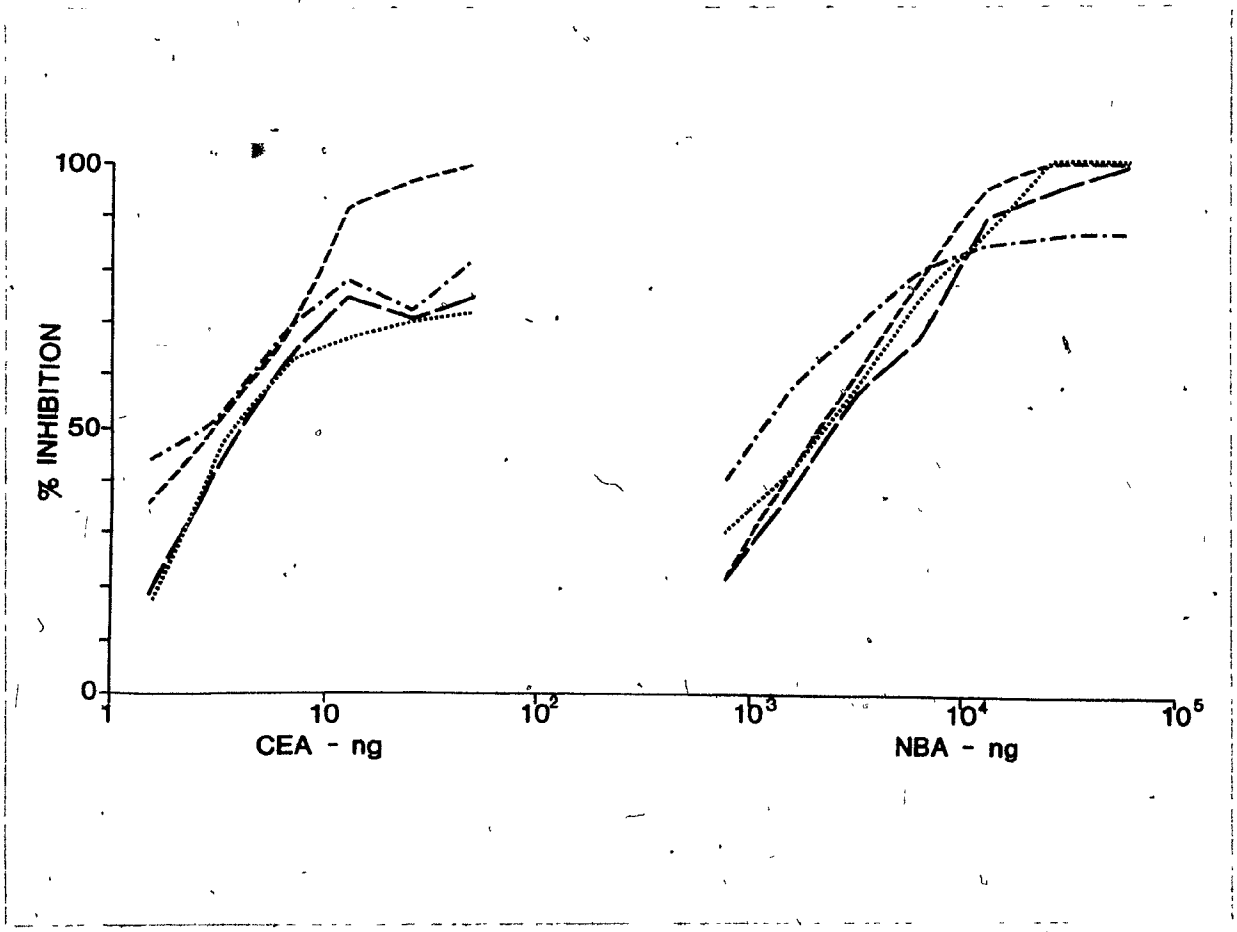


Fig. 24: Inhibition curves established betweeen either CEA or NBA (Peak 1) with ^{125}I-CEA and the immunoabsorbed horse anti-CEA antisera eluted with 0.3 M glycine-HCl pH 2.8 following a 1st cycle (—·—·—); a 2nd cycle (— — —); a 3rd cycle (—————); and a 4th cycle (·····) on four separate NBA (Peak 1) affinity columns. The curves were obtained using the double antibody method.

useful working ranges of the two sets of curves are between 3.0 ng and 12.5 ng for CEA and 1000 ng and 10,000 ng for NBA. Table 16 gives the dilutions, at 35% binding, for each of the four elutions and the I^{50} values subsequently determined from the inhibition curves.

