ISOLATION AND CHARACTERIZATION OF ORGAN SPECIFIC NEOANTIGENS FROM THE URINE OF LUNG CANCER PATIENTS

by

© Aaron Fink

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TO MY WIFE

ABSTRACT

Organ specific neoantigen (OSN) is shed from cancer of patients with metastatic lung cancer and is filtered into the urine. The OSN was purified and characterized by physicochemical methods. OSN activity was detected by the blocking of the tube LAI assay. Two different methods are described for isolating the OSN. By both procedures material was isolated that consisted of three to four polypeptide chains in the molecular weight range of 43,000 to 31,000 dalton and that had high OSN activity. Urinary protein isolated from normal subjects by the same methods did not have OSN activity and also lacked the 31,000 mol. wt. protein. Immunoprecipitation studies with specific antisera did not show the presence of β_2 microglobulin or HLA molecules in the OSN material. Furthermore, lung tumor associated antigens such as carcinoembryonic antigen, lactoferrin and α_1 antichymotrypsin were absent from the final isolate.

Material containing the lung tumor OSN is glycosilated and has a pI of 5.0 to 6.0. By two dimensional gel electrophoresis the OSN material was heterogeneous showing many spots from a pH of 6.2 to 7.2 but all had a molecular weight of 31,000 dalton.

RÉSUMÉ

Un néoantigène spécifique à un organe (NSO) est libéré par des cellules cancéreuses de patients porteurs d'un cancer métastatique du poumon et est filtré dans les urines. Le NSO a été isolé et purifié par des méthodes physicochimiques. L'activité du NSO a été décelée par bloquage du test d'inhibition d'adhésion leucocytaire. Deux méthodes différentes ont été décrites pour isoler le NSO. On a pu isoler par ces deux procédures des substances consistant de trois à quatre chaines polypeptidiques d'un poids moléculaire de 31,000 à 43,000 dalton avec haute activité NSO. Des protéines urinaire obtenues de sujets normaux par les mêmes moyens n'avaient pas d'activité NSO, et ne contenaient pas la protéine de poids moléculaire 31,000. Des études d'immunoprécipitation avec des anti-sérums spécifiques n'ont pas démontré la présence de β_2 microglobuline ni de molécules HLA dans le matériel NSO. De plus, les antigènes associés au cancer du poumon tels, l'antigène carcinoembryogénique, la lactoferrine et l'α_1 antichymotrypsine étaient absents du matériel isolé.

Le matériel contenant le NSO du cancer du poumon est glycosilé et a un point isoélectrique de 5.0 à 6.0. Au moyen de l'électrophorèse sur gel à deux dimensions le NSO parait hétérogène, montrant plusieurs taches variant d'un pH de 6.2 à 7.2 mais ayant un poids moléculaire de 31,000 dalton.

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CHAPTER I

INTRODUCTION

Since the turn of the century when Ehrlich speculated that tumors may be recognized as foreign by their host and subsequently rejected, efforts have been made to identify the relationship of the immune response to malignant disease. In its early years the field of tumor immunology was hampered because in the early experiments which used tumor rejection as an assay, the rejection was probably due to incompatibility in the major histocompatibility complex (MHC) rather than the tumor per se. The development by Gorer of syngeneic mice enabled Foley (1) to show that mice immunized with syngeneic chemically induced tumors could reject a graft of the immunizing tumor, while normal adult tissues were accepted as a graft. The antigens responsible for this rejection are commonly referred to as tumor transplantation antigens. In the following years Prehn and Main (4), Baldwin (2), Thomson and Alexander (3) and many others have shown that in experimental models, using mice and rats, several categories of tumor antigens may be found. If the criteria for tumor antigens in rodents is followed, it seems that tumor antigens may be expressed by human neoplasia. The hallmark of a true tumor antigen as shown in the experimental work is that it should not be expressed by normal adult tissues and should be immunogenic in the primary host as detected by in vivo and in vitro assays. This strict definition will apply in this manuscript, although for historical and clinical reasons, tumor association antigens (TAA) which are not immunogenic in the primary host will be discussed in some detail. Although confusion exists in the literature concerning the classification of tumor antigen, some definitions can be made.

1. Tumor specific transplantation antigen (TSTA)

a. These antigens are found in syngeneic rodent tumors induced by chemical and viral carcinogens and differ from antigens found on normal cells.

b. Those antigens induced by chemical carcinogen are unique to the individual tumor and differ from other tumors induced by the same carcinogen.

c. Viral carcinogenesis will induce common TSTA in tumor cells induced by the same virus.

d. TSTA are recognized by their ability to induce tumor rejection in syngeneic animals when immunized with the same tumor.

2. Oncofetal or oncodevelopmental antigens

a. These antigens are found in rodent tumors induced by chemical and viral carcinogens.

b. These antigens are expressed by embryonic and fetal tissue.

c. Oncofetal antigens are immunogenic in the primary host.

3. Organ specific neoantigen (OSN)

a. These antigens may be the human counterpart of oncofetal tumor antigens found in experimentally induced tumors in rodents

b. Human organ specific neoantigens are possibly expressed in embryonic organs.

c. Organ specific neoantigens are immunogenic in the primary host as determined by in vitro assays.

4. Tumor associated antigens or oncodevelopmental products.

a. These molecules are expressed on tumor cells but also on normal cells; the difference is only quantitative.

b. Tumor associated antigens are often found in embryonic tissue.

c. Tumor associated antigens are recognized by immunizing a xenogeneic host, for they are not immunogenic in the primary host.

Nature of Tumor Antigens

a. Chemically induced antigens

Foley (1) demonstrated in 1953 that subcutaneous grafts of 3 methylcholanthrene (MC) induced sarcomata in syngeneic mice which, on ligation of their blood supply, resulted in the development of transplantation immunity to subsequent challenge by the same tumor. The rejection of the MC induced sarcoma in the syngeneic host as demonstrated by Klein et al (5) points to the fact that alien tumor constituents are present. These foreign constituents are termed tumor specific transplantation antigen (TSTA). TSTA have been shown to be specific and characteristic of cells transformed by chemical carcinogens and are found on the outer cell surface. Two major systems are used for tumor induction.

1) 3-methylcholanthrene (MC)

Historically, sarcomata in mice induced by MC were the first to be examined. It is generally accepted that many MC induced sarcomata are highly immunogenic, which is probably acquired during neoplastic transformation.

One of the most interesting features of the TSTA of the MC sarcoma is that they are distinctive components of an individual tumor even within different tumors arising in the same animal, so that immunization against one tumor does not confer resistance against similar tumors induced by the same carcinogen (36). The heterogeneity of tumor antigens induced by MC was recently studied by Pimm et al (6), who showed that sublines established from opposite poles of a primary sarcoma induced by MC in WAb/Nst rats were antigenically distinct, indicating that primary tumors comprise heterogeneous populations of cells. Moreover, heterogeneity may also be produced in tumor cells clonally derived as shown by Basombrio and Prehn (7). These facts may suggest that the carcinogen induces a mutation in the cell's DNA which product is expressed on the cell membrane (2).

2) 4-dimethylaminobenzene (DAB)

In the rat this carcinogen produces hepatomata. The hepatoma associated antigens are also individually distinct as indicated by the specificity of the resistance elicited against transplanted hepatoma cells in syngeneic recipients (2). This was further emphasized by Ishidate (44) showing that four distinct hepatoma nodules arising in a single rat were antigenically distinct. The individual specificity of different DAB induced hepatoma was further demonstrated by Baldwin et al (46) by comparing the capacity of the intact tumor to absorb antibody from tumor immune serum. The same authors further showed that serum from tumor immune rats was cytotoxic for cells of the immunizing tumor when tested in complement dependent inhibition of colony formation of cultured hepatoma cells. Baldwin and

Embelton (85) also showed the existance of cell mediated immunity specific for the individual hepatoma induced by DAB. When lymphocytes from lymph nodes of immunized animals were tested for colony inhibition, only cells of the immunizing hepatoma were inhibited, while the same lymphocytes were ineffective for cells of other hepatomas. It wasconcluded that tumor antigens associated with DAB induced rat hepatomata are individual membrane components.

Although for the last decade tumor rejection antigens of chemically induced tumors have been generally considered to be individually specific, this doctrine has been challenged. Leffel and Coggin (8) have demonstrated that previously described unique TSTA of two MC induced fibrosarcomata in balb/c mice were in fact mutually cross-reactive. The observed cross-reaction was dependent on the method of prior immunization and the dose of secondary challenge. Specificity was obtained only in experiments in which initial immunization was achieved by excision of the tumor followed by rechallenge with either the homologous or reciprocal tumor line. Cross-protection (cross-reactivity) was observed at low doses of secondary challenge while high dose conferred specificity. Moreover, mice immunized with irradiated tumor cells and then challenged with live tumor cells showed in all cases significant cross-protection between the two sarcoma lines. Jamasbi and Netteshein (9) demonstrated that in five different rat squamous cell carcinomata of the respiratory tract induced by DMBA and MC, in vivo cross-protection can be induced with 4 out of 5 carcinomas. This suggests

that these tumors have a common TSTA. In vitro cytotoxicity with sera from tumor immune hosts showed cross-reactivity among 3 of the carcinomata studied.

One of the possible interpretations of cross-reactivity between different chemically induced tumors is that the carcinogen activates endogenous RNA virus whose proteins are expressed on the cell surface. Brawn et al (10) in MC induced fibrosarcomata in balb/c mice have shown that the viral protein gP70 is detected at the cell surface of most sarcomata studied. Tumors which expressed gP70 induced the formation of antibodies specific for proteins gP70 and p15 of the murine leukemic virus (MULV), while tumors lacking gP70 did not. Likewise, Zbar et al (11) showed that cross-reactivity inbetween two MC induced tumors is due to endogeneous infection with MULV. Since uninfected allogeneic (to the MC induced sarcoma) embryo cells did not release infective virus or gP70, mice immunized with these cells did not inhibit tumor growth in vitro. On the other hand uninfected syngeneic (to the MC induced sarcoma) embryo cells release only gP70 into supernatant fluids, and mice immunized with these cells were immune to tumor growth in two of seven experiments. Virus infected syngeneic and allogeneic cells release both viable viruses and gP70; in mice immunized with these cells, tumor growth is inhibited in 100% of experiments. These results indicate that antigen coded for by endogenous MULV may function as common TSTA on chemically induced murine fibrosarcoma.

Physiochemical properties of chemically induced TSTA

Tumor specific transplantation antigens have been isolated and partially characterized. Natori et al (12) solubilized membranes of MC induced sarcomata in the mouse with detergent and, after several purification steps on gel filtration, ion exchange and affinity chromatography determined the molecular weight of the TSTA on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) to be 70,000 dalton with an electrophoretic mobility of γ globulin. On the other hand, Thomson and Alexander (3), analyzing plasma membrane of a MC induced sarcoma of the rat, demonstrated that, after antigen extraction and molecular sieve chromatography, the molecular weight of the TSTA was of 40,000-50,000 dalton. Likewise Bowen and Baldwin (13) isolated TSTA from the serum from rats bearing hepatoma: serum was fractionated by gel filtration and affinity chromatography by using syngeneic rat hepatoma IgG. The purified TSTA had a molecular weight of 55,000 and an isoelectric point of 4.6.

Another approach that was employed by Thomson et al (14) was limited protein digestion of membrane protein from the MC induced sarcoma in male hooded rats. Solubilized membrane proteins were fractionated on ion exchange, molecular sieve chromatography, and affinity chromatography using IgG fraction of syngeneic rats hyperimmunized to MC-1 sarcoma. TSTA activity was monitored by inhibition of membrane immunofluorescence. Maximal TSTA activity was found to reside in the molecular weight range of 40-60,000 daltons. These proteins, on reduction and SDS-PAGE separation, were found to be 3 polypeptide chains with molecular weights of 12,000, 18,000 and 25,000 daltons. The major peak had an apparent molecular weight of 12,000 daltons and an electrophoretic mobility similar to that of β_2 microglobulin.

b. Antigens induced by viruses

In contrast to chemically induced tumors, murine virally induced tumors in experimental animals share the same tumor antigen for all neoplasms induced by the same virus in the same host strain (15,16).

1) Antigens induced by DNA viruses

The most intensively studied DNA viruses for inducing tumors are the simian virus-40 (SV-40), polyoma adenovirus and herpes virus. DNA viruses can either infect or transform the cell. In the case of lytic infection the virus directs the cells biosynthetic processes to reproduce new virions which eventually lead to cell lysis and death. In the case of transformation, the viral DNA is incorporated into the hosts genome, inducing changes in growth properties and the appearance of virally induced tumor antigens on the cell surface. The best studied virus in this group is SV-40 and it will serve as an example of the role of DNA viruses in tumorgenesis.

SV-40 infection causes the expression of viral genome in two phases. In the first phase, T antigen is produced and is expressed in the nucleus. In the second phase, which only appears in lytic infection, there is the appearance of structural proteins followed by cell lysis and death. Cells from many species transformed by SV-40 not only express the nuclear T antigen but also the following cell surface proteins: 1) TSTA that do not cross-react with similar antigens from cells transformed by chemicals or other viruses (17); 2) oncofetal antigens that cross-react with embryonic tissue of up to 11 days gestation. The cross-reacting embryonic tissue can

confer transplantation resistance in hamsters against a challenge with SV-40 transformed hamster sarcoma cells (18).

Cell surface expression of SV-40 TSTA coincides with nuclear expression of T antigen and occurs not only in transformed cells but also cells infected with the lytic form of virus (19). In view of this it was proposed that T antigen and TSTA are related, and possibly a posttranscriptional modification of T antigen leads to the appearance on the cell surface of a protein that functions as TSTA (20).

Rogers and Law (21) describe a method for isolating TSTA of SV-40 induced sarcoma by injecting the isolate into mice and monitoring for transplantation immunity to the sarcoma in the mice. Cells were disrupted, differentially centrifuged and chromatographed, the fraction having TSTA activity had an apparent molecular weight of 70,000-100,000 daltons.

2) Tumor antigens induced by RNA viruses

Transforming RNA viruses act differently from DNA tumor viruses in that infection with RNA virus usually does not lead to cell lysis but to transformation. RNA viruses are in general retroviruses since they contain reverse transcriptase. Not all viruses which possess this enzyme are oncogenic. The genome of a typical retrovirus contains 1) SARC gene which is comparable to a gene in the DNA virus in that it is required for transformation; 2) gag and env genes which code for cell surface proteins which make up group specific antigens of the virus. The most extensively studied RNA virus is the murine leukemia virus (MULV), and as such will serve as an

example for this group. Genetic hybridization has demonstrated the presence of MULV genome in mice and may be transmitted vertically and horizontally. Furthermore, viral antigens may be expressed in spontaneous and chemically induced tumors in rodents as demonstrated by Aoki et al (22) who also found that this expression is dependent on the age and strain of the animal.

As the viral products on the cell surface are highly immunogenic, antibodies against viral determinants are readily produced. The antigen on the cell surface to which these antibodies are directed is the gP70. Recently Elder et al (23) compared the tryptic maps of 27 virion associated gP70 molecules, and gP70 isolated from sera and seminal fluid on nine different strains of mice. It was found that C type viruses of murine origin can be divided into several groups, each group manifesting some degree of polymorphism. The free gP70 from the serum of all mice is conserved pointing to the fact that this molecule may be a product of the same viral genome, regardless how many other proviruses the mouse harbors. Interestingly, the gP70 from seminal fluid is different structurally from its counterpart found in the serum, perhaps indicating that unique provirus may be expressed at different sites. The data available today enables us to question the presence of true TSTA on tumor cells produced by RNA viruses. Indeed Hogg (24) using the [^{125}I] antiglobulin binding assay showed that Moloney virus completely blocked the binding of mouse and rat sera to virus shedding target cells, thus suggesting that mice and rats recognize viral determinants. When gP70, P30, P15, were prepared using guanidine HCl method, they were able to block the binding of the rat anti-Moloney immune serum to Moloney virus shedding target cells. It was demonstrated that the rat serum

contained specificities for viral protein gP70 and P30, but no antibody to TSTA was demonstrated. Moreover, Rogers et al (25) using these virion proteins failed to induce transplantation resistance to Friend and Raucher induced leukemia cells.

From the above it seems that true TSTA in RNA induced tumors probably do not exist, or if present, their immunogenicity may be too weak in the primary host to detect a response with the present techniques, especially when a massive response is generated towards virally induced proteins.

Oncofetal Antigens

Tumors may express on their cell surface proteins which are normally detectable only in embryos and fetuses (26). It is believed this reappearance of fetal proteins is the consequence of gene derepression (24) (28).

These antigens have been observed to be cross-reactive with antigens present on chemically and virally induced tumors but are distinct from TSTA induced by the chemical carcinogen or the virus. This fact was demonstrated by Thomson and Alexander (3) who analyzed the constituents of plasma membrane of a methylcholanthrene induced sarcoma (MC-I) in the rat. The authors described four tumor associated macromolecules. One was the TSTA found only in MC induced sarcoma and not on embryonic tissue. This antigen was immunogenic in the primary host. Three other antigens, of which only one was immunogenic in the primary host, were found to be cross reactive with embryonic tissue and with many rat sarcomata, thus fulfilling the criteria of oncofetal antigens.