Passage through the first NBA immunoabsorbent resulted in dissociation of 3.2% of the CEA-reactive antibodies from the normal bowel ligand, for a dilution of 1/8000. The next elution yielded a dilution of 1/2300, representing dissociation from the second immunoabsorbent of 5.8% of the recycled antibodies. Chaotropic dissociations of the third and fourth passages from the last two NBA affinity columns yielded antibody dilutions of 1/3000 and 1/200 for elution of 11.5% and 7.7% respectively of the recycled anti-CEA antiserum.

Reactivity to CEA, of each of the four bound antibody fractions, varied slightly with each successive elution, giving I^{50} values of 2.8 ng, 3.0 ng, 4.3 ng and 3.9 ng for the first, second, third and fourth elutions respectively. Reactivity to NBA also showed little difference with each passage, yielding I^{50} values of 1000 ng, 2200 ng, 2540 ng and 2340 ng for the four separate dissociations with the glycine-HCl.

TABLE 16

Reactivity of Bound Horse Antibody Fraction

Eluted with 0.3M Glycine-HCl

	Dilution of Eluted Fraction at 50% Binding	I^{50} Inhibitory Activity (ng - CEA)	I^{50} Inhibitory Activity (ng - NBA)
1st cycle	1/8000	2.8	1000
2nd Cycle	1/2300	3.0	2200
3rd Cycle	1/3000	4.3	2540
4th Cycle	1/200	3.9	2340

Discussion

The aim of this study was to modify, by means of affinity chromatography, the specificity of an anti-CEA antiserum. Two anti-CEA antisera from different species, that of horse and guinea pig origin, were subjected to immunoabsorption in an attempt to improve their specificity to CEA by reducing their reactivity to normal bowel antigens.

Experimental Approach

The method currently used to absorb anti-CEA antiserum for use in clinical assays is by the addition to the antiserum of various normal tissue extracts. The resulting precipitate is removed by centrifugation. However, when the resulting absorbed antiserum is reacted with CEA (Fig. 6, p. 73) or NBA (Fig. 9, p. 99) in the RIA, no significant change in immunoreactivity is observed when compared with the unabsorbed serum. This residual normal bowel activity probably leads to the lack of specificity of the clinical assays. Hence, in the attempt to modify the anti-CEA antisera, the effect which was desired was a decrease in the reactivity of the immunoabsorbed antisera to normal bowel material with little or no change in its sensitivity to CEA isolated from tumor tissue.

In the first part of these studies the optimum conditions to be used for solid phase immunoabsorption were defined. Two different types of matrices, glass beads and agarose, and two methods of coupling ligand to the carrier arms were examined. Several parameters were investigated, including the

stability of the linkage between the carrier and the ligand, the efficiencyof the method of coupling ligand to the carrier and the performance of the ligand-coupled matrix as an immunoabsorbent, with interest taken in observing the nonspecific absorption by the matrix itself.

From these studies, it was concluded that the performance of the glass bead matrices, both the aryl amine and the longchain alkylamine glass beads, was superior to that of the sepharose. This is in part due to the high resistance and stability of the glass as well as the silyl coating rendering the beads quite inert.

Once it was determined that the glass beads were to be the matrices for the subsequent studies, one last parameter was investigated - the determination of the optimum pH of 0.3M glycine-HCl to be used for the dissociation of the ligand from the affinity column. Several parameters must be considered for the optimum conditions to prevail. To minimize the denaturation of both the ligand and the absorbed material, a single incubation with the dissociating agent was preferred. It was also important to choose an optimum pH in order to remove, quantitatively, all of, or as much as possible, those molecules immunologically bound to the ligand, especially in those cases where the unbound fraction from the first passage through the immunoabsorbent was recycled through the same column. As seen in Table 5, a pH of 2.8 was determined to be the optimum value.

Complete recovery of the immunoabsorbent was not achieved, possibly due the presence of high affinity antigen-antibody complexes that are not readily dissociated by the chaotropic treatment, and a certain degree of denaturation

of the eluted absorbed material.

Experimental Data

Immunoabsorption studies were performed with horse and guinea pig anti-CEA antisera. Both were chosen due to their strong immunoreactivity with CEA.

Two types of immunoabsorptions were performed with the guinea pig antiserum. The first one involved the use of, initially, a stool-coupled affinity column followed by recycling the recovered unbound fraction twice through an NBA-coupled affinity column. The aqueous extract of stool material was employed due to a previous report in which the efficiency of this material in removing "normal" antibody populations from guinea pig anti-CEA antiserum was described (89). The second immunoabsorption involved recycling the antiserum through a single NBA-coupled affinity column. A comparison of both these absorptions showed that the stool column performed in virtually the same manner as the first NBA column in the second absorption. Both these initial columns yielded unbound fractions with similar inhibitory immunoreactivities to CEA (6.25 ng for the stool column and 8.5 ng for the NBA column) and to NBA (7000 ng for both columns), as well as producing identical residual antiserum titres. The fractions eluted with the chaotropic agent also showed no difference with respect to antibody titre and reactivity to both antigens in the RIA. The stool material was originally suggested as a good source of normal bowel material because during stool formation and passage through the intestines, it would collect the NBA as it

was sloughed off the epithelial tissue into the lumen of the intestines. However, this present study indicated that the NBA activity in stool is not different immunologically from that obtained by dissection of tissues obtained at autopsy. The remainder of both absorptions yielded almost identical results, showing that further recycling of the guinea pig antiserum did not improve its specificity to CEA. Rather, as the number of cycles increased, the sensitivity decreased to CEA as well as to the NBA.

Two different absorptions with the horse anti-CEA antiserum were performed. The first one involved recycling the antiserum five times through one NBA immunoabsorbent. The first passage of the antiserum through the column resulted in a large decrease in titre, as was also seen with the guinea pig antiserum. The reactivity of the unbound fraction to CEA remained about the same, while that to the NBA decreased almost four-fold. However, subsequent recycling to further modify the antiserum indicated that as antibodies to NBA are sequentially removed, causing a decrease in sensitivity to that antigen, the sensitivity to CEA also lessened.

The second absorption with the horse anti-CEA antiserum involved recycling the serum through four different NBA columns, each one coupled with the material from Peak 1 of the Ultragel chromatographic step. This material, as determined by its reactivity in the radioimmunoassay, represented a two-fold increase in purity to the NBA employed as a ligand in the three previous immunoabsorptions. When further purification was attempted, recovery of the reactive antigenic moieties was minimal, as was its ability to couple to the carriers on the glass bead matrices.

The use of a DEAE cellulose preparation of normal bowel material in these three previous attempts at antiserum modification was chosen due to the possibility that further purification might eliminate weakly antigenic moieties which, simply by virtue of being attached to a solid support system, may become more immunogenic and absorb out antibody population otherwise missed by a purer NBA preparation. However, due to the results of these three previous immunoabsorptions, an Ultragel chromatographic step was included in the NBA isolation procedure to obtain a further degree of purification, this time, in the hope that this concentrated ligand would extract, in a more specific manner, a greater amount of antibody populations with each serum recycling.

Furthermore, multiple affinity columns, each one coupled with an Ultragel preparation of NBA, were prepared, as opposed to a single column used in the other three instances. The reason for this procedure was the possibility of the presence of high affinity antibodies in the antiserum, which when tightly complexed with its ligand, would not be eluted with the glycine-HCl and, thus, would be masking strong immunogenic sites on that column upon recycling of the antiserum. In addition, the possibility existed that the ligand might experience mild denaturation due to repeated exposure to the chaotropic agent, a factor that would be omitted with the use of multiple columns.

The results from this last immunoabsorption were similar to those obtained from the previous attempts at serum modification, in that the first cycle of immunoabsorption resulted in the greatest drop in serum titre, as well as a greater decrease in NBA reactivity relative to that of CEA. However, as seen before, additional recycling caused further decrease in

reactivity to the NBA with a concommitant decrease towards CEA.

Elution of the bound fraction from each immunoabsorbent gave antibody populations whose reactivity to NBA remained similar to that of the unabsorbed horse antiserum, while that to CEA decreased about two-fold following the third and fourth cycles. When these eluted fractions are compared with those obtained from both of the guinea pig chaotropic elutions, similar results are seen. The reactivity of those fractions of guinea pig antibodies eluted with the glycine-HCl also showed a two-fold decrease in sensitivity to CEA while that to NBA remained the same as the unabsorbed antiserum.

A comparison of the two types of absorptions done with the horse antiserum showed that recycling the antiserum through different affinity columns was not more effective than recycling through the same column. This indicated that, most probably, little or no denaturation of the ligand occurred and the percentage of high affinity antibodies masking the ligand was minimal.