The demonstration that multiparous pregnant rodents mount cellular and humoral immune response to fetal constituents which in turn cross-react with malignant tissue lends support to the existence of oncofetal antigens. Steel and Syogren (38) demonstrated that peripheral blood lymphocytes of multiparous pregnant Wistar/Furth rats were cytotoxic to plated cells derived from chemically induced (N-methyl-N-nitro-N-nitroso- guanidine) rat colon adenocarcinoma as well as fetal kidney, liver, and colon cells, but not to normal adult rat colon mucosal cells. Conversely, blood lymphocytes from rats bearing colon carcinoma induced by nitroso-guanidine and 1,2 dimethyl-hydrazine were cytotoxic to fetal colon but not to fetal liver, fetal kidney or normal adult rat colon mucosa cells. Cytotoxicity of lymphocytes from colon carcinoma bearing rats was blocked by sera of multiparous pregnant rats as well as by sera from carcinoma bearing rats when fetal colon or colon carcinoma cells were used as targets. Blocking activity could be absorbed by incubating multiparous rat sera with colon carcinoma and fetal gut cells but not by polyoma virus induced sarcoma, normal colon mucosa, fetal kidney or whole embryo cells. Blocking activity was recovered from colon carcinoma cells and fetal gut cells by elution at low pH.

More recently Coggin et al (31) have shown that in two MC induced balb/c sarcomata, oncofetal antigens are recognized by cytotoxic lymphocytes derived from pregnant mice. A similar fetal antigen determinant was observed on two DMBA induced sarcomata of hamsters, which were likewise recognized by maternal cytotoxic lymphocytes.

Using 4-dimethylaminoazo benzene (DAB) induced hepatoma and 3 Methylcholanthrene induced sarcomata in syngeneic rats, Baldwin et al (29) have presented similar data, and have shown that embryonic antigens are present on the cell surface by their reactivity in membrane immunofluorescence tests with sera from multiparous rats. The same sera also showed complement-dependent cytotoxicity for target tumor cells, and lymph node cells from multiparous rats when compared to lymphocytes from virgin age matched rats were also cytotoxic. The embryonic antigens could be demonstrated on embryonic cells but not on adult cultured rat liver cells or lung fibroblasts. Since these embryonic antigens were common to both tumor types, they differ from the individually distinct TSTA. In other experiments Baldwin and Embelton (30) showed that in spontaneous mammary carcinoma of the rat, tumor bearer lymph node cells were cytotoxic for spontaneously arising and chemically induced tumors. This cytotoxicity was blocked by pretreatment with serum of tumor bearing rats and also with sera of multiparous rats. Conversely tumor bearer sera were able to block sensitized lymph node cells from multiparous rats from attacking embryo cells. This not only suggests the presence of oncofetal neoantigens but also their immunogeneicity in the primary host. Brawn (32) showed that lymphocytes from lymph node of pregnant balb/c mice inhibited the in vitro colony formation of primary syngeneic MC induced sarcoma, while lymph node cells from matched virgin females did not. Parker and Rosenberg (33) demonstrated by the microcytoxicity assay that cross-reactivity among three MC induced sarcomas in C57 Bl/6N female mice is probably due to a common fetal antigen, which is distinct from the individual TSTA. However, in experiments of in vivo challenge with the tumors, cross-reactivity was not exhibited, so it seems to be that the fetal antigen may not provide for in vivo immune protection.

On the other hand Hedlund and Sjogren (34) have succeeded in inducing transplantation immunity to chemically induced rat colon carcinoma by immunizing with fetal colon. Likewise, Lemevel and Wells (35) showed that if B57/B16N mice were immunized with syngeneic fetal tissue, they generated antibodies to MC induced tumor cells from the same mice.

Both simian virus-40 (SV-40) induced viral oncogenesis and growth of transplantated SV-40 transformed tumor cells can be prevented in hamsters by prior immunization with intact fetal cells. The fetal determinant responsible for inducing transplantation resistance is detectable by this technique up to the 11th gestational day but not thereafter. Crude plasma membranes from 10 to 14 days old embryos are capable of inducing protection against challenge with SV40 transformed hamster sarcoma cells. In addition, spleen cells from adult hamsters immunized against SV40 surface antigen will undergo blastic transformation on exposure to homologous tumor cells and to 10 and 14 day embryonic cells (18).

Some generalizations can be made from the data presented:

1) Oncofetal antigens exist as separate entities in chemically and virally induced tumors in rodents.

2) Oncofetal antigens differ from TSTA.

3) Oncofetal antigens are not usually specific for any given tumor but are shared by tumors induced by different methods.

4) There is no evidence as to whether experimental oncofetal antigens are organ-specific.

5) Specific immunity to oncofetal antigens may be generated by immunizing adults with fetal tissue or by pregnancy.

Tumor associated antigen - Oncodevelopmental products

It has been frequently noted that cancer tissue often closely resembles fetal or embryonic tissue on histological examination. The morphological resemblance of cancer tissue of adults to non-malignant but less differentiated tissue has been extended to biochemical and immunological similarities. An increasing number of tumor products which resemble fetal or developmental pfoducts have been described. These oncodevelopmental products are not true tumor antigens since there is no evidence that they are immunogenic in the primary host. Moreover, all are recognized by immunizing xenogeneic animals. However, these products do serve as markers of tumor presence and growth. The two most widely recognized oncodevelopmental products are carcinoembryonic antigen (CEA) and Alpha-feto Protein (AFP).

Carcinoembryonic Antigen (CEA)

CEA was first identified in colonic tumor tissue by means of antisera (raised in rabbits) against colonic tumor. The antiserum contained antibodies specific to colon carcinoma after extensive adsorption with normal colon tissue (39). When described by Gold et al in 1965 it appeared to be specific for adenocarcinomas derived from the digestive tract. However, after a radioimmunoassay was devised by Thomson et al (40), it was found that elevated CEA levels were also identified in other types of tumors, such as breast, lung, and ovarian carcinomas.

The CEA molecule is a glycoprotein having a molecular weight of 200,000 dalton. The carbohydrate content accounts for 40-70% of its weight. The molecule is intimately associated with the cell glycocalyx. The CEA

molecule has a β-electrophoretic mobility at pH 7-8.4 (41) and an isoelectric point of 6.8.

The biological significance of the CEA molecule is not well understood, but its prevalence in various cancers enables it to be used for the monitoring of patients for cancer recurrence.

Alpha-feto Protein (AFP)

Alpha-feto protein is produced by the liver. It is a normal serum constituent during fetal life but disappears rapidly within two weeks post partum (42).

This protein has a molecular weight of 70,000 daltons and a carbohydrate content of 40% (42). Its sequence suggests homology with serum albumin (43). The biological function of AFP is not understood. It may be the fetal counterpart to albumin and have the same functions. However, AFP has been described by Murgita and Tomasi to have some inhibitory effect on immune function, in vitro (45). This was disclosed by monitoring primary and secondary immune responses in the presence of AFP (purified and derived from amniotic fluid) using the plaque forming assay and sheep red blood cells (SRBC) as antigen. Other functions such as protection of the fetus from maternal estrogen and as an organizational signal for liver lobule formation is still debatable (42). As primary hepatocellular carcinoma and regenerative processes in the liver produce high levels of AFP, measurements of AFP in these diseases can diagnose and monitor these conditions.

Organ specific neoantigens of human cancer (OSN)

The development of neoplasia is gradual and involves alteration of the normal phenotype during preneoplastic phases. The cancer phenotype is usually associated with some loss of specialized function along with the appearance of proteins that are usually present at earlier stage of development. This notion as presented by Potter (26) explains the phenomenon that certain human cancers have the phenotypic expression of embryonal or fetal cells. Potter hypothesized that the neoplastic transformation could be looked upon as "blocked ontogeny". Organ specific neoantigens (OSN) are probably expressed by stem cells of the developing organs, but not by the adult organ. When cells become neoplastic they once more reexpress the OSN as though they are blocked at the point were OSN are usually expressed. Evidence for the presence of organ specific neoantigens (OSN) of human cancer is based upon certain premises. 1) Immunogenicity in the primary host as disclosed by in vitro and in vivo assays; 2) Specificity of the immune response evoked by a particular tumor's OSN; 3) Demonstration that the corresponding fetal organ (of a particular type of neoplasm) is recognized by the cancer bearer's immune system.

Experimental results that support these premises will be described.

1) Delayed type skin hypersensitivity

Hebermann (49) tested cancer patients with allogeneic as well as autologous tumor antigen by means of delayed type skin hypersensitivity. Soluble tumor antigens were prepared from tumor cell membranes by low

frequency sonication followed by separation by molecular sieve chromatography, ion exchange chromatography and by gradient polyacrylamide electrophoresis. Tumor antigens were obtained from surgical specimens of lung and colon carcinoma and from the corresponding fetal organs. When antigens were injected intradermally in the appropriate patients, all these antigens elicited positive delayed type hypersensitivity responses while comparable preparation from normal tissue gave negative reactions. Positive reactions persisted after elimination of carcinoembryonic antigen by gel electrophoresis.

2) Cell mediated cytotoxicity

Vose and Moore (48) using a cytotoxicity assay exposed cells from various organs from 13 week old fetuses to PBL from healthy donors, cancer patients, (malignant melanoma, lung carcinoma, colon carcinoma, bladder carcinoma) and patients with non-malignant chest conditions. Peripheral blood leukocytes from patients with cancer, when reacted with fetal organs, showed greater cytotoxic potential compared to leukocytes from patients with no malignant conditions. Interestingly, cytotoxicity did not reveal selectivity with regard to fetal target cell derivation.

3) Leukocyte migration inhibition assay (LMI)

Zoller et al (89) used the LMI assay to demonstrate that leukocytes from cancer patients are sensitized to fetal tissue of 10-22 weeks gestation: whole fetal antigen and antigen derived from colon, lung and gastric carcinomata (extracted with 3M KCl). In 50% of the tests, leukocytes

derived from colorectal, lung and gastric cancer demonstrate migration inhibition in the presence of whole fetal antigen while normal donors' leukocytes did not. In 81%, 67.4% and 69% of the tests, leukocytes from gastric colorectal and lung cancer patients respectively demonstrated migration inhibition when exposed to the respective antigens. In only 2% to 5% of the tests, did leukocytes of normal donors disclose migration inhibition. Specificity of these reactions was not clear because in 50% of the tests leukocytes from cancer patients (breast, thyroid, kidney, brain) showed leukocyte migration inhibition in the presence of an unrelated tumor antigen extracts.

4) Blastogenic response of PBL from cancer patients

Levine et al (130) studied the blastogenic response to an ovarian cancer crude cell extract in 48 patients with ovarian cancer. The controls were 26 healthy females matched for age and 18 female patients with other types of cancer in remission from the disease. The blastogenic response to fetal ovary, lung, and liver and normal adult ovary were also assessed in all 3 groups. The blastogenic response to ovarian cancer and fetal ovary were found to be significantly greater in ovarian cancer patients than in controls, but not in the group having other types of cancer in remission. A few ovarian cancer patients responded to adult ovarian tissue, but the overall reactivity of PBL from the ovarian cancer patient group was not detected when exposed to normal ovarian tissue.

5) Leukocyte adherance inhibition (LAI)

Based on many experiments and large numbers of cancer patients (colon, breast, melanoma, pancreas) as well as patients studied with out cancer in Thomson's laboratory (63) (65) (66) (67) (68) a consistant pattern emerges: 1) The OSN is recognized by allogeneic or authochtonous leukocytes from patients with the same cancer; 2) antitumor immunity is present in most patients with early cancer, while in disseminated disease antitumor immunity is seldom detected. 3) the immune response, evoked towards the OSN of the tumor, is specific and does not exist to normal tissue of the same organ; 4) the organ specific neoantigens are probably fetal antigens as 13 to 19 week old fetal organs are able to substitute for tumor extracts and to generate positive LAI responses.

Leukocyte Adherence Inhibition (LAI)

a. General

Evidence for the presence of neoantigens in human tumors is based on data derived from different *in vitro* tests. Difficulties have been encountered with various methods for measuring *in vitro* cytotoxicity of peripheral blood leukocytes from cancer patients (53) (47) (48) (55), and as a result, other tests have been devised to measure cellular immunity: cutaneous delayed *in vivo* hypersensitivity to cancer tissue extracts (56) (54) (83), leukocyte stimulation by tumor cells or tumor extracts (57) (58), migration inhibition of leukocytes by tumor extracts (59) (60) (61), (62) or inhibition of adherance of leukocytes to glass by tumor extracts (51).

The most promising *in vitro* assay is leukocyte adherance inhibition (LAI). The assay was described originally by Halliday and Miller (51) and was later modified for test tubes and for automation by computerized image analysis (63) (50). The LAI assay is based on the phenomenon of tumor antigen induced leukocyte adherance inhibition. Leukocytes derived from patients bearing cancers, after being incubated *in vitro* with extracts of cancer tissue arising from the same organ and of the same histogenesis, lose their ability to adhere to glass.

Since the original description, the validity of the assay has been strengthened by numerous studies done in Thomson's laboratory and elsewhere (64) (52) (51) (65) (66) (134). The specificity and sensitivity of the tube LAI assay was studied with coded samples of PBL from patients with breast

carcinoma of stages I, II, and III (67) (65); positive tests were found in 83%, 72% and 29% of patients, respectively. In stage IV, only 15% were positive. Of control subjects only three percent were positive. Tumor specific immunity to carcinoma of the colon, pancreas and lung was also studied (63) (66) (68). Of 1,292 control subjects with benign disorders or other malignancies, one percent had a positive test. Of patients with inflammatory conditions of colon, pancreas and lung three percent were positive. By contrast, of 351 patients with cancer of the above mentioned organs, 80% were positive. Antitumor immunity in malignant melanoma patients (69) was also measured by means of the tube LAI assay. Of the patients with malignant melanoma, 80% responded to extracts of malignant melanoma while of 475 control subjects less than five percent had positive tests.

In the above studies it was noted that the antitumor immune response to malignant melanoma and colon antigens was reduced with increasing tumor burden, and patients with advanced cancers seldom exhibited LAI reactivity (70) (63). The diminished LAI response appears to be the result of free tumor antigen released from the tumors.

b. Blocking the LAI assay

In early experiments, Thomson et al speculated that free, excess tumor antigen in the serum of advanced cancer patients blocked the host's immune response. It was postulated that the excess of tumor antigen is responsible for the abrogation of the LAI response. In subsequent experiments, incubation in vitro of known positive LAI cells with excess tumor antigen caused

the cells to become unresponsive to subsequent challenge with the antigen, thus mimicking the in vivo situation of advanced cancer where LAI positive leukocytes revert to LAI negative with increased tumor burden. This observation lead to the development of the blocking LAI assay. The blocking assay is currently used to monitor the isolation of tumor antigen. The presence of tumor antigen is recognized by the ability of an isolate to block specifically the positive response of peripheral blood leukocytes (PBL) previously active in the LAI assay.

The blocking of a positive response was proven to be immunologically specific because blocking of leukocytes' positive LAI response is achieved only when leukocytes are incubated with isolates derived from patients with cancer of the same organ as the leukocyte donor. Besides its immunologic specificity, the blocking assay has the advantage that it is possible to use very small quantities of isolates, and the minimum quantity of protein in each sample that is able to block the LAI response can be determined, thus allowing the enrichment and specific activity of an isolate to be calculated.

c. Mechanisms mediating the LAI response

The reactive cell in the LAI assay is the monocyte (70). The mechanism whereby the specific tumor antigen is recognized is by cytophilic antitumor antibodies which are bound to Fc receptors on the cell surface of the monocyte (70). Normal peripheral blood monocytes can be "armed" by incubation with serum or IgG from a reactive cancer patient, as these monocytes were found to be active when tested in the LAI assay. It is believed that when antigen is bound, there is cross-linking of cell bound IgG molecules

with bridging of IgG-Fc receptors. This phenomenon probably initiates physical and chemical changes in the membrane such as methylation of membrane phospholipids with subsequent translocation of these lipids from the inside to the outside of the membrane (71). This in turn leads to Ca^{++} influx, release of arachidonic acid, lysophosphaticylcholine and prostaglandins. These biochemical changes facilitate transmission of signals by the influx of Ca^{2+} and the generation of cyclic AMP. New experimental evidence has been obtained demonstrating that mediators are produced by the monocytes secondary to antigen binding and these mediators are responsible for the LAI phenomenon. Indeed, in Thomson's laboratory it was found that supernatants from monocytes that were armed by positive LAI serum and exposed to tumor antigen inhibited the glass adherence of normal leukocytes, whereas appropriate control supernatants had no effect. The link between the stimulus and the physiologic response seems to be Ca^{++} influx. In a mammalian cell Ca^{++} concentration in a steady state is in the ranges from 10^{-8} to 10^{-7}M, and cell stimulation will increase the influx of Ca^{++} or release from the cell membrane. Increase of Ca^{++} to 10^{-6}M in the cytosol will activate Ca^{++} binding proteins such as calmodulin to form a complex with Ca^{++}. This complex in turn with effector proteins will trigger biochemical reactions (72) which result in physiological effects. Calcium ionophore increases the permeability of the cell membrane to Ca^{++}. When normal leukocytes are incubated with calcium ionophore, they will exhibit enhanced nonadherance while positive LAI leukocytes lose their reactivity to the sensitizing tumor antigen. Ouabain, which increases intracellular calcium concentration by inhibiting sodium potassium ATPase, was also shown

to enhance the non-adherance properties of normal leukocytes and to negate the positive LAI response. Conversely blocking Ca^{++} influx into the cell by using cromolyn sodium, lidocaine and nifedipine was shown to nullify in a dose response fashion the reactivity of positive LAI leukocytes to the sensitizing tumor antigen, but no effect was noted on the glass adherance properties of normal leukocytes. These experiments demonstrate that increase of intracellular concentration of Ca^{++} triggers changes which are manifested by a loss of the leukocytes' glass adherance properties.

It was noted that cancer patients with previously positive tests converted to negative tests for up to two weeks after surgery (69). Experimental evidence suggested that cortisol produced because of stress at surgery was responsible for this change in LAI response. Cortisol added to the tube LAI assay had a biphasic effect: at a concentration two-fold above physiological levels sensitization of PBL by cytophilic antibodies was inhibited while at higher levels the reactive cells' response with tumor antigen was inhibited even if the cells were armed (73). From this latter experiment it was evident that high cortisol levels act directly on the monocytes and prevent their activation. Steroids are anti-inflamatory drugs in part at least because of their ability to inhibit membrane bound phospholipase A_2 of the leukocytes and to prevent the conversion of membrane phospholipids into free arachidonic acid which is the substrate for prostaglandin (PG) and leukotrienes synthesis. Since the monocyte is the cell whose membrane contributes mostly to PG production, it was felt that cortisol may act at this level. The first link between PG synthesis and LAI reactivity was proposed by Faeby and Appelboom (81) who studied the LAI response in rheumatoid arthritis. These authors noted that the LAI response

is inhibited by indomethacin, an inhibitor of the cyclooxygenase pathway of PG synthesis. But if leukocytes were incubated with both prostaglandin E_2 (PGE_2) and indomethacin, they retained their reactivity. Similarly, when PGE_2 was incubated with leukocytes from cancer patients with negative tests (either after surgery or with high tumor burden), it was found to convert their response to positive. The response to PGE_2 was dose-dependent, effects were undetected below 10^{-6}M and maximal between 10^{-6}M and 10^{-5}M, and declined above 10^{-5}M (133).

PGE_2 stimulates adenyl cyclase to raise intracellular cyclic AMP. In fact cyclic AMP levels were elevated immediately after PGE_2 incubation. Substances which elevate intracellular cyclic AMP such as dibutyryl cyclic AMP, or inhibit its degradation like aminophylline also increases the LAI response. Phospholipase A_2 is the enzyme which provides endogenous arachidonic acid which serves as a substrate for cyclooxygenase and lipoxygenase metabolic pathways. Blocking phospholipase A_2 (as is the case with steroids) negates the LAI response while blocking the cyclooxygenase by indomethacin has no effect on the LAI response. The later metabolic pathway does not mediate the response but it seems to have an important role in modulating the LAI response. The products from the 5-lipoxygenase pathway are responsible for mediating the LAI phenomenon. Nordihydroguairetic acid (NDGA) is an inhibitor of 5-lypoxygenase pathway and it negates the positive LAI response in a dose response fashion (133). Leuktrienes C and D, which are equivalent to the slow reacting substances of anaphylaxis, are products of 5-lypoxygenase pathway. FPL 5512 is a specific inhibitor of leukotrienes C and D. Incubation of LAI positive PBL with FPL 5512 inhibited in dose response fashion their response.

The pattern that emerges is as follows: Ca^{++} ion and cyclic nucleotides serve as second messengers to the triggering event initiated by bridging of the Fc receptors. Ca^{++} complexed to calmodulin probably activates phospholipase A_2 (72) which in turn releases arachidonic acid from cell membrane phospholipids. The arachidonic acid is metabolized along two pathways leading to prostaglandin and leukotrienes, the first modulates the LAI phenomenon and the latter mediates it. This system seems to be governed by feed back mechanisms which operate through cyclic nucleotides perhaps by regulating the Ca^{++} ion flux and its accumulation in the endoplasmic reticulum. These results have led to a better understanding of why PBL from advanced cancer patients show low or no reactivity. Excess tumor antigen in the circulation repeatedly activates the monocytes which are not able to be reactivated as cyclic nucleotides are not generated at the same rate. Addition of PGE_2 or aminophylline will raise cyclic nucleotide levels and restore the cells capacity to react. Likewise leukocytes from cancer bearing patients immediately after surgery are not able to respond in the LAI assay as prostaglandin synthesis is blocked by cortisol and hence cyclic nucleotides are not formed in sufficient amount to modulate the LAI activity.

Release and fate of tumor antigen

Circulating soluble TSTA have been demonstrated in chemically induced rat sarcoma by Thomson et al (74). The levels of circulating TSTA were elevated immediately after tumor cell injection probably as a result of cell autolysis; subsequently the levels of TSTA dropped only to rise again between 10 and 16 days after injection. This second elevation of TSTA level was attributed to a specific immunological attack on tumor cells and shedding of the TSTA. Evidence for the immune mechanism for TSTA shedding was suggested by Thomson et al (75) because rats X-irradiated before MC-1 tumor implantation had less tumor antigen in their sera after 10-12 days. A similar pattern of fluctuation of the concentration of TSTA in serum was found in DBA/2 mice bearing spontaneous SL2 lymphomas (76).

Shedding of TSTA from the tumor cell surface also occurs spontaneously as shown by Davey et al (77) in the DBA/2 murine M-lymphoma. These authors have found that metastizing mouse lymphoma cells spontaneously shed H-2 antigens that are readily detectable in soluble form in sera of tumor bearing mice.

TL antigen is a cell surface antigen that is detectable on certain mouse leukemia cells and on normal thymocytes in certain mouse strains. It was shown that TL antigen present on the cell surface disappears following passive immunization of the TL positive host with anti-TL antiserum or following in vitro incubation of TL positive cells with the respective antiserum (78) (79). To describe this phenomenon, Old and Boyse coined the term "antigenic modulation". Antigenic modulation is reversible in the in vitro and in vivo experiments (80) after placing the cells in medium which is free of the respective antibody. The cellular process that operates during

modulation is energy dependent. After change of their spacial configuration by binding antibody, the antigen disappears through internalization by pinocytosis.

Tumor antigen, after being shed from the tumor cell surface, finds its way into the lymph and the systemic circulation (75,76). Some of the protein or proteins which constitute the tumor antigens are filtered through the kidney and find their way into the urine. The possibility that antigens from tumors originating outside the urinary tract might be found in the urine is well documented. Jehn et al (82) reported that a melanoma patient filtered melanoma associated antigen into the urine since this protein, when incubated with lymphocytes of the same patient and six other melanoma patients, caused blastic transformation of the homologous and autologous lymphocytes. This urinary protein was similar to an extract from malignant melanoma, when compared for biologic activity and immunochemical identity by gel diffusion using rabbit antiserum. Using the leukocyte adherance inhibition assay (LAI), Lopez et al (84) were able to detect OSN of breast cancer both in serum and in urine of patients with metastatic cancer. Using serum from a source autologous with the source of urine, Gupta et al (86) and Rote et al (87) demonstrated in the complement fixation assay that tumor antigen is present in urine of patients bearing solid tumors. The same authors found that the amount of tumor antigen in the urine is dependent upon tumor mass, and after surgical excision the amount of tumor antigen in the urine decreased. From the evidence presented, it seems that the quantity of free tumor antigens which circulates in serum and is eventually filtered in the urine is dependent on tumor mass. The shedding of tumor antigen is probably important because of its ability to alter the host's immune

response by combining with specific receptors on the surface of the immune cells or with antitumor antibody. Shed tumor antigens may provide a mechanism by which the tumor escapes the host's immune rejection.

Hellstrom et al (88) claimed that serum from a tumor bearing host blocked the in vitro cytotoxicity of the hosts' lymphocytes for the authochtonous tumor cells. They suggested that the blocking factor was an antibody since serum from mice with growing Moloney sarcomas but not from mice with spontaneous mammary carcinomas or MC induced sarcoma abrogated the inhibitory effect of lymph node cells derived from mice who spontaneously recovered from the Moloney-induced tumor. This hypothesis was seriously challenged by Baldwin et al (90), for they demonstrated with the rat hepatoma model that levels of cytotoxic antibody rose in the serum after tumor excision and correlated with the loss of serum blocking activity of the cytotoxic effect produced by immune lymph node cells. On the other hand, serum from rats repeatedly immunized to hepatoma blocked lymph node cell mediated reactions in vitro despite the immune status of the donor. Similarly, Sjogren et al (91) demonstrated that sera from mice carrying sarcoma induced by either Moloney virus or methylcholanthrene blocks the cytotoxic effect of lymphocytes immune to the tumor specific antigen of the respective neoplasm. When the serum was fractionated by adsorption onto tumor cells and recovered by elution with low pH, it was shown to contain high and low molecular weight fractions. When the high molecular weight fraction was incubated with the target tumor cells or lymphocytes, there was no blocking. But after 1:1 admixture of both the high and low molecular weight fraction, there was blocking activity. These experiments suggested that antigen-antibody complexes and free antigen possess blocking activity. Subsequently, Baldwin et al (92) drew the distinction between "blocking"

which involves interaction between serum and tumor cells, and "inhibition" in which interactions were being made between serum and tumor sensitized lymphocytes. The same authors showed that tumor antigen obtained by papain digestion of rat hepatoma membrane when mixed with specific antiserum manifested blocking activity only after antigen-antibody complexes were formed in the zone of equivalence. On the other hand, using hooded rats bearing chemically induced MC-1 sarcoma, Thomson et al (75) have demonstrated by means of indirect immunofluorescence and mixed hemoadsorption with a [^{51}Cr] labeled indicator cells that, after tumor excision, anti-TSTA antibody was measurable in serum and lymph. By contrast, in the tumor bearing animal, anti-TSTA antibodies were not detected. It was hypothesized that in the presence of a growing tumor the serum contained antigen-antibody complexes with antigen in excess. This hypothesis was confirmed by the same authors after the complexes were split by salt or acid and fractionated by ultrafiltration to high and low molecular weight fractions. It was found that the low molecular weight fraction inhibited the anti TSTA antibody when tested by either membrane immunofluoresecence or mixed hemoadsorption, indicating the presence of antigen from antigen-antibody complexes in the circulation of the tumor bearing host. Additional support for tumor antigens as inhibitors of cellular antitumor immune responses was obtained by Currie and Basham (93) and Currie (94). Lymphocytes from cancer patients were assayed in microcytotoxicity assay against autologous and allogeneic tumor cells. Extensive washing of the lymphocytes from various cancer patients (melanoma, bladder carcinoma, hypernephroma, fibrosarcoma, transitional cell carcinoma) was found to enhance their cytotoxic effect. This effect was restricted to autologous tumor cells and allogeneic cells of

similar histological origin. Cytotoxicity evoked by washing was abolished by adding the patients' serum. The serum component responsible for inhibiting lymphocyte cytotoxicity had no detectable affinity for the target cells and appears to act on the lymphocyte surface, implying that tumor antigen may well be implicated.

Major Histocompatibility Complex

The major histocompatibility complex (MHC) in the mouse is known as the H-2 complex. This complex is found on chromosome 17 and contains a series of closely linked loci which code for cell surface proteins. According to Klein (95), the cell surface proteins coded for by these genes can be divided in 3 classes.

Class I loci

K and D loci in the mouse each consist of multiple alleles coding for serologically detectable alloantigens. The alloantigens are glycoproteins, having a molecular weight of 44,000, and are associated noncovalently with a 12,000 dalton polypeptide coded for on chromosome 15 known as β_2 microglobulin. In the cell membrane, the class I molecule has its amino terminal end away from the cell surface to the periphery. The amino-terminal end contains the epitope responsible for immunological recognition and diversity. The remainder of the molecule spans the cell membrane, and the carboxy-terminal is found inside the cell. This end is believed to be reversibly phosphorylated (96), which may be the means by which outside signals are transduced internally.

There is some degree of sequence homology between class I molecules, β_2 microglobulin and the CH_3 domain of the immunoglobulin molecule (97, 98, 99, 100, 101). This fact argues in favor for a common ancestral gene which diverged early in evolution, and explains similarity in primary and secondary structure.

Biological function of class I gene products

The products of class I genes are the K and D antigens. Functionally, these alloantigens are mainly involved in regulation of lymphocyte receptor and effector functions such as rapid allograft rejection, cell mediated lympholysis (CML), certain weak mixed lymphocyte reactions (MLR), and the specificity of effector T lymphocytes directed against virally infected cells.

Class II loci

According to the unitarian theory of the major histocompatibility complex in the mouse put forward by Klein et al (102, 103), the Ia molecules of the cell surface are the products of the immune response (IR) genes located in the I region of the MHC. Structurally the Ia molecules contain two polypeptide chains α and β having a molecular weight of 32,000 and 28,000 respectively. The chains are synthesized separately and linked non-covalently on the cell surface. The polypeptide chains are integrated in the cell surface and span it, so that the amino-terminal end protrudes to the outside and the carboxy terminal end is found inside the cell. The allotypic site is exclusively found on the α chain.

Biological function of class II gene products

The Ia molecules are mainly involved in vivo in the control of immune responsiveness, T-B cell collaboration, and antigen presentation by macrophages, while in vitro they govern the mixed lymphocyte reaction.

Class III genes

In this locus there are genes which code for the synthesis of soluble glycoproteins which are part of the complement sequence, including C_4; functionally this locus, according to Klein, should be omitted from the MHC.

TSTA and H-2 Histocompatibility antigens (HA)

Histocompatibility antigens seem to be markers of self recognition. This hypothesis was presented by Zinkernagel and Doherty (117), who have demonstrated that virally infected cells were lysed only by cytotoxic T lymphocytes sharing the same H-2 determinants as the target cells. For this phenomenon, they coined the term "H-2 restriction," and Blanden et al (129) have proven that this depends upon the D and K gene products. The mechanism by which antigens associate with MHC gene products is not clear, but two concepts are favored: 1) Dual recognition - implies that cytotoxic T cell recognizes separately at the same time on the target cell surface specificities of self and foreign antigen (128); 2) Altered self - implies that self MHC gene product is altered by a foreign antigen and as such is recognized by cytotoxic T cell.

Parmiani and Invernizi were the first to note that some MC induced fibrosarcomata in balb/c mice grew easily in a single allogeneic strain but others did not (104). Adopting the hypothesis forwarded by Klein and Klein (105), they suggested that TSTA may originate from an alteration of histocompatibility antigen (HA) caused by the chemical or viral carcinogen. According to this hypothesis, chemical and viral carcinogens may cause the expression of genes whose products are antigenically cross-reactive with the histocompatibility antigens of normal allogeneic tissue.

Evidence derived from chemically and virally induced tumors, as well as human neoplasms, suggests the existence of some relationship between the MHC coded molecules and tumor antigens (104) (111) (114) (122) (125). The main question that arises is whether this relationship could be looked at as if tumor antigens are: 1) the consequence of an expression of alien MHC coded molecule; 2) tumor antigens are altered MHC coded molecules or 3) tumor antigens are not related to MHC coded molecules but are noncovalently linked to β_2 microglobulin; 4) tumor antigens are not related to MHC coded molecules and merely copurify with them.

1) Transplantation studies

Invernizzi and Parmiani (107) (106) and Parmiani et al (139) have found that the balb/c mouse sarcoma (C-1) apparently cross-reacts with H-2^k normal tissue. The evidence supporting this finding was suggested by experiments in which balb/c mice that were made immune to C3Hf and AKR mice tissue (all share K^k coded antigens) demonstrated in subsequent experiments transplantation resistance to challenge with 10^5 cells of C-1. The immunologic specificity of this tissue cross-reaction was further demonstrated by the use of congenic mice. It was shown that balb/c mice developed antitumor transplantation immunity when immunized against H-2^k and K^kIA^k tissue. The same type of immunization was ineffective with other antigenically unrelated balb/c sarcomas. The fact that C-1 cross-reactivity is restricted to only certain H-2 antigens, that alloimmunization

was ineffective against tumors lacking alien H-2 determinants, that anti C-1 immunity would be passively transmitted with balb/c anti H-2^k lymphocytes, strengthen the evidence of immunological specificity.

Martin and Gipson (109) and Gipson et al (108) showed that immunization of C3Hf mice with normal B10A tissue induced transplantation immunity to subsequent challenge of C3Hf lung tumors expressing H-2^k like determinants. These authors concluded that there is an extra H-2 antigen in the K region of the H-2^k haplotype of the C3Hf lung tumor which is different from the K region of the C3Hf mice.

Serologic studies

Using monospecific antisera produced in congenic mice, Meschini et al (110, 127) were able to show that C-1 sarcoma adsorbed from the serum the cytotoxic activity to antigenically related normal lymphocytes, not only anti H-$2D^d$ and K^d but also H-$2K^k$. After immunization of balb/c mice with C-1 cells, antisera obtained contained antibodies against K^k specificity. The concomitant existance of K and D specificities was demonstrated by immunofluorescence, indicating that H-2^d and H-2^k are simultaneously expressed on the same C-1 tumor cell. Recently Roman and Bonavida (111) reported that SJL (H-2^s) reticulum cell sarcoma expressed extra H-2^d and H-2^k antigens which were detected by complement dependent cytotoxicity of the tumor cells. Bowen and Baldwin (112) had found that tumor specific antigens of the MC-induced fibrosarcoma have immunochemical and structural similarity to normal H-2 antigens of the same species.