Conclusions

The hypothesis adopted at the start of this research is that the CEA molecule carries both tumor and nontumor determinants and that immunization with this molecule would produce a heterogeneous antiserum containing anti-tumor and anti-normal antibody populations. The purpose of this research study was to attempt to isolate the anti-tumor population from the rest of the heterogeneous antiserum.

Two high-titre anti-CEA antisera were employed, both able to produce sensitive radioimmunoassays. The two antisera were from different origins,

due to the fact that diverse species of animals may react immunologically in a different fashion to the same antigen.

The results of these studies indicated a number of things which, while not disproving the starting hypothesis, do nothing to further support it. The first observation is that immunoabsorption of both horse and guinea pig anti-CEA antisera did not result in a tumor-specific modified antiserum. The results of the immunoadsorption studies can best be summarized by Table 17. This table lists the ratio of NBA activity in the RIA to the CEA reactivity of the antisera before application onto the affinity columns and those fractions obtained following each passage through the immunoadsorbents. A greater ratio indicates a larger decrease in the antiserum's sensitivity to NBA relative to its sensitivity to CEA. A lower ratio demonstrates a lack of the antiserum's ability to distinguish between the two antigens. With respect to both of the guinea pig immunoadsorptions, only the first passage through the immunoadsorbents produced any increase in the discriminating ability of the serum. The ratio of 1120/1 for the stool column and 820/1 for the NBA column show a two to three-fold increase in CEA sensitivity relative to NBA as compared with the unabsorbed guinea pig antiserum, which had a ratio of 480/1. However, subsequent recycling only served to destroy these increased ratios.

The first immunoabsorption performed with horse antiserum, which involved recycling the antiserum through the same affinity column five times, gave its greatest ratio after the first passage. The succeeding three passages, though, all yielded absorbed sera with larger ratios than that for the unabsorbed antiserum, showing a two to three-fold increase in the antiserum's

TABLE 17

Comparison of the NBA:CEA I^{50} Values

from the Horse and Guinea Pig

Immunoabsorption Studies

	Guinea Pig: Stool and NBA Columns†	Guinea Pig: Single NBA Column†	Horse: Single NBA Column†	Horse: Multiple NBA Columns*
1st Cycle	1120/1	820/1	2300/1	2230/1
2nd Cycle	270/1	270/1	1680/1	715/1
3rd Cycle	580/1	560/1	1530/1	1060/1
4th Cycle	-	-	1530/1	920/1
5th Cycle	-	-	500/1	-
Guinea Pig Unabsorbed	480/1	480/1	-	-
Horse Unabsorbed	-	-	785/1	1250/1
Horse Absorbed (Standard)	-	-	1830/1	6000/1

† All the calculations in this column were obtained from the RIA using the Farr Technique.

* All the calucaltions in this column were obtained from the RIA using the double antibody method.

sensitivity to CEA relative to NBA. However, when these ratios are compared with that obtained using the standard absorbed antiserum, it can be seen that passage through the immunoadsorbent could not achieve a more CEA-specific antiserum. Only the first passage yielded a serum whose ratio was just slightly greater than that of the standard absorbed; the remaining four cycles could not improve this specificity towards CEA.

The second immunoadsorption with horse anti-CEA antiserum, similarly, yielded its most discriminating antiserum, when compared with the unabsorbed horse serum, following the first application onto the first column. Subsequent recycling followed the pattern observed in the three previous attempts at serum modification, in that passage through the next three affinity columns only served to destroy this specificity. However, when compared with the standard absorbed antiserum, whose ratio of 6000/1 was obtained from the RIA using the more sensitive double antibody method, the affinity chromatography of unabsorbed horse anti-CEA antiserum was not successful in producing a more modified tumor-specific anti-CEA antiserum than that previously prepared by our laboratory. Thus, of all the antisera tested, both before and following immunoabsorption, the one most sensitive to CEA and most insensitive to NBA, as determined by inhibition in the RIA, is the standard absorbed horse antiserum, obtained by the batch method of reacting the antiserum with water extracts of various normal tissues followed by precipitation of these immune complexes. Multiple attempts at serum immunoabsorption was incapable of modifying two different anti-CEA antisera to a greater extent than that observed with the standard horse.