Biochemical purification of the alien H-2 antigens (118) demonstrated that it is a glycoprotein having a molecular weight of 48,000 dalton, and that it is noncovalently linked to β_2 microglobulin. Chemical stability to papain digestion and different elution position on gel filtration distinguishes the alien from the original haplotype.

Roman and Bonavida (111) have also purified a 45,000 dalton molecule solubilized from SJL ($H\text{-}2^s$) tumor and shown that it is reactive with $H\text{-}2^d$ sera, and the anti-$H\text{-}2^d$ antisera does not react with normal SJL lymphocytes. The presence of alien H-2 specificities seems to be well documented. The unanswered question is whether they are the TSTA?

Using cell hybridization with subsequent karyotype analyses of selected hybrids, Klein and Klein (112) have shown that the gene coding for TSTA is not present on chromosome 17; they therefore concluded that the tumor antigen is not related to H-2 products. Likewise, Bowen and Baldwin (119) have shown that in rat hepatoma D23, the TSTA is not directly associated with the rat histocompatibility complex as there is no reactivity with an alloantisera raised against syngeneic erythrocytes. Furthermore, the same authors (120) could not find any association between TSTA and β_2 microglobulin. Recently, Parmiani's group has shown that passage in vitro of C-1 cells causes them to lose their serologically detectible H-2 antigens, while TSTA is retained (121). Indeed purification on gel filtration and lens culinaris affinity columns of detergent solubilized membrane indicate that the TSTA and alien H-2 antigens are separate entities. In conclusion, it seems that alien H-2 specificities are found on certain tumor surfaces in close proximity to the native haplotype. The alien haplotype is not the TSTA and probably is an epiphenomenon of malignant transformation.

Cocapping and immunoprecipitation experiments favor the altered self hypothesis (128). The same type of experiments were also presented as evidence for the relationship between tumor antigens and H-2 antigens vis à vis the same hypothesis. Fujimoto et al (113) detected soluble tumor associated antigen and histocompatibility antigens in sera of A/J mice bearing spontaneous L1117 lymphoma by the means of the inhibition of the cytotoxicity of rabbit anti-L1117 serum and of alloantisera to normal A/J antigens, respectively. It was shown that both antigens copurified from serum of tumor bearing animals using reverse immunoabsorbents prepared by insolubilization of either rabbit anti L1117 antibodies or of the anti A/J alloantibodies. It was suggested by the authors that the determinants responsible for these two antigenic specificities were associated with the same molecular moiety. Gooding and Edidin (114) using immunofluorescence techniques demonstrated that teratoma antigen co-caps with H-2D and H-2K molecules in various transformed fibroblasts and teratoma cell lines. Bubbers and Lilly (115) have shown that in serum of balb/c mice infected 10 - 14 days previously with Friend virus (FV), there are viral particles whose envelope contains H-2K and H-2D determinants. This was demonstrated by the ability of virions[c] isolated from infected sera of balb/c mice to inhibit the cytotoxic activity of various H-2 antisera. The same authors also have shown that capping of FV antigens on $H\text{-}2^b$ tumor cell surfaces caused partial co-capping of H-2D but not H-2K antigens.

Recently Callahan et al (116) characterized a plasma membrane antigen of the murine lymphoma 6C3 Hed. Syngeneic C_3H/Hej anti-6C3 Hed antiserum was shown to be specifically reactive with the lymphoma cells. Alloantisera to H-2, 2.3 were found to react in immunoprecipitation and cytotoxicity adsorption with lymphoma cell lysates. SDS PAGE electrophoresis of radiolabeled immunoprecipitates of tumor cell extracts obtained with alloantiserum and anti 6C3 Hed were identical. Proteins with molecular weight of approximately 70,000, 45,000 and 12,000 daltons were apparent in both.

From the data presented above, altered H-2 antigen may be the tumor antigen (in conformity with the altered self hypothesis). It cannot be excluded, however, that tumor antigens and H-2 antigens are separate entities found in close proximity on the tumor cell surface, and thus co-purify and co-cap as one.

Tumor antigens of human cancer and histocompatibility complex (HLA)

Extrapolating from the rodent system, it seemed logical to investigate whether human tumor antigens are related to HLA antigens. Reisfeld's group isolated tumor specific antigen from a melanoma cell line, the tumor antigen in the spent medium from a melanoma cell line was precipitated by ammonium sulfate, separated by Kbr floatation, CM cellulose ion exchange and lentil lectin Sepharose affinity chromatography. The melanoma specific antigen detected by means of the LAI assay, antiserum, and delayed type hypersensitivity was not associated with material containing HLA antigens, nor was it associated with β_2 microglobulin (122, 123). Cell kinetic studies done by the same group demonstrated that the HLA antigen profile of cultured melanoma cells from nine patients, measured by a quantitative microadsorption technique, does not differ from that of autologous lymphoblasts (131).

Malley et al (124) also could not find a correlation between melanoma associated antigen and HLA antigen, but demonstrated close association with β_2 microglobulin. These authors demonstrated that the majority of melanoma tumor antigen activity (assessed by the LAI assay) from melanoma extracts derived from fresh tumors binds to Sepharose anti-β_2 microglobulin adsorbent. Activity of the tumor antigen was retained after HLA antigen removal by KBr floatation. On the other hand, Thomson et al (125, 133) and Rauch et al (126) have demonstrated that tumor antigens are associated with HLA antigens and β_2 microglobulin. Breast carcinoma, melanoma, and hepatoma membrane were solubilized and digested by papain. Membrane proteins were then subjected to molecular sieve chromatography and anti human β_2 microglobulin affinity chromatography. Tumor antigen activity was assessed by

means of the LAI assay, while the presence of β_2 microglobin and HLA antigens (A, B) were determined by radioimmuneassay. Indeed, it was found that tumor antigen activity and HLA antigens were found in the same bound fraction from the β_2 microglobulin affinity column. On SDS polyacrylamide gel electrophoresis, the material containing tumor antigen activity was composed of two major subunits with molecular weight approximately 12,000 and 40,000. Although the evidence from Thomson's laboratory points to the coisolation of tumor antigen and the HLA molecule, it should be borne in mind that tumor antigen may not be related to HLA. Rather OSN may be strongly bound to the HLA molecules and only copurify with them, or OSN could be a different family of molecules that are also linked to β_2 microglobulin.

Human lung tumor antigen

The definition of lung tumor antigens in this review is restricted to proteins derived from tumor tissue that are capable of eliciting an immune response in the primary host. Molecules often associated with lung cancer such as carcinoembryonic antigen (CEA), antidiuretic hormone (ADH) and human chorionic gonadotropin (HCG) are not considered. In accordance with the above statement, the presence in the primary host of humoral and cellular immunity directed against lung cancer constituents is mandatory. Several *in vivo* and *in vitro* assays are employed in order to disclose immunity in the host against lung tumor cells.

1) Delayed hypersensitivity

Hollinshead et al (56) tested in vivo delayed hypersensitivity reactions to membrane preparation of human lung cancer cells, "normal" lung cells from the same lung and normal lung cells from a healthy accident victim. These membranes were sonicated and subjected to molecular sieve chromatography. Fractions were tested by intradermal injections. Of 106 patients tested with fractions of lung cancer sonicates, 50 reacted positively. Of 106 patients tested with normal lung sonicates fraction from the cancer patients, 17 produced positive delayed type hypersensitivity skin reactions in autologous and allogeneic lung cancer patients. Allogeneic tests on a patient with epidermoid lung cancer were performed with the soluble antigens, both from epidermoid cancer cells and from metastatic tumors growing in the lung of patients with intestinal and cervical cancers; only the epidermoid lung cancer soluble antigens gave positive tests. Recently, Sega et al (54) employed a lung tumor associated antigen prepared by homogenization of surgical specimens of adenocarcinoma and epidermoid carcinoma. The pooled crude extracts were submitted to gel filtration on Sephadex S-200. Tumor antigen activity was initially determined by means of xenoantisera on double immunodiffusion.The active fraction was utilized for skin testing for detection of delayed type hypersensitivity. Skin reaction occurred in 32% of lung cancer patients; 48% of those with limited disease and 17% with extensive disease were positive.

2) Cell mediated cytotoxicity

Vose et al (47) obtained peripheral blood lymphocytes (PBL) from patients with pulmonary neoplasia and tested them for cytotoxicity against

cultured cells derived from lung tumor of different histological types, fetal and normal adult tissue and tumors arising in other organs (colon, breast, bladder). Leukocytes from 75% of patients were cytotoxic for cells from lung tumor while reactivity against normal adult lung cells and unrelated tumor cells (colon, breast, bladder) was 42% and 18%, respectively. Leukocytes from lung cancer patients were also cytotoxic for cells from fetal lung with maximal cytotoxicity (88%) observed for fetal cells at 13-14 week gestation. However, 35% cross-reactive cytotoxicity of leukcytes from non lung tumor bearing patients was observed to lung cancer cells.

In a study by Pierce and Devald (53), PBL from patients having bronchogenic carcinoma, but clinically disease free, were found to be more cytotoxic to bronchogenic carcinoma cells than leukocytes from normal donor or patients with advanced disease. In control experiments the same PBL were more cytotoxic to bronchogenic carcinoma cells than to cultured fibroblasts.

3) Leukocyte migration inhibition assay (LMI)

Vose et al (60) examined the immune reactivity of PBL from healthy donors, from patients with lung cancer, from patients with malignant disease outside the lung by the leukocyte migration inhibition assay. The antigens were homogenates of lung tumor tissue, lung tissue material derived from tumor free area, and material from non malignant lung lesions. PBL migration was inhibited in the lung cancer patient group in response to lung cancer extracts, but also to tumor free and normal lung homogenates. They concluded that in some lung cancer patients sensitization is not only against tumor specific antigens but also against antigens of normal lung tissue.

McCoy et al (59) used as a source of antigen a 3M KCl extract of a tissue cultured squamous cell lung tumor cell line and a fresh pleural effusion from a patient with adenocarcinoma of lung. Of patients with lung cancer, 60% reacted in the direct capillary tube LMI assay, whereas of normal donors, 10% did. Some cross-reactivity was noted with breast carcinoma patients. Interestingly, there was no correlation between LMI reactivity and disease stage, nor between LMI reactivity and histological type, since PBL from patients with oat cell, squamous cell, and adenocarcinoma were reactive against the same extracts. Thus, most patients with carcinoma of the lung had cell mediated immunity against a common lung cancer organ-specific neoantigen which was not restricted to a given histologic type of lung carcinoma.

4) Blastogenic response of PBL of lung cancer patients

Sega et al (83) have purified lung tumor antigen from lung tumor tissue and tissues from human 3 to 5 months fetuses by homogenization and ammonium sulfate precipitation. Proteins were further purified by DEAE chromatography. Elution of an antigen from fetal lung tumor was monitored in a double immunodiffusion test with antiserum raised in rabbit. The isolated protein was used as an antigen for inducing blastogenic response of Ficoll separated PBL as measured by [^{3}H]thymidine incorporation. PBL from lung tumor patients showed a significant blastogenic response in 60% of the cases. PBL of healthy individuals and from patients having other malignant disease and chronic inflammatory lung disease were completely unreactive.

5) Leukocyte adherence inhibition (LAI)

Antitumor immunity to human lung cancer was measured by the LAI assay. (68) (63). A positive test to lung cancer antigen was detected with PBL from 56% of patients with lung cancer and 4% of patients with inflammatory lung disease. The LAI response was related to the extent of the tumor, as 80% with stage I, 66% with stage II, 54% with localized stage III and 35% with widespread stage III were LAI positive. Leukocytes from patients with adenocarcinoma, oat cell carcinoma, and squamous cell carcinoma reacted to a common tumor antigen shared by all lung tumors.

Recently, Sanner et al (64) modified the LAI assay by trypsinizing normal donor leukocytes and then incubating them with serum from patients before they were exposed to antigen extracts derived from lung and breast human carcinoma cell lines. Otherwise, the assay was conducted in a similar manner to that of Halliday's hemocytometer method, with which it was compared. Of 21 lung cancer patients studied, 95% (20) gave a positive response in the modified assays when breast cancer antigen was employed. None of the control serum tested gave positive responses.

From the data presented, the following conclusions may be drawn:

1) Lung tumor antigen exists and is immunogenic in the primary host.
2) Lung tumor antigen is probably a fetal component.
3) Lung tumor antigen expressess a common epitope on histologically different lung cancers.
4) There is a direct correlation between spread of disease and the amount of immune response detected.

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CHAPTER II

Significance, rational and objectives of present study

Certain human tumor antigens are organ specific and are probably expressed in embryonic tissue, suggesting that they may have an important role in differentiation. Tumor antigens in human are also found to circulate free and are excreted in the urine. Attempts to define and isolate human tumor antigens in the past employing xenoantisera to tumor products have failed mainly because of inability of xenogeneic animal to respond to the specific epitope which evokes an immune response in the human host. Conversly, the LAI assay for detecting and monitoring the isolation of the putative tumor antigen has the advantage that it measures the host's immune response which is directed to a molecule containing the epitope or epitopes that are sufficiently unique to evoke an antitumor immune response.

Since past attempts to isolate and characterize lung tumor antigen have failed, it was felt that an attempt to isolate lung tumor antigen by using the LAI assay to monitor its purification might prove successful.

Urine of lung cancer patients was chosen as a source for the tumor antigen for the following reasons:

1) Ease of collection with no difficulty in obtaining large quantities.
2) No dependency on pathological specimens.
3) The putative tumor antigen is partially purified as proteins are filtered through the kidney.

The objective of this study was to isolate, purify and characterize the tumor antigen from lung cancer patients and thus resolve the debate about the nature of tumor antigens. Once isolated and characterized it may be possible to develop simple serologic assays with the specificity and sensitivity of the LAI assay, so that routine laboratories can avail themselves of this diagnostic tool.

CHAPTER III

Isolation and Purification of Lung Tumor Organ-Specific Neoantigen

Material and Methods

Patients:

Twenty patients having advanced lung cancer were asked to collect their daily urine output. Thirteen patients had squamous cell carcinoma and seven oat cell carcinoma. Eighteen were males and two females. The mean age was 62 years. During urine collection patients were not receiving chemotherapy or radiotherapy.

Urine collection:

Urine was collected in 2 litre sterile plastic bottles, containing 10 ml of 1.0 M Tris HCL, 0.002% sodium azide, stored at 4°C, and picked up every morning. Urine from each day's collection was pooled and concentrated in the cold to 1/10 of its original volume in an Amicon concentrator mounted with 150 mm PM-10 membrane. A total of 300 litres of urine was collected over a period of 4 months.

Ammonium sulfate precipitation

Urine concentrate of each day was precipitated with ammonium sulfate. The amount of salt that was added to the urine was such as to bring the

solution to 80% saturation. Ammonium sulfate was added to urine at 4°C with constant stirring over a period of 1 hr. The urine, after the ammonium sulfate was completely dissolved, was left to stir in the cold for another hour. The precipitate was collected by centrifugation for 30 minutes at 15,000 g on a Sorval centrifuge cooled to 4°C. The precipitate was re-suspended to 3 ml by adding 0.1 M sodium phosphate buffer pH,7.3, then placed in a dialysis bag Spectrapor® No. 1 with a molecular weight cutoff of 10,000 daltons - (Spectrum Medical Industries, L.A.-U.S.A.) and dialyzed at 4°C against 100 times the volume of 0.1 M sodium phosphate buffer pH,7.3, and 0.002% sodium azide. Dialysis buffer was changed twice in 24 hours. Insoluble material was discarded after centrifugation on a Sorval centrifuge at 10,000g for 10 min. The supernatant was concentrated to 2 ml in an Amicon concentrator (Amicon Corporation, Lexington, Mass. - U.S.A.) using a PM-10 membrane with a molecular cut off of 10,000 dalton. The concentrate was stored in -70°C until further use.

Affinity chromatography

a) In order to remove albumin from the urine precipitate, Cibacron blue covalently attached to Sepharose CL-6b® was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). 25 gm of gel was swollen in 0.1 M phosphate buffer, pH,7.3, + 0.002% sodium azide according to manufacturer's instructions.

The swollen gel was poured into a 5.0 cm x 60 cm column (Pharmacia, Uppsala, Sweden). The bed volume thus achieved was 200 ml; the flow rate

used was 50 ml/hour. Elution was performed with 0.1 M sodium phosphate buffer pH,7.3, + 0.002% sodium azide. The column effluent was continuously monitored at 280 nm. The unbound fraction was concentrated using an Amicon PM-10 membrane. The bound fraction was eluted with 3 column volumes of 3M KSCN and then 3 column volumes of 0.1 M phosphate buffer pH,7.3 + 0.002% sodium azide was used to return the column to starting conditions.

b) The anti-human IgG, anti-human K light chain and anti-human λ light chain affinity column (will be referred as anti-human light chain affinity column) was prepared by coupling commercially available antibodies (directed against human Ig, K and λ light chains raised in rabbit - Schering - U.S.A.) to AH Sepharose 4B® (Pharmacia Fine Chemicals, Uppsala, Sweden) using the method of Cambiaso (3). A 30 ml volume of gel was prepared containing 500 mg of anti-IgG, 50 mg of anti λ and anti κ light chain and 10 ml of protein A bound to Sepharose CL-4B®. The gel was poured into a 5.0 cm x 60 cm column creating a bed volume of 40 ml. Thereafter the gel was equilibrated with phosphate buffered saline (PBS) pH,7.3, + 0.002% sodium azide. Specificity and capacity of the column was established with [^{125}I] labeled IgG. Elution of the urinary protein was carried out in PBS pH,7.3, + sodium azide 0.002% at a flow rate of 30 ml/hour. Four passages through this column were necessary. The unbound fraction was collected and concentrated by ultrafiltration to 2 ml volume on a PM-10 membrane.