Two major questions are constantly raised with respect to the RIA for CEA. The first one concerns the antigenic nature (specificity) which the antibody populations are recognizing. Do they react with a tumor-specific site or a "normal" site on the molecule; are there different populations for each site, or are both determinants so similar that the antibody molecules cannot differentiate between the two, if indeed two distinct sites exist? Separate areas may exist on the molecule, but the determination of their presence is dependent on the antiserum used, which is dependent on the way the immunizing animal visualizes the CEA molecule and produces antibodies to it. If the animal cannot distinguish, specifically, between normal and tumor areas on the CEA, then it will make similar antibody populations to both determinants. On the other hand, the animal may be able to "see" two distinct epitopes, but react immunologically stronger to the normal epitope than to the tumor one, thereby producing a preponderance of normal antibodies and only a minor amount of tumor-specific antibodies. The second question, equally as important as the first, concerns the presence of CEA in normal bowel tissue. If it does exist, it is present in minute quantities. However, its immunoreactivity to specific anti-CEA antibodies may be great enough so that only a very small amount of the antigen would be sufficient to hinder the successful modification of an unabsorbed anti-CEA antiserum.

The studies performed in this thesis attempted to answer these questions. However, even after numerous immunoabsorptions, these questions are still left unanswered, mainly, because one can only speculate as to what antibody populations exist in the antisera, and what is really happening on the affinity column between the ligand and the antibody. Several alternatives

can be examined in light of the results which were obtained.

As stated before, the hypothesis adopted at the start of this work was that there is a tumor-specific site on the CEA molecule to which xenogeneic hosts are able to produce antibodies. Thus, in each of the animal antisera preparations, there would be a tumor-specific population of antibodies, most probably a very small percentage of the total. If this population was specific only for the tumor epitope on the CEA molecule, then each cycle of immunoabsorption should concentrate these antibodies and a trend of lower CEA I^{50} values and higher NBA I^{50} values would be observed. However, this type of result was not obtained. Only the first cycle of immunoabsorption produced any noticeable increase in CEA specificity, albeit a minor one in some cases, when compared with the corresponding unabsorbed antiserum. Subsequent recycling, though, only served to diminish the increased discriminatory ability of the immunoabsorbed sera.

Thus, if one does postulate the existence of tumor-specific antibody molecules, then the only way these results can be explained is if minute amounts of CEA are present in normal bowel tissue. Thus, the first absorption on the columns would take out some tumor-specific antibodies, but a larger proportion of anti-normal antibodies would be removed, allowing for the increase in the immunoabsorbed antiserum's sensitivity to CEA. However, subsequent recycling would only serve to absorb out those residual CEA-specific antibodies, thereby leaving only anti-normal populations, which would react with NBA and CEA in a similar manner, as seen by the ratios in Table 17 for all column absorptions following the first one.

The other case to consider is that there were no antibodies specific for only the tumor epitope on the CEA molecule. Here, two alternatives can be discussed. The first is the presence of antibodies which, by virtue of the close similarity of the normal and CEA antigenic determinants on the molecule (assuming two distinct ones occur), react with both antigenic sites. They would be distinct antibody populations, but would have similar sensitivities to both CEA and NBA. Thus, the first cycle of immunoabsorption would preferentially remove only those antibody populations directed against the coupled NBA, thus leaving, in the unbound fraction, those populations which were more reactive to the tumor-specific site on the CEA molecule. However, upon the succeeding immunoabsorptions, this tumor-specific population, due to a large decrease in the competitive anti-normal bowel population, would now react with the coupled ligand. Thus, one would see the parallel reactivities, as observed in the RIA, to CEA and NBA with subsequent recycling.

The second alternative is that there are no tumor-specific antibodies present in the unabsorbed sera. Even if CEA does have tumor epitopes, the immunized animal is unable to recognize them; the immunized host would only see the normal antigenic determinants and mount immune reactions solely to those sites. Thus, this alternative would interpret the results seen in this study, as determined by the RIA, to be simply quantitative. Certainly, the majority of the specificities observed on the affinity columns are NBA directed; however, the increases in CEA sensitivity produced by the first cycle on immunoabsorption, although minor, are definite increases and would not totally support this alternative.