Ion exchange chromatography

The concentrated urinary protein was dialyzed and equilibrated with Tris HCl 0.003 M pH,7.8. DEAE Sephadex A-50® (Pharmacia, Uppsala, Sweden) was swollen and equilibrated with the same buffer. The anion exchange gel was poured into a column 5.0 cm x 60 cm resulting in a bed volume of 500 ml

and a flow rate of 80 ml/hour. After the gel had settled, the sample was applied and eluted in a stepwise fashion increasing the ionic strength in each step. The volume of the eluent was 3 times the bed volume at each step.

Fraction I - Tris HCl 0.003 M pH,7.8,

Fraction II - Tris HCl 0.003 M pH,7.8, + 0.013 M NaCl

Fraction III - Tris HCl 0.003 M pH,7.8, + 0.03 M NaCl

Fraction IV - Tris HCl 0.003 M pH,7.8, + 0.05 M NaCl

Fraction V - Tris HCl 0.003 M pH,7.8, + 0.1 M NaCl

Fraction VI - Tris HCl 0.003 M pH,7.8, + 0.15 M NaCl

Each fraction was concentrated to 3 ml in an Amicon concentrator using PM-10 membrane.

Molecular sieve chromatography on Sephadex G-75® superfine

The active fraction from the DEAE column as defined by the LAI assay was then chromatographed on a calibrated 5 x 100 cm Sephadex G-75® superfine column, equilibrated with phosphate buffered saline at pH,7.3, containing 0.002% sodium azide. The column effluent was continuously monitored at 280 nm in an LKB spectrophotometer (LKB, Sweden).

Fractions corresponding to the elution positions of standards for molecular weight of 48,000, 30,000, 12,000 were pooled and concentrated separately to 2 ml on an Amicon mounted with PM-10 membrane.

Adsorption chromatography

Adsorption chromatography was achieved using Hydroxyapatite (LKB, Sweden). The swollen gel was layered in a disposable glass column 0.7 x 10 cm (Bio-Rad Laboratories, Mississauga, Canada) to create a 2 ml bed volume. The gel was washed with 10 ml of 0.005 M Tris HCl pH,6.8. The sample from the molecular sieve column was dialyzed against the same buffer. After sample application the proteins were eluted in a stepwise fashion using an increasing ionic strength of potassium phosphate ($K_2H\ PO_4$). Five fractions were generated:

1. 0.005 M Tris HCl pH,6.8,
2. 0.005 M Tris HCl pH,6.8, + 0.001 M K phosphate
3. 0.005 M Tris HCl pH,6.8, + 0.005 M K phosphate
4. 0.005 M Tris HCl pH,6.8, + 0.01 M K phosphate
5. 0.005 M Tris HCl pH,6.8, + 0.1 M K phosphate

Each fraction was concentrated to 1 ml volume in an Amicon cell using PM-10 membrane.

Protein determination

Protein determination was accomplished by using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie blue, shifts from 465 nm to 595 nm when binding to protein occurs.

Standards used were gammaglobulin and albumin. Briefly the assay was performed as follows:

1. Standard dilutions containing from 0.2 to 1.4 µg/ml are prepared.
2. 0.2 ml of standards and diluted sample are placed in separate tubes. 0.1 ml sample buffer in "blank" is placed in a separate tube.
3. 5.0 ml diluted dye reagent is added to each tube.
4. Vortex.
5. After 5 minutes OD_{595} measured versus reagent blank.
6. OD_{595} versus concentration of standards is plotted. Unknowns from standard curve are read.

Radioiodination of proteins

Proteins were iodinated with [^{125}I] (Amersham, England) using the lactoperoxidase-glucose oxidase enzyme bead method employing the manufacturer's conditions (Bio-Rad, Mississauga, Canada). The reaction was quenched by adding 100 µl of a solution of 0.1 µg/ml KI. The labeled protein was separated from free iodine by passage on a prepacked PD-10 column (Pharmacia, Uppsala, Sweden). A 20 µl sample of the separated and labeled protein was precipitated with 20% TCA to determine the amount of radioactivity incorporated into the protein. Usually 95% of the protein was labeled.

Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1). The running gel (10 cm x 12 cm x 2 cm) consisted of continuous gradient of 20 to 10%

bisacrylamide (Bio-Rad, Mississauga, Canada) and was overlayed with stacking gel (3% bis-acrylamide). Before application the protein was reduced by addition of 2-mercaptoethanol and boiling. The gel was run over night, removed, stained for 2 hrs at 37 °C with Coomassie blue and destained in 7.5% acetic acid, 10% methanol. The radioactive gel was later autographed for 72 hours at room temperature on x-omat R2 film (Eastman-Kodak Company, Rochester, New York, U.S.A.).

Preparative SDS-PAGE and elution of protein bands

Standard gradient SDS-PAGE according to Laemmli (1) was run over night. Elution of proteins, SDS removal, and recovery of biologic activity was achieved using the method of Hager et al (2). The proteins in the gel were visualized by precipitation with cold 0.25 M KCl (0°C) + 1 mM of dithiothreitol (DTT) (Pierce Chemical Company, Rockford, Illinois). The various bands were cut out with a blade and the protein eluted in buffer containing 0.1% SDS, 0.05 M Tris pH,7.9, 0.1 mM DDT, 0.1 µg/ml, BSA, and 0.2 M NaCl. The acrylamide was crushed with a teflon pestle (Knotes, K 886001 size 19) and the protein was eluted over night. After 5 minute centrifugation at 500 g the supernatant was transferred into 15 ml Corex tubes. SDS was removed by acetone precipitation in the cold (-20 °C) for 30 min. The acetone precipitate was allowed to dry, then resuspended with 20 µl 6M quanidine HCl. The solution was then diluted 50-fold with buffer, 0.05 M Tris HCl pH,7.9, 0.15 M NaCl 0.1 mM DTT, 20% glycerol, 0.1 µg/ml BSA. Proteins were permitted to renature for 1 to 2 hrs and then extensively dialyzed against distilled water. Radiolabeled and cold proteins were processed in the same manner. Homogeneity of the eluted proteins was determined by SDS-PAGE. Biologic activity was measured in the LAI assay.

Computerized antigen induced tube LAI assay

Donors of leukocytes

A 20 ml sample of heparinized venous blood was drown from Stage I lung, colon, and melanoma cancer patients. The blood was incubated in a vertical position for 45 min at 37 °C. The leukocyte rich plasma was aspirated with a Pasteur pipet, particular attention was paid not to include erythrocytes in the aspirate, and then centrifuged at 200 g for 5 min. The pellet of cells was resuspended in 3.5 ml of Tris-buffered ammonium chloride solution (90 ml 0.83% aqueous ammonium chloride, 10 ml 0.1 Tris buffer) pH.7.2, at 4 °C. The suspension was left for 10 min at room temperature to lyse contaminating erythrocytes. The procedure was terminated by the addition of 6.5 ml of Medium 199; the cells were then centrifuged, and the supernatant was removed and discarded. The cells were washed twice with 10 ml of Medium 199 and resuspended at a concentration of $1x10^7$/ml of medium.

Tube LAI Assay

The tube LAI assay was performed as previously described by Grosser and Thomson (4). Twenty ml, 16x150 mm glass test tubes in triplicate for each antigen were used. To each tube was added 0.3 ml of Medium 199, 0.1 ml of tumor extract containing ≈100 µg protein, 0.1 ml of the suspended PBL (initial concentration 1 x 10^7 cells/ml). Well agitated, the tubes were laid horizontally so that the contents covered four-fifths of the length of the tube and were incubated at 37 °C in a 5% CO_2, humidified atmosphere. Two hours later, the tubes were stood upright, and the contents at the

bottom were gently agitated with a Pasteur pipette. And then a sample was withdrawn and pipetted onto a hemocytometer with a surface marked only by a single square, outlining an area of 16 mm^2.

Within the square the cells were counted automatically by a computer-linked image analyzer. The computer calculates the mean number of nonadherent cells, standard deviation, coefficient of variation, and expresses the results as a Nonadherence Index (NAI):

$$NAI = \frac{A-B}{B} \times 100$$

A = Number of nonadherent cells in the presence of lung carcinoma extract (specific antigen)

B = Number of nonadherent cells in the presence of colon carcinoma or melanoma extracts (nonspecific antigen)

Based upon studies of a large number of patients with malignant and non malignant diseases, an NAI of 30 or greater was established as a positive test (4) (5) (6).

"Blocking" tube LAI assay

A blocking tube LAI assay was performed as previously described by Lopez et al (7). The isolated antigen was diluted in 0.5 ml of Medium containing 1% fetal calf serum. This solution was added to 1.3×10^7 peripheral blood leukocytes (PBL) suspended in 0.5 ml of Medium 199. The PBL were from patients that showed reactivity in the tube LAI assay. The mixture was incubated for 30 min at 37 °C in a 5% CO_2 atmosphere with frequent agitation of the tubes. At the end of this period, the cells were centrifuged at 500 g for 10 minutes, washed twice with Medium 199, and transferred

separately to glass test tubes with the specific and nonspecific tumor extracts. Specific antigen was accepted as present in an isolate when the sample was able to abrogate specifically the LAI response of leukocytes from patients with tumors from the same organ from which the antigen was derived (lung) but not that of PBL derived from patients with other cancers. A NAI value of greater than 30 was positive and indicated no blocking, whereas a NAI value of less than 30 was negative, indicated blocking. NAI values of blocking experiments were expressed as the percent of the positive NAI and blocking was considered to be present when the response was inhibited by 50% or more. All samples were coded, unknown to the person performing the test.

RESULTS

Isolation and Purification of Lung Tumor OSN from Urine of Lung Cancer Patients

Pilot Studies

In preliminary experiments, 46 liters of urine were collected from four lung cancer patients. These experiments were designed to determine the conditions for collection, storage and purification of lung tumor OSN from the urine from metastatic lung cancer patients. Table 1 shows that 2.8 gm of urinary protein was recovered. This protein at 50 µg and 20 µg negated the LAI response of PBL from lung cancer patients but not the positive response of PBL from patients with unrelated cancers.

The LAI active urinary protein was chromatographed on a Blue Sepharose affinity column (5.0 cm x 60 cm) having 200 ml bed volume. Protein quantity eluted in the unbound fraction from this column was 1.85 gm. This fraction displayed activivity in the blocking LAI assay as shown in Table 1. To determine the elution profile of the LAI active urinary protein from an ion exchange column, the urinary protein was equilibrated with the starting buffer for the DEAE ion exchange column (5.0 cm x 60 c), that had a 200 ml bed volume, and was eluted from the column with a step wise gradient of NaCl. Table 1 shows the protein recovered in each fraction. Only fraction II eluted with 0,013M NaCl demonstrated activity in the LAI assay and contained 58.52 mg protein. Other eluted fractions did not display activity in the blocking LAI assay (Table 1).

TABLE 1

Pilot Study/LAI Activity and Protein Recovery

Step		Protein recovered in mg	LAI Activity*
Ammonium sulfate precipitation		2800	+
Blue Sepharose affinity chromatography (unbound fraction)		1852	+
Ion Exchange Chromatography			
eluted with starting buffer	Fr I	112.2	-
eluted with 0.013M NaCl	Fr II	58.52	+
eluted with 0.03M NaCl	Fr III	85.8	-
eluted with 0.05M NaCl	Fr IV	136.8	-
eluted with 0.1 M NaCl	Fr V	177	-
eluted with 0.15M NaCl	Fr VI	153	-

*The fractions that specifically blocked a positive response of leukocytes from lung cancer patients by more than 50% and did not alter the positive response of leukocytes from patients with an unrelated cancer.

+ = blocked

- = no effect

Isolation and Purification of Lung Tumor OSN from Urine of Lung Cancer Patients

Based on these studies a purification scheme for the isolation of lung tumor OSN in a large scale was undertaken as outlined in Table 2. From 300 liters of urine 10 gm of protein was precipitated by 80% saturated ammonium sulfate. The urinary protein was chromatographed on a Blue Sepharose affinity column (5.0x60 cm, having 200 ml bed volume). The unbound fraction was eluted in two peaks (Fig. 1). The two peaks were pooled and concentrated. Table 3 shows that this fraction contained 5.1 gm protein.

A) DEAE A50 Ion Exchange Chromatography

The unbound protein was equilibrated with the starting buffer for the DEAE ion exchange and the proteins were eluted from the column with a step wise gradient of NaCl. Table 3 and 4 shows the amount of protein recovered in each fraction. DEAE fraction II (eluted with 0.013M NaCl) had 270 mg of protein and was the LAI active fraction (Table 3). Other fractions presented in Table 4 were eluted with the starting buffer and higher ionic strength buffers and did not display activity in the blocking LAI assay since all these fractions were not able to block the positive response of leukocytes from lung cancer patients.

Blocking the LAI assay with urinary protein isolate DEAE Fraction II

Fractions that demonstrated no LAI activity are presented in Table 4. The fraction or fractions containing LAI inhibiting activity were further analyzed and a dose-response inhibition of LAI was obtained. If there was greater than 50% inhibition of the positive NAI value, this was considered

TABLE 2

Purification of a lung tumor organ-specific neoantigen

1. Urine protein precipitation
   by 80% ammonium sulphate
   Redissolved precipitate in 0.1 M sodium
   phosphate buffer pH,7.3

2. Affinity column: Blue Sepharose
   Unbound fraction dialyze and
   equilibrate with 0.003 M Tris
   HCl pH,7.8

3. Ion-exchange column: DEAE A-50
   Fraction II eluted by 0.013 NaCl
   0.003 M Tris pH,7.8

4. Affinity column: Anti-human light chain
   Unbound fraction

5. Molecular sieve chromatography:
   G75 superfine
   Collect P44 Fraction

6. Adsorption chromatography: Hydroxyapatite
   Collect unbound fraction eluted by
   0.005 M Tris HCL pH,6.8

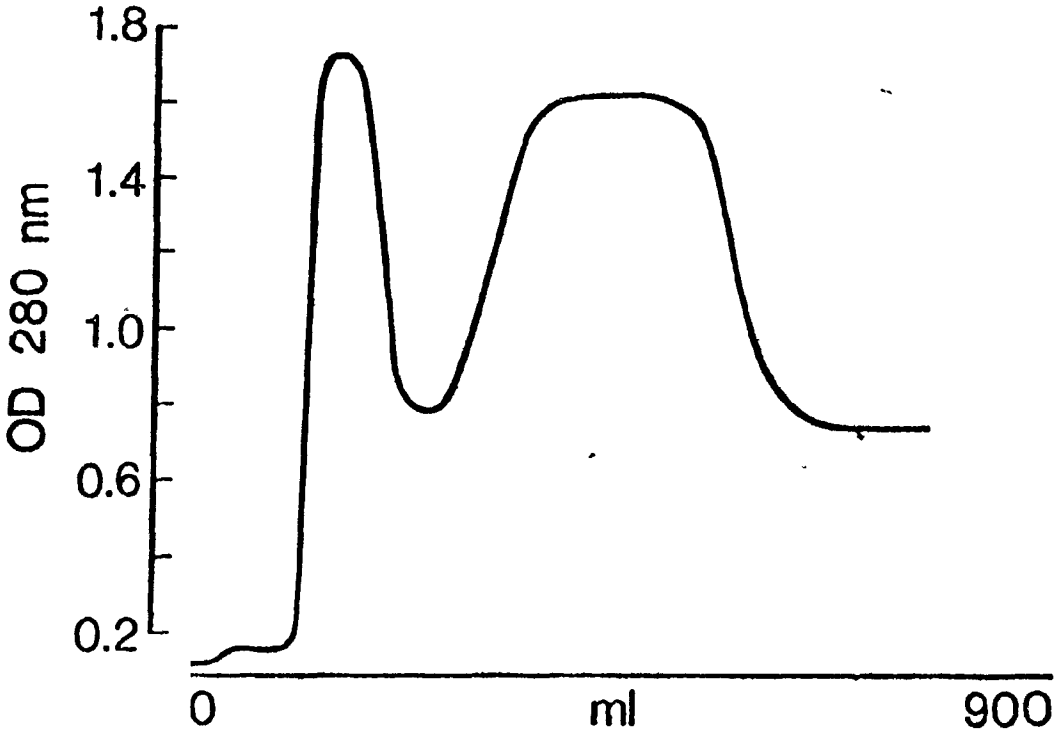

Figure 1

Elution profile from the Blue Sepharose affinity chromatography. Elution recorded at 280 nm. Both peaks collected and pooled.

TABLE 3

Yield of lung tumor OSN purification from urine of patients with metastatic lung cancer

Purification step	Total protein (mg)	µg/unit*	lung OSN units recovered	% recovery	sp. act u/mg	enrichment (fold)
Urine protein precpitation	10,000					
Blue Sepharose- Unbound fraction	5,100	25	204,000	51	40	
Ion exchange chromatography-DEAE-A50 fraction II	270	0.5	540,000	2.9	2000	50
Human anti-light chain affinity chromatography - unbound fraction	40	1	40,000	0.4	1000	25
Molecular sieve chromatography - P44 fraction	4	0.1	40,000	0.04	10,000	250
Hydroxyapatite unbound fraction	1.9	0.05	38,000	0.019	20,000	500

*The minimal amount of protein required to specifically block the LAI response.

†One unit of activity is defined as the amount of material which will negate the LAI response

TABLE 4

Results of LAI blocking with fractions at various isolation steps, demonstrating no activity in the assay

Step	Fraction	Protein Recovered in mg	% of positive NAI
Ion exchange chromotography DEAE-50			
eluted with starting buffer	I	1500	100
eluted with 0.03M NaCl	III	525	100
eluted with 0.05M NaCl	IV	230	100
eluted with 0.1M NaCl	V	41	100
eluted with 0.15M NaCl	VI	110	100
Anti human light chain affinity			
chromatography	bound	230	100
Molecular sieve chromatography			
6-75 superfine	P25	4	100
Adsorption chromatography			
hydroxy-apatite	II		100
	III		100
	IV		100
	V		100

to be evidence for blocking activity of the isolate. Accordingly, the isolate must not block the positive response of leukocytes from patients with unrelated cancers by more than 50%. Figure 2 shows the titration of activity present in fraction II eluted at low ionic strength from the ion exchange chromatography column. Blocking activity is evident at protein concentration ranging from 20 µg to 0.5 µg. At a concentration of 0.1 µg no blocking activity was detected. The blocking effect was specific since no blocking was shown when 20 µg of the same isolate was incubated with reactive PBL from colon carcinoma patients.

B) Anti-Human Light Chain Affinity Chromatography

Immunodiffusion with anti-human light chain antisera and anti-human IgG antisera have demonstrated that the DEAE fraction II contained substantial amounts of IgG and free light chain determinants. To remove the gamma globulins, this fraction was subjected to anti-human light affinity chromatography using 5.0 cm x 60 cm column having a bed volume of 40 ml. After four passages through this column, 40 mg of protein were recovered and 230 mg lost in the bound fraction.

Blocking LAI Assay with Urinary Protein Isolate Unbound Fraction from Anti-Human Light Chain Affinity Chromatography

Figure 3 shows the titration curve of the unbound fraction, after passage through anti-human light chain affinity column. Activity was present in protein concentration ranging from 50 µg to 1 µg, while at concentration of 0.5 µg no blocking activity was demonstrated. No blocking was evident when 50 µg of the same urinary isolate was incubated with PBL from colon carcinoma patients.

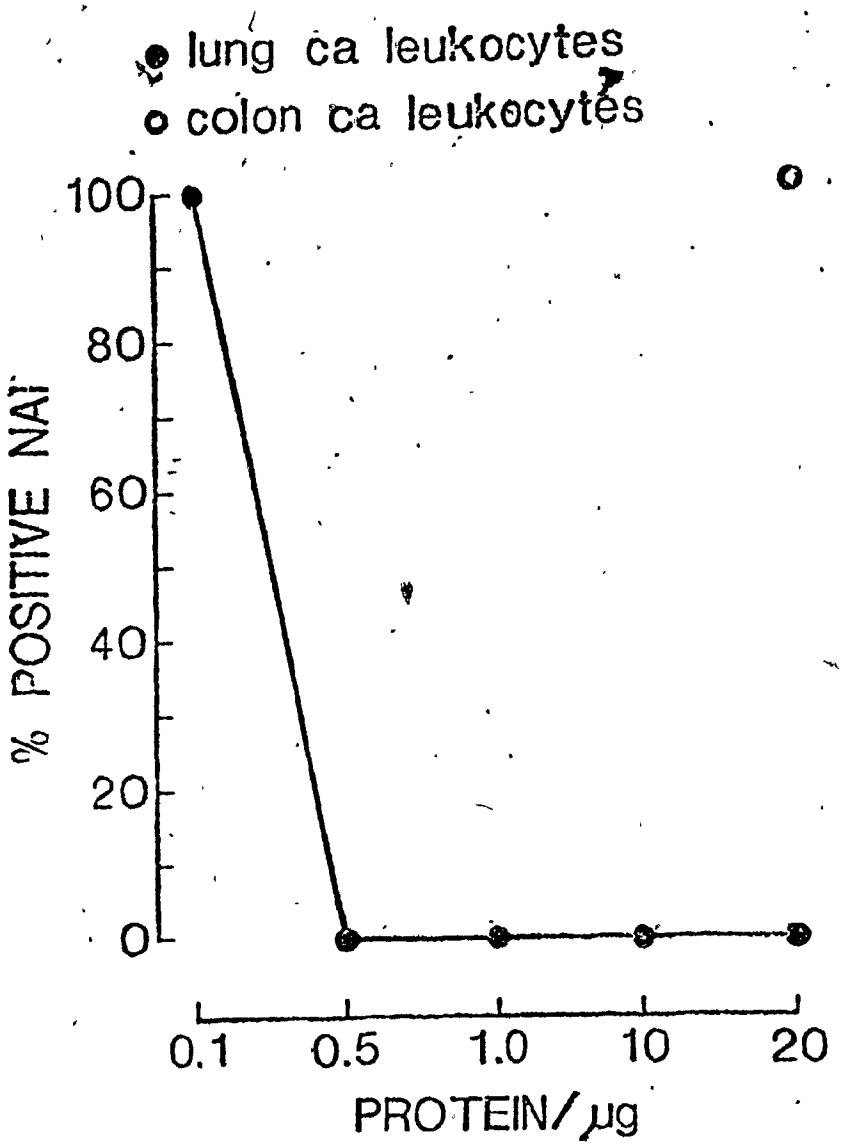

Figure 2

Titration of LAI activity of urinary isolate, eluted with low ionic strength from the ion exchange chromatography DEAE A50 ● — blocking with PBL from lung cancer patients. Blocking is evident with protein concentration ranging from 20 µg to 0.5 µg. No activity is evident at concentration of 0.1 µg. ○ - blocking experiments with PBL from colon cancer patients - no activity is evident at 20 µg protein that previously abrogated the LAI activity when incubated with PBL from lung cancer patients.

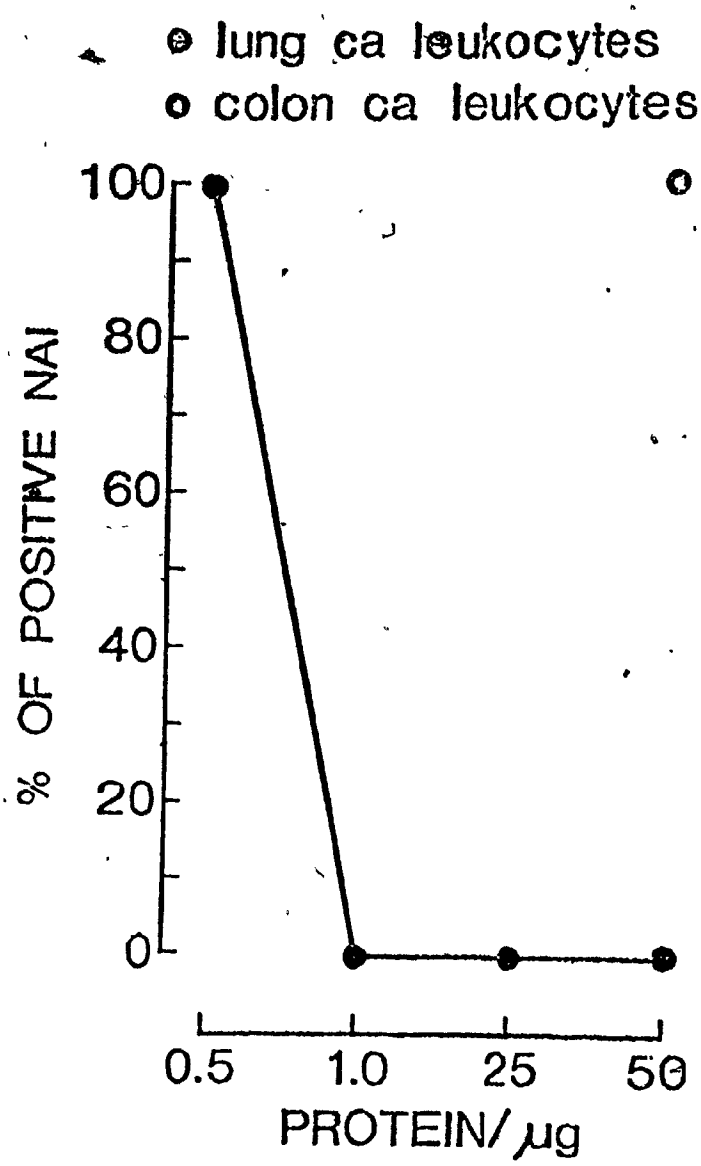

Figure 3

Titration of activity of urinary protein isolate after passage through anti-human light chain affinity chromatograph ⊙— bocking with PBL from lung cancer patients. Activity is demonstrated at protein concentration from 50 µg to 1 µg. O — blocking with PBL derived from colon cancer patients. No activity is demontrated at the maximal (50 µg) concentration that abrogated the assay when PBL from lung cancer patients were employed.

SDS PAGE

Fractions previously shown to be active in the LAI assay were iodinated with [^{125}I]. DEAE fraction II and the same fraction after passage through anti-human light chain affinity column were analyzed on SDS gels with a continuous gradient of 10% to 20% polyacrylamide. Figure 4 shows the pattern of the DEAE fr II in Lane 1. Several major protein bands were present ranging from molecular weight of 14,000 to 68,000 daltons. Of note is the high intensity staining of proteins at 28,000 and 54,000 daltons. In Lane 2, the same urinary protein are shown after passage through the anti-human light chain affinity column.

C) Molecular Sieve Chromatography - Sephadex G-75 Superfine

The concentrated unbound fraction of the anti-human light chain affinity chromatography column was further purified by subjecting the urinary protein to molecular sieve chromatography on a calibrated Sephadex G-75 super fine. The elution profile was obtained by monitoring at 280 nm and is shown in Figure 5. Two peaks were obtained. The first peak (P44) was eluted at the elution volume of ovalbumin, corresponding to molecular weight of 43,000 dalton. The second peak (P25) was eluted at the elution volume of chymotrypsinogen, corresponding to a molecular weight of 25,000 dalton. From each peak 4 mg of protein was recovered.

Blocking LAI Assay with Urinary Protein Isolate - Ovalbumin Fraction

Most of the LAI active material resided exclusively in the P44 fraction, (corresponding to the elution volume of ovalbumin). Figure 6

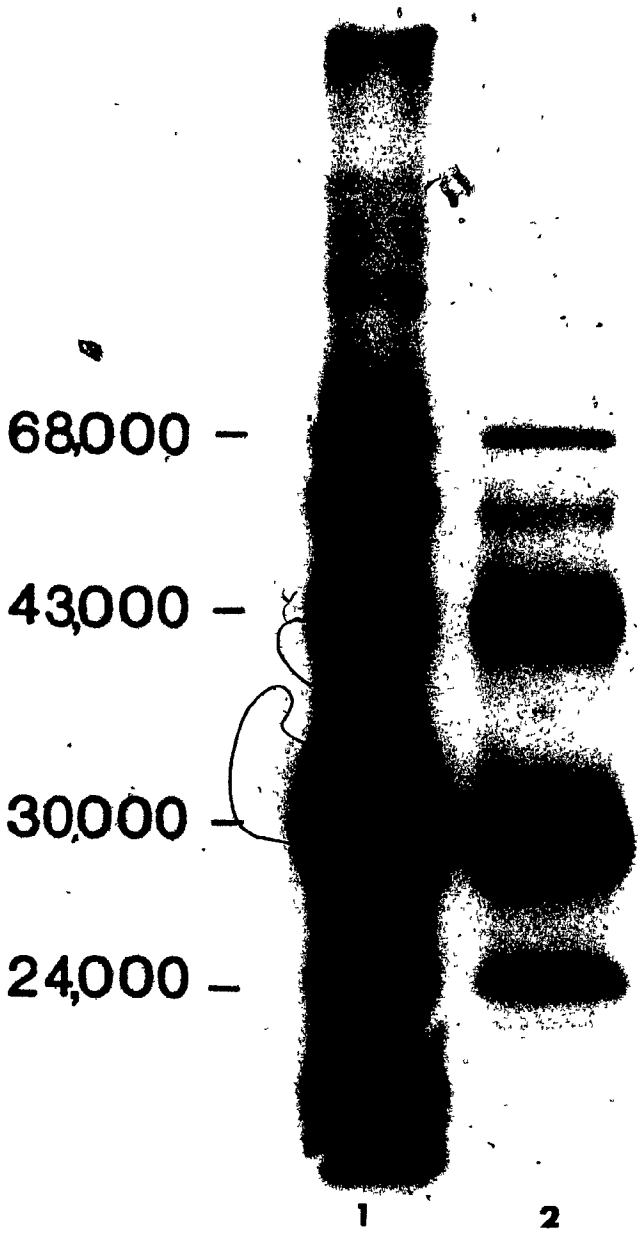

Figure 4

SDS PAGE analysis (autoradiogram) of [^{125}I] labeled isolate containing LAI activity. LANE 1–urinary protein eluted with low ionic strength from the DEAE A50 ion exchange column. LANE 2 – the same material after affinity chromatography on anti-human light chain.

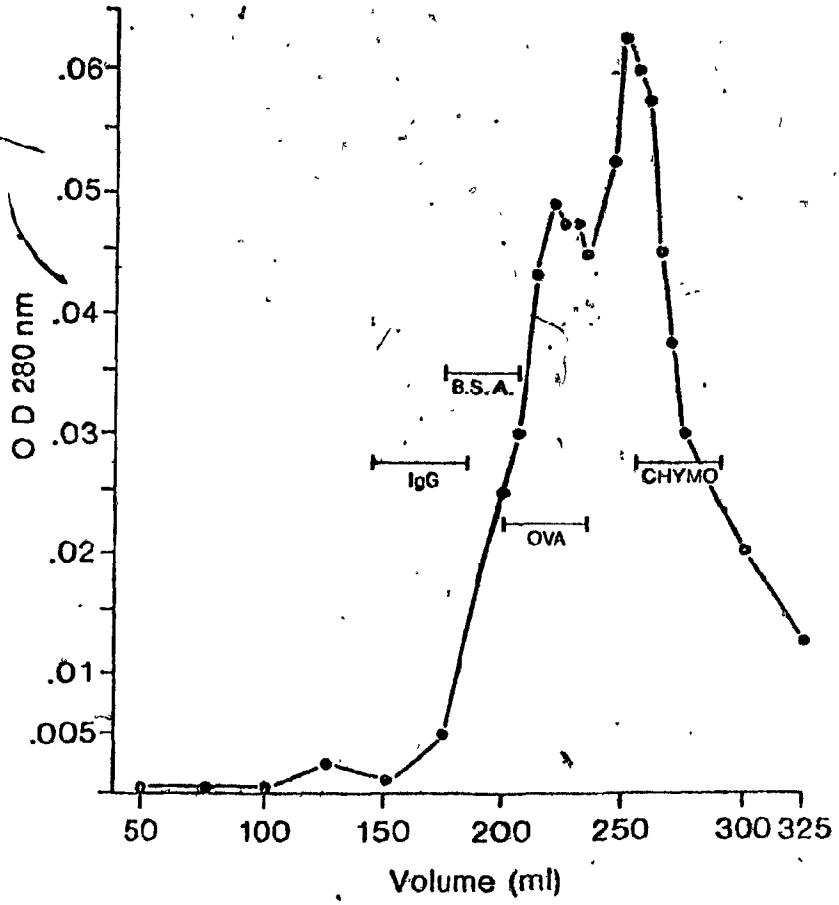

Figure 5

Elution profile of urinary protein sieved on molecular sieve chromatography - Sephadex G-75 superfine. Elution recorded at 280 nm peaks corresponding to elution volume of ovalbumium (OVA) P44 and chymotrypsinogen (chymo) P25 collected separately and concentrated.