Thus, these studies cannot conclusively say whether or not tumor-specific antibodies to CEA exist. The results strongly indicate a very close similarity between NBA and CEA; the immunizing host only barely discerning a difference. The results do demonstrate that the affinity columns are showing a certain degree of specific immunoabsorption, and are not randomly absorbing out antibody populations. In all the immunoabsorptions performed, the first cycle consistently produced antisera whose sensitivity to CEA was increased when compared to their homologous unabsorbed antisera. (See Table 17). However, as seen from the results, extensive immunoabsorption of anti-CEA antiserum with normal material leaves residual antibody populations that do not show any tumor specificity. The conclusion reached is that this similarity is too close for affinity chromatography to achieve a significant amount of anti-CEA antiserum modification that would obviate the use of the present standard absorbed antiserum.

Future Prospectives

At first, anti-CEA reactivity of a serum was defined by the presence of a precipitation line with CEA in agar and was equated with tumor specificity. However, with the advent of more sensitive detection techniques, such as radioimmunoelectrophoresis and radioimmunoassays, the specificity of these antisera has had to be qualified, and the question asked whether or not CEA specificity and tumor specificity are the same. Due to the heterogeneity of both the antigen and the antiserum, this question has not yet been resolved.

The work done in this study had attempted to better define anti-CEA

antiserum by means of affinity chromatography. However, this method of approach does not seem feasible. One approach that is currently being investigated and which may be the only way of obtaining a modified, more tumor-specific antiserum, is the production of monoclonal antibodies, by which one can selectively choose specific antibody populations. Of course, the ultimate determination of the choice of clones would be deduced from their performance in a clinical assay.

Another method of approach, which would also gain further insight into the question of similarity between CEA and normal bowel tissue and the concept of families of CEA-related molecules, is the fragmentation of purified CEA. Systematic, carefully controlled breakdown of the molecule may be able to separate the tumor-specific antigen (if one exists) from the other "normal" determinants. If the problem with the antiserum is the poor immunogenicity of this moiety, then, perhaps, by itself or by conjugation as a hapten, the immunogenicity may increase to the point where a much more tumor-specific xenogeneic serum may be produced.

Both the above approaches lead to another problem currently inhibiting progress in the area of CEA specificity - that of the need for standardization of both the antigen and antiserum preparations. The standards for the antibody may not necessarily be such that they are completely tumor-specific. If one knew exactly what type of antibody population is present in the serum used, then all further determination can be made with respect to that serum. The same type of standardization procedure could be applied to the CEA molecule.

Clinical and research assays vary from lab to lab, thus results from one lab may be deemed different from those obtained in another, but when compared with one specific assay, these results may turn out to be identical. Hence, a reference point for CEA and anti-CEA antiserum is required in order for information coming from different research centres to be meaningful.

STATEMENT OF ORIGINALITY

This thesis was concerned with the modification of anti-CEA antisera, primarily an attempt to increase its tumor specificity, in the radioimmunoassay, to the CEA molecule. The original contributions made during this investigation may now be considered.

1) The use of solid phase immunoabsorption for the modification studies. The matrices used were two types of glass bead. The longchain alkylamine beads used a Schiff's base formation with glutaraldehyde to couple ligand, while the amino aryl glass beads employed diazotization. The glass bead matrices were found to be more stable and chemically more inert, with respect to nonspecific absorption, than agarose beads, using amino-hexyl Sepharose 4B as an example. The ligand, which was normal bowel material partially purified by ion exchange chromatography and, in one case, by an additional step on an Ultragel column, was coupled to the carriers of both glass bead matrices with a high degree of efficiency. The immunoabsorption procedure involved the recycling of unabsorbed anti-CEA antisera on these affinity columns, either numerous times through the same column or only once through successive columns. In each case, the unbound fraction from the affinity columns was analyzed in the radioimmunoassay for its tumor specificity.

2) The demonstration that most of the antibodies in the anti-CEA antisera tested reacted with normal bowel material and were not tumor-specific. The recycling of the antisera produced various degrees of immunoabsorbed sera, each of which was tested in the radioimmunoassay against CEA and normal bowel

material. The ratios of their reactivities served as a measure of the degree of modification achieved by the immunoabsorption technique. In all cases, the ratios showed that most of the antibody populations were directed primarily against the normal bowel antigenic determinants and suggeted tht some of these populations may be recognizing the tumor epitopes on the CEA molecule, but are not specific for them.

3) The work presented in this thesis demonstrated that the method of affinity chromatography is not suitable for the successful modification of the anti-CEA antiserum into one more specific for the determination of tumor epitopes on the CEA molecule. The close similarity of the CEA molecule with the normal bowel materials hinders the specific immunoabsorption of normal antibody populations without also removing those reactive towards CEA.

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