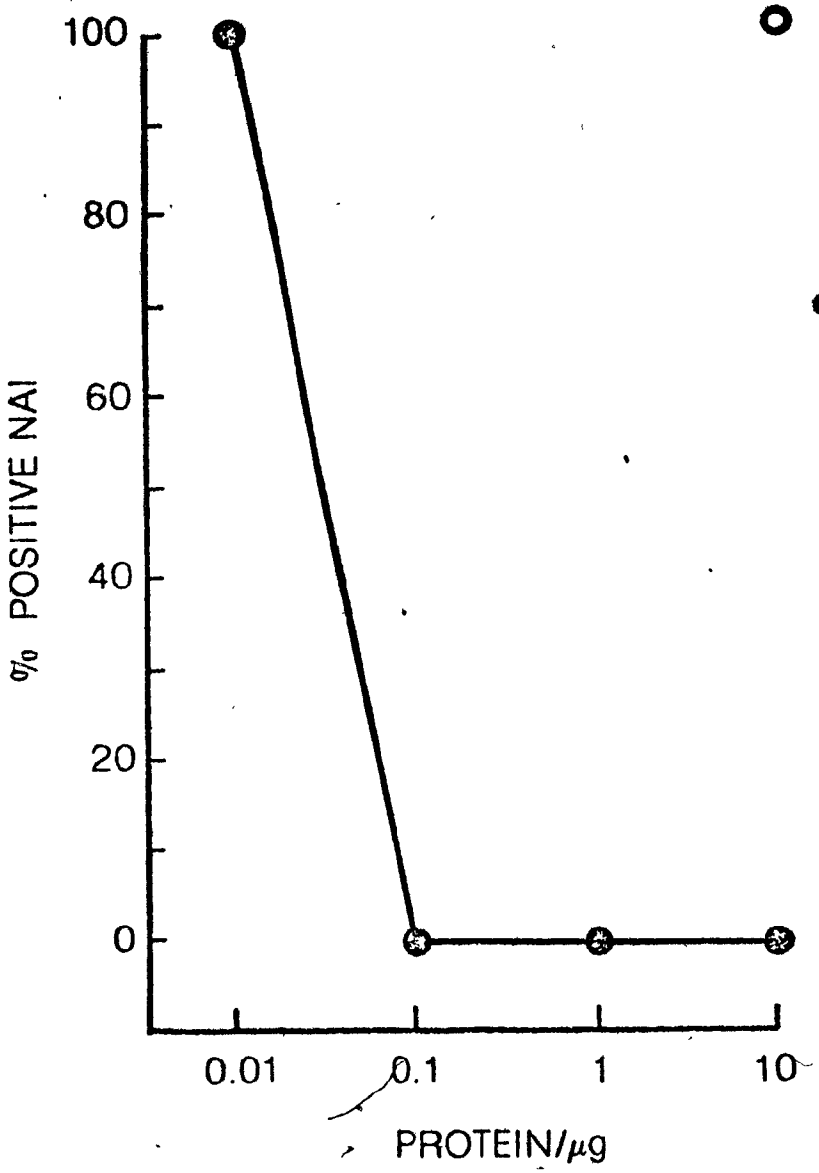

Figure 6

Titration of the activity of urinary protein isolate after molecular sieve chromatography. ●—●blocking with PBL derived from lung cancer patients. activity in the blocking assay is demonstrated in protein concentration ranging from 10 μg to 0.1 g. ○—○ blocking with PBL derived from colon cancer patients. No activity is evident at the concentration of 10 μg.

shows the titration curve for activity in the blocking assay of the LAI material eluted from the molecular sieve chromatography. Activity was evident at protein concentration of 10 µg to 0.1 µg while at concentration of 0.01 µg no blocking activity was demonstrated. No blocking was evident when 10 µg of the same urinary isolate was incubated with PBL from colon carcinoma patients. No activity was demonstrated in the P25 fraction (Table 4).

SDS PAGE

The active fraction from the molecular sieve chromatography was radiolabeled with [^{125}I] and analyzed by SDS-PAGE. Figure 7 shows proteins ranging in molecular weight from 68,000 to 14,000 daltons.

D) Adsorption Chromatography on Hydroxyapatite

To achieve further purification, 4 mg of protein which contained the LAI activity (P44) was subjected to adsorption chromatography on hydroxyapatite. Proteins were eluted in a stepwise gradient with increasing ionic strength of potassium phosphate. Figure 8 shows the amount of protein eluted in each batch. In the unbound fraction eluted with 0.003 M Tris HCl buffer 1.9 mg of protein was obtained. When this fraction was tested in the assay, it was found to contain LAI activity. Other fractions from this column were devoid of LAI activity (Table 4).

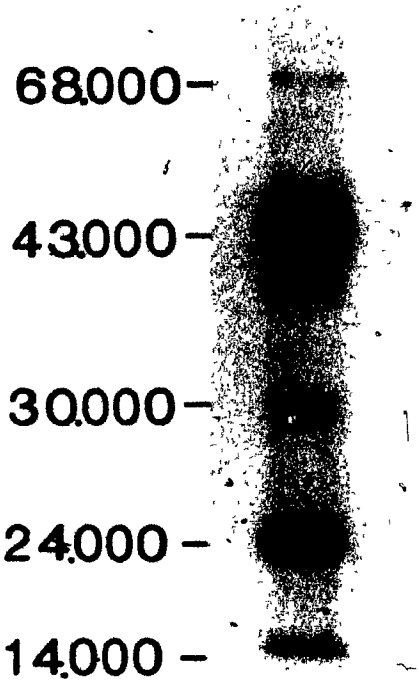

Figure 7

SDS PAGE analysis (autoradiography) at [^{125}I] labeled urinary isolate demonstrating LAI activity. Proteins eluted off the molecular sieve chromatography (Sephadex G-75 superfine) at elution volume of ovalbumin (P44).

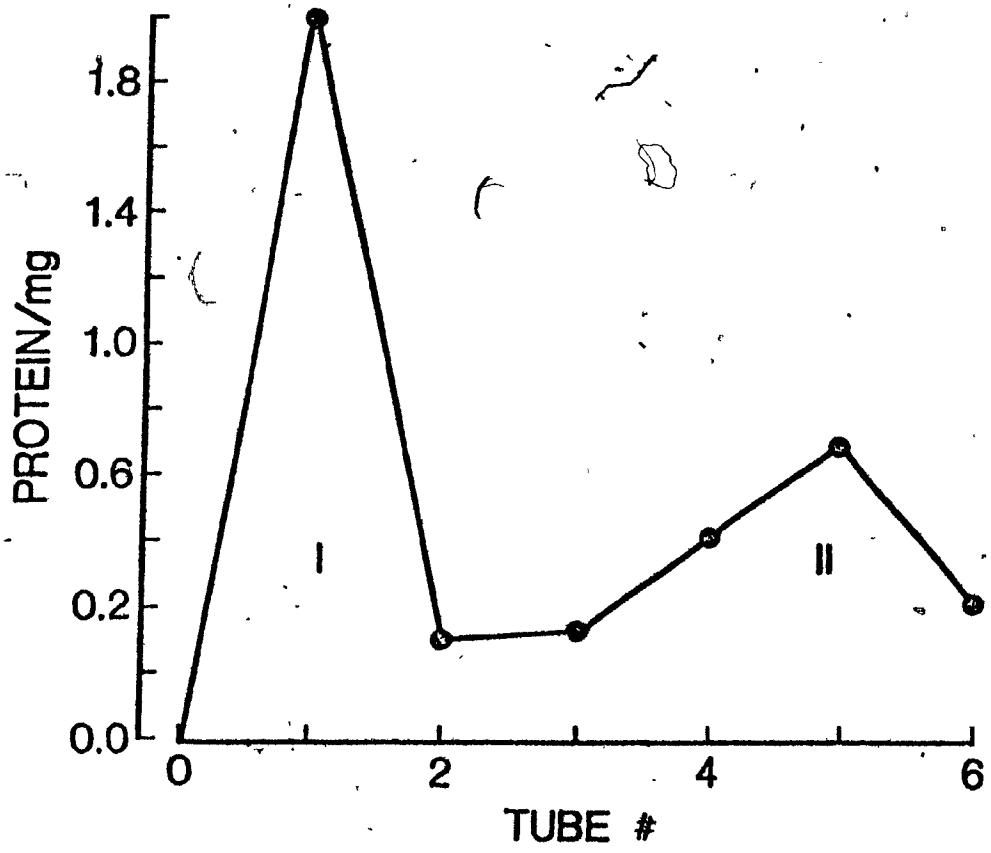

Figure 8

Eluted proteins from adsorption chromatography (Hydroxyapatite). Amount (in mg) of protein eluted with different ionic strength of K_2HPO_4 was determined by the Bio-Rad assay. Greatest amount is present in the unbound fraction (Tube 1).

Blocking LAI Assay with Urinary Isolates - Unbound Fraction

Figure 9 shows the titration of LAI activity of urinary protein eluted in the unbound fraction from the adsorption chromatography column. LAI activity was present at concentrations ranging from 10 µg to 0.05 µg, but 0.01 µg urinary protein did not demonstrate any LAI activity. Specificity was shown by the inability of 10 µg of the same protein to nullify the LAI response of PBL derived from malignant melanoma patient.

SDS PAGE

Figure 10, Lane 1 shows the protein on SDS PAGE which contain the LAI active material from the adsorption chromatography column. Lanes 2, 3 and 4 show the homogeneous single proteins eluted from the gel corresponding to molecular weights of 31,000, 28,000 and 25,000 daltons, respectively.

E) Elution of Protein from SDS Gels

To determine which protein was responsible for LAI activity, the LAI active fraction from the absorption chromatography was run on SDS gels and the protein bands were precipitated by 0.2 M KCl, cut out and eluted from the gel. Homogeneity of the protein isolated from the gel was shown by running a small portion of it again on SDS PAGE.

Blocking the LAI Assay with Isolated Single Band Proteins

Activity of each protein was determined in the LAI assay. Figure 11 shows the titration curve of LAI activity for the 31,000 dalton protein

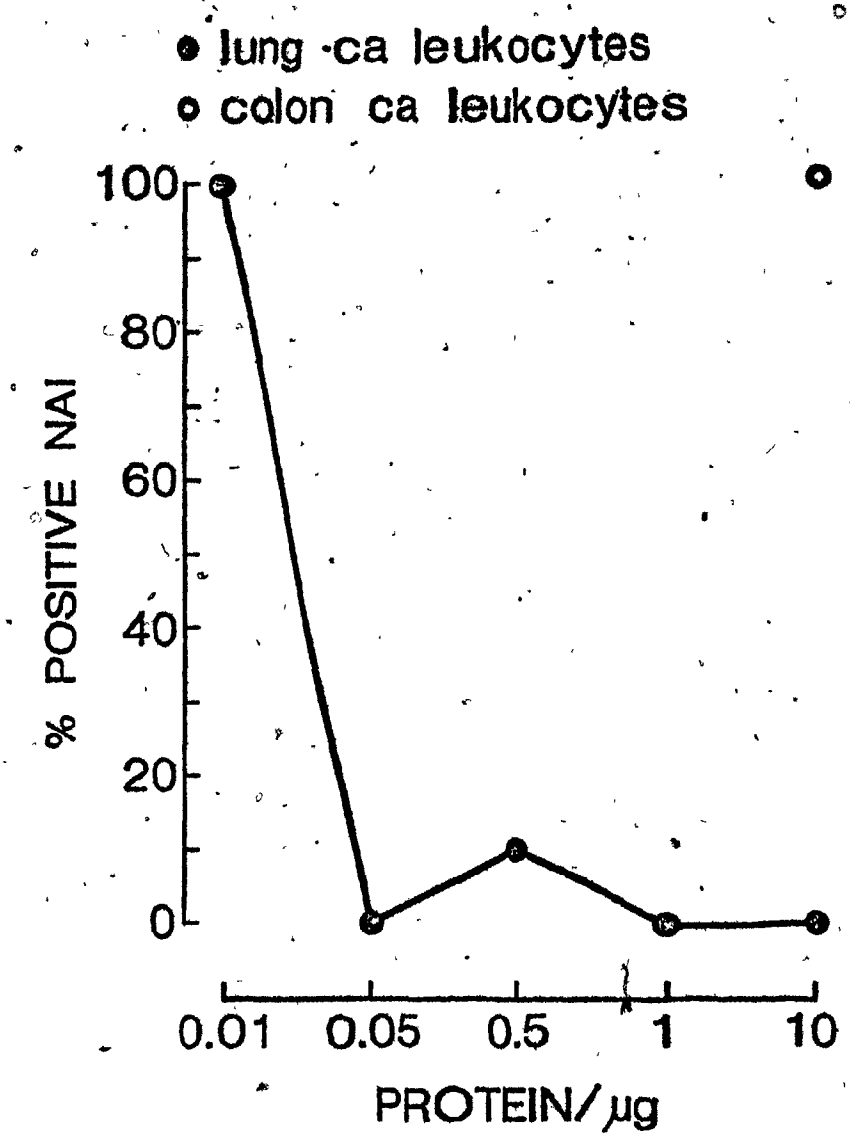

Figure 9

Titration of activity of urinary protein isolated off the adsorpiton chromatography column (unbound fraction). ●— blocking activity of the urinary isolate when incubated with PBL derived from lung cancer patients. Activity demonstrated at protein concentration ranging from 10 µg to 0.05 µg. ○— blocking of the urinary isolation when incubated with PBL derived from melanoma patients. No activity is evident, as protein concentration of 10 µg failed to negate the LAI response.

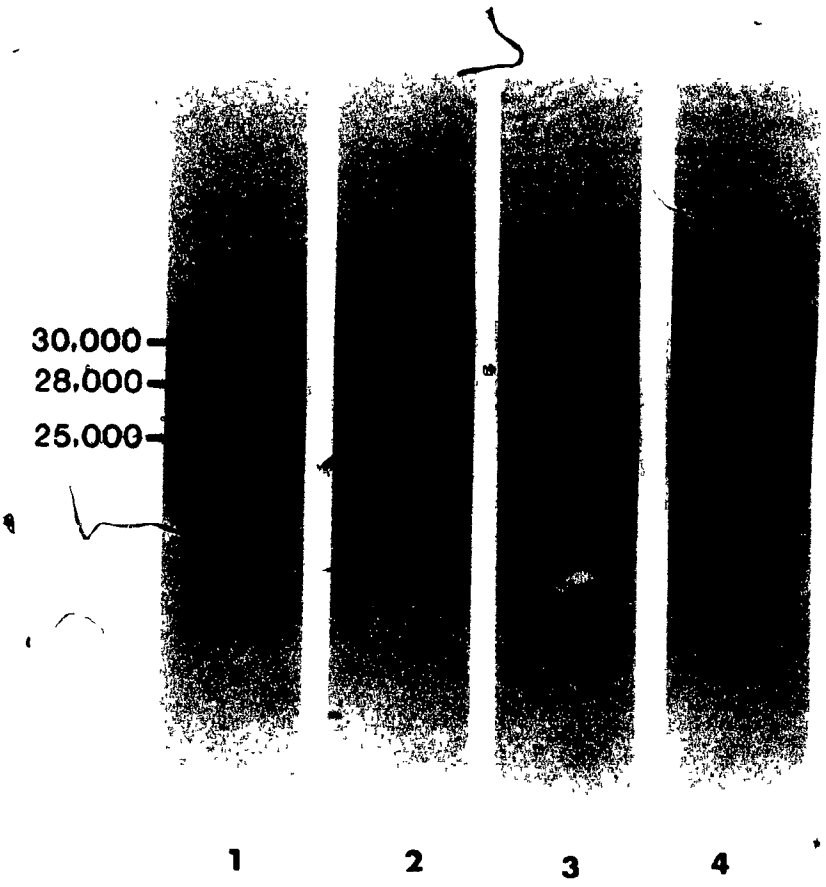

Figure 10

SDS PAGE-analysis (autoradiogram) of [^{125}I] labeled urinary isolate containing LAI activity. LANE 1 - urinary protein eluted with low ionic strength from the adsorption chromatography column. LANE 2 - single band protein eluted from an SDS gel corresponding to molecular weight of 30-31000 daltons. LANE 3 - single protein eluted from SDS gel corresponding to molecular weight of 28,,000 daltons. LANE 4 - single band protein corresponding to molecular weight of 25,000 daltons.

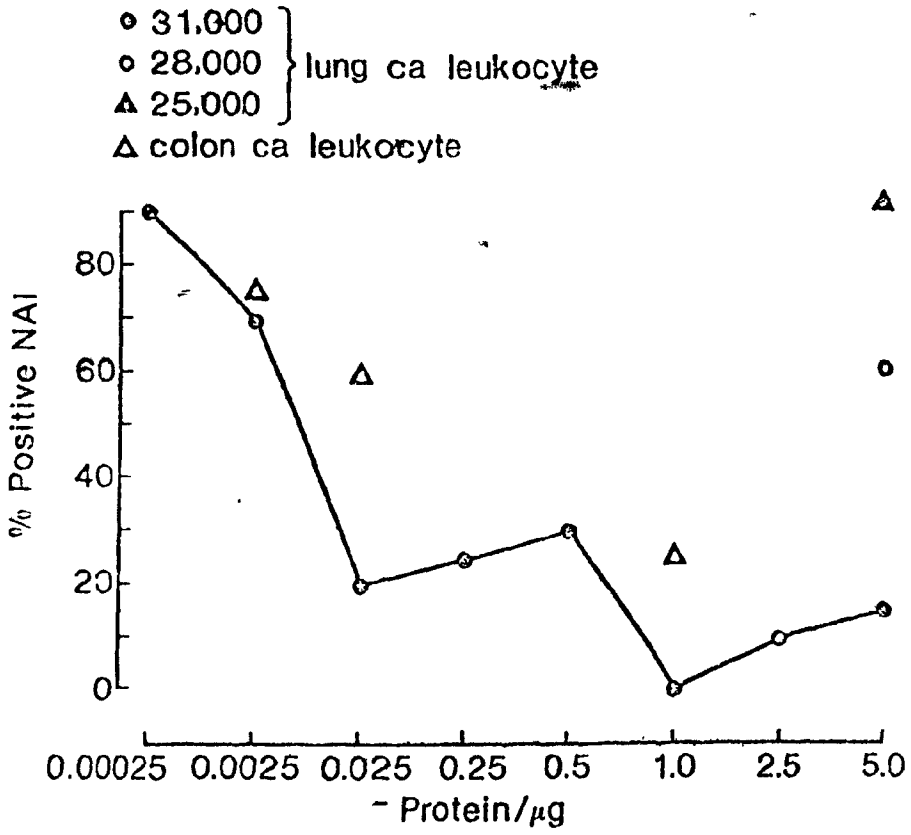

Figure 11

Titration of activity of single band proteins eluted from SDS PAGE gel. o— Blocking of the LAI of PBL from lung cancer patients. With the 31,000 dalton protein blocking is demonstrated in protein concentration ranging from 5.0 μg to 0.025 μg. o— blocking of the LAI of PBL from lung cancer patients, with the 28,000 dalton protein. No blocking is demonstrated in protein concentration of 5.0 μg. ▲Blocking of the LAI of PBL from lung cancer patients with the 25,000 dalton protein. No blocking is demonstrated in protein concentration of 5.0 μg. △Blocking of the LAI of PBL from colon cancer patients with the 31,000 dalton protein. Partial specificity is demonstrated as blocking of PBL from colon cancer patients was achieved in protein concentration of 1.0 μg, however, lower concentration (0.025 μg) failed to negate the LAI response.

that was found to be active in low concentration (0.025 µg). The 28,000 and the 25,000 dalton protein did not show any blocking activity when incubated in the presence of leukocytes derived from lung cancer patients since percent of positive NAI was still 60% and 80%. Unfortunately, the 31,000 dalton protein, LAI active material, lacked specificity since this protein at 1.0 µg abrogated the LAI response when incubated with leukocytes from colon cancer patients. The nonspecific blocking of the LAI response was reproducible on two additional experiments. On the other hand, lower concentrations of the protein clearly showed specificity in that 0.025 µg of the 31,000 dalton protein negated the LAI response when incubated with leukocytes of lung cancer patients but failed to do so when incubated with leukocytes derived from colon cancer patients.

SDS PAGE

The radiolabeled protein on SDS PAGE showed three protein bands by autoradiography and corresponded to molecular weights of 31,000, 28,000 and 25,000 dalton (Fig. 10, Lane 1). In Lanes 2, 3, and 4, a single band of protein corresponding to molecular weights of 31,000, 28,000 and 25,000 dalton, respectively were observed.

Yield, recovery and enrichment of lung tumor OSN from urine of patients with metastatic lung cancer

Table 3 shows the yield, specific activity and the enrichment factor at each purification step. It is shown that the specific activity increases with each isolation procedure and ranged from 40 u/mg to 20,000 u/mg. The overall enrichment achieved at the last purification step is 500 fold. The greatest increase in specific activity was achieved at the molecular sieve and adsorption chromatography purification steps. Likewise, it was also evident that the human anti-light chain affinity chromatography purification step was associated with a loss of specific activity.

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CHAPTER IV

Purification of Normal Urine Protein

Introduction

It has been demonstrated that the OSN is an integral protein of the tumor cell membrane (Chapter I), and is shed into the urine as a result of tumor cell metabolism and possibly from tumor cell death secondary to tumor cell destruction by the host.

In accordance with this statement, urine from normal subjects should not contain a protein that demonstrates activity in the LAI assay.

The following study was undertaken to prove that:

1) Urinary protein from normal subjects, processed in the same manner as that from lung cancer patients, did not demonstrate activity in the LAI assay.
2) Urinary protein, derived from normal individuals, differed from that of lung cancer patients when isolated and compared by SDS PAGE.

Material and Methods

Subjects

Ten normal healthy volunteers were asked to collect their daily urine output. All subjects were males, with a mean age of 35.

Urine collection and methods employed for urinary protein precipitation and purification were the same as presented in Chapter III.

RESULTS

One hundred liters of urine were collected. Table 5 shows that 2 gm of urinary protein was recovered and shows the relative amounts of protein recovered at each purification step.

The fractions identical to urinary protein from lung cancer patients that contained LAI active material were purified. Urinary protein was precipitated with 80% ammonium sulfate and subjected to affinity chromatography on Blue Sepharose by DEAE-ion exchange chromatography at low ionic strength (0.003 M NaCl) and chromatographed on anti-human light chain affinity column.

Molecular sieve chromatography - Sephadex G-75 superfine

The isolated urinary protein was chromatographed on Sephadex G-75 and figure 12 shows the elution profile. Two main peaks were present corresponding to the elution volume of ovalbumin (P44) and chymotrypsinogen with an apparent molecular weight of 43,000 and 23,000 daltons. There were 3 minor peaks at the elution volume of gamma globulin and bovine serum albumin. The general outline of the elution profile of normal urine protein was similar to that shown for urinary protein from lung cancer patients urine at the same stage of purification (see Fig. 5, Chapter III).

Adsorption chromatography on hydroxyapatite

The P44 fraction was concentrated and further purified by adsorption chromatography on hydroxyapatite. The fraction contained in the 0.005M Tris HCl pH6.8 was concentrated and found to contain 0.16 mg protein.

TABLE 5

Yield and Recovery of Normal Urinary Protein

Step	Protein Recovered	% Yield
Urine protein precipitation	2 gm	
Blue Sepharose affinity chromatography unbound fraction	980 mg	49%
Ion exchange column DEAE A-50 fraction II - eluted with 0.013M NaCl	40 mg	2%
Affinity chromatography anti-human light chain - unbound fraction	6 mg	0.3%
Molecular sieve chromatography - G-75 P44 fraction	0.6 mg	0.03%
Adsorption chromatography-hydroxyapatite eluted with 0.005M Tris HCl	0.16 mg	0.008%

Blocking the LAI assay with normal urine isolate - adsorption chromatography - hydroxyapatite

The fraction eluted in the 0.005M Tris HCl buffer from the adsorption chromatography column was assayed in the blocking assay. No blocking occurred when 1 µg of this fraction was incubated with reactive PBL from lung cancer patients.

SDS PAGE

Radiolabeled urinary protein from normal subjects and lung cancer patients from the adsorption chromatography step were analyzed by SDS PAGE. The same number of radioactive counts for both samples were placed in two separate lanes. Figure 13 shows an overexposed autoradiogram of the SDS gel. Lane 1 contains [^{125}I] urinary protein from the lung cancer patients. A 31,000 and a 25,000 protein were prominent. In Lane 2 is the [^{125}I] urinary protein from normal subjects. No protein was visible at 31,000 dalton while the protein at 25,000 was present.

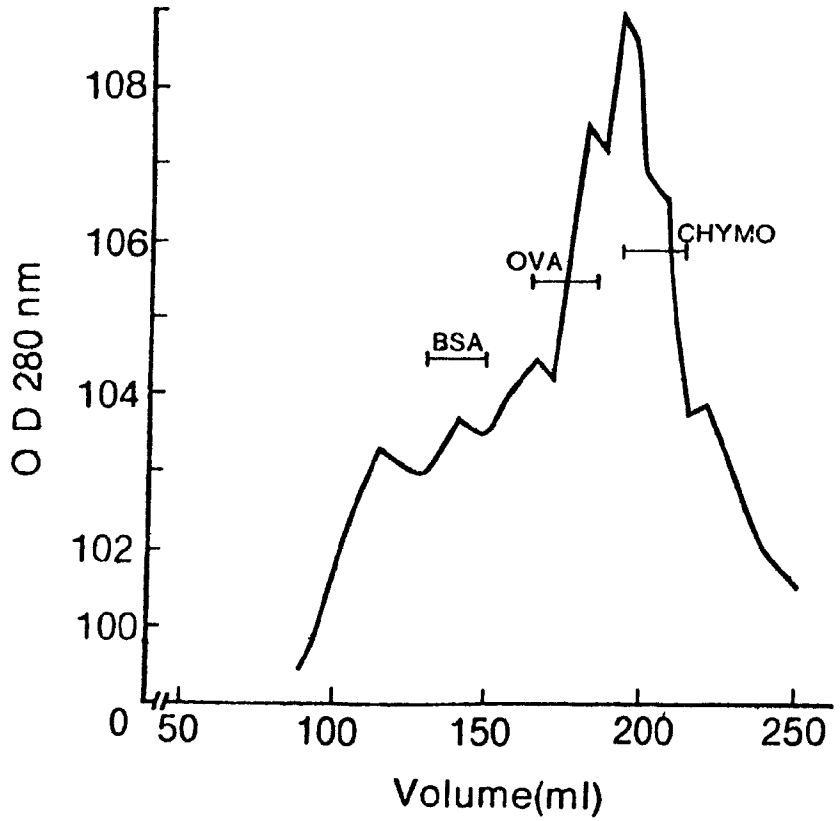

Figure 12. Elution profile of normal urinary protein sieved on molecular sieve chromatography Sephadex G-75 superfine. Elution recorded at 280 nm, (P44 corresponding to elution volume of ovalbumin) and P25 (corresponding to elution volume of chymotrypsinogen) were collected separately and concentrated.

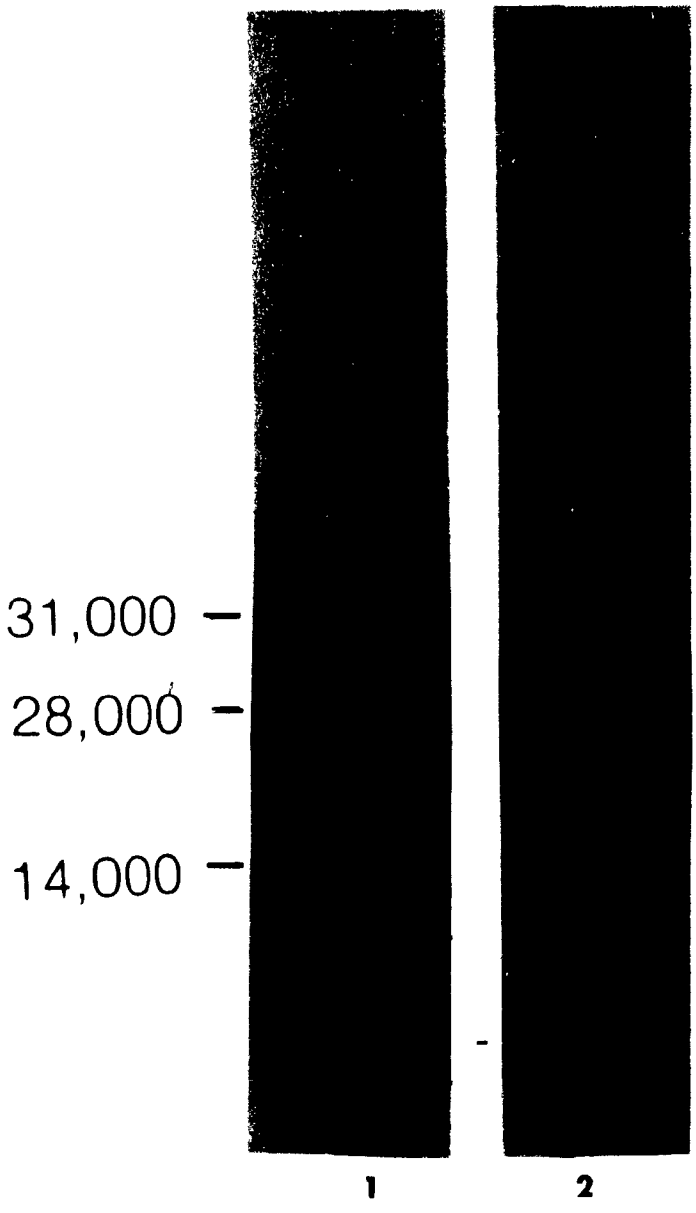

Figure 13. SDS PAGE analysis (autoradiogram of [^{125}I] urinary isolate). Lane 1 - urinary protein derived from lung cancer patients - hydroxyapatitite unbound fraction. To note the presence of 31,000 dalton protein band. Lane 2 - urinary protein derived from normal subjects (same purification step). No protein band is evident at 31,000 dalton. Gels were developed by autoradiography.

CHAPTER V

Characterization of Lung Tumor Organ-Specific Neoantigen

Material and Methods

1) Identification of glycoproteins

The urinary isolate was resolved on SDS-PAGE with a continuous gradient of 10-20% polyacrylamide. The gel was fixed overnight in a solution containing 25% (V/V) isopropyl alcohol and 7% (V/V) acetic acid. Glycoproteins were determined using the technique described by Rostas et al. (1) namely: [^{125}I]-labeled concanavalin-A (Con-A) (Pharmacia, Uppsala, Sweden) was diluted in 50 ml of 0.5 M NaCl, 0.05 M phosphate buffer pH,6.8. The gel was incubated in this solution for 20 min, then washed thoroughly, dried and autoradiographed. The same was done for a negative control, placing a gel in an incubation mixture containing [^{125}I]-concavalin-A with 0.2 M α methyl-manoside.

2) Peptide mapping by limited proteolysis

The previously isolated, eluted and [^{125}I]-labeled single protein bands were digested by trypsin and protease V-8 according to the method of Cleaveland et al (2). Trypsin was purchased from Worthington, Freehold, N.J., and Staphylococcus aureus-V8 protease was purchased from Pierce, Rockford, Illinois. Each single protein was dissolved in sample buffer, consisting of 0.125 M Tris, 0.5% SDS, 10% glycerol, pH,6.8, and trace amount

of bromphenol blue. The sample was boiled for 2 min and then digested with 30 µg/ml of enzyme for 30 min in a water bath at 37 °C. The digestion was stopped by boiling for 2 min, and the sample was loaded on a continuous SDS-PAGE. After completion of the run, the gel was fixed and stained in 40% methanol and 10% acetic acid and 0.1% Coomassie blue. After destaining (50% methanol, 7% acetic acid and 43% H_2O) the gel was dried on gel drier (Bio-Rad, Mississauga, Canada), and autoradiographed.

3) Flat bed isoelectric focusing

Separating proteins according to their isoelectric point was performed on a flat bed using Bio-Rad cell model 1415 (Bio-Rad, Mississauga, Canada). A 20 cm bed was used, and was cast with 85 gm Biogel® (Bio-Rad), containing 5 ml of 20% solution of Biolyte® (Bio-Rad) pH,3-10, previously swollen and equilibrated in distilled water. After the slurry had been degased, it was laid on the tray confined on each side by paper wicks (Whatman, England).

Excess moisture was removed with filter paper placed on each side wick. The gel was considered ready when it exhibited no reflection of light. The [^{125}I] radiolabeled sample was mixed with 100 µl of ampholyte (Biolyte) pH,3-10 and a trace amount of crystal violet and applied with a sample applicator into the gel.

The power supply was initially set to 250 V and after an hour to 700 V overnight. The chamber was cooled to 4°C throughout the focusing. The gel was cut with a grid creating 24 slices 0.8 cm each and the pH of each section was measured. The slices were suspended in distilled water and counted in a gamma counter.

4) Two-dimensional electrophoresis

Two-dimensional gel electrophoresis was performed according to the technique of O'Farrell (3).

First dimension

[^{125}I] radiolabeled protein, was run in 30% acrylamide 4% Ampholites (Bio-Rad) pH,3-10 and pH,4-6 in ratio 1:2. The gels were cast in 128 mm long, 5 mm diameter tubes and overlaid with 8 M urea solution. After polymerization, the 8 M urea solution was replaced with "lysis buffer" which contained 9.5 M urea, 8% Biolyte® (Bio-Rad) pH,3.5-10, pH,4-6 and pH,6-8 in ratio 1:2:2 and 10% mercaptoexphanol.

The gel was electrophoresed for 1 1/4h in the following order: 200 V - for 1/4 h, 300 V - for 1/2 h, 400 V - for another 1/2 h. The sample was applied to the top of the gel in the tubes, overlaid with a solution containing 8 M urea and 10% Biolyte® (pH,4-6, pH,3-10) and electrophoresed overnight at 300 V.

After the gel was squeezed from the tubes, the blank gel was cut into 8 mm slices which were placed in 1 ml distilled water and the pH of each slice was determined by measuring the pH of the water containing the gel slice. From the determined pH, a curve of pH versus distance was constructed.

Second dimension

A continuous gradient of 10-20% acrylamide was cast between a glass rectangular plate and a glass notched plate (Bio-Rad model 220). The gel of the first dimension was placed in the grove aligned properly according to polarity. The gel was fixed to the grove with 1% agarose solution in SDS

sample buffer. While the hot agarose solution was being poured, a plastic "tooth" was placed between the glass plates to create a well for running molecular weight standards (Pharmacia, Uppsala, Sweden). After the agarose had solidified, the gel was prerun for 20 min at 10 milliamper. Molecular weight standards were applied to the well and the gel was run at 200 milliampere at a constant current until the tracking dye, Bromphenol blue, that was placed in the upper chamber reached the bottom of the gel. After the run was completed, the gel was removed and soaked in 20% T.C.A. for 1/2 h and then stained in Coomassie blue stain, (40% methanol, 10% acetic acid and 0.7% Coomassie blue) for four hours. The gel was destained in 50% methanol, 7% acetic acid, 43% H_2O.

5) Immunoprecipitation

Commercial antisera that was raised in rabbits to human serum and urine proteins were purchased from Dako, Denmark: anti-albumin, and free light chains (k,λ) anti-lactoferin, anti-β_2m, anti-normal urine protein, anti-α chymotrypsin. Other anti-sera used were:

a) Anti-HLA-A and B heavy chain, common site, raised in rabbits and characterized by inhibition of cytotoxicity (6), (kindly donated by Dr. J. Rauch).

b) Monoclonal antibody HLA-A, B heavy chain, common site (P44), clone W6/32, the specificity was determined by tissue distribution, immunoprecipitation, inhibition of binding by soluble antigen (4).

c) Monoclonal antibody to HLA-D (P32), clone DA-2 the specificity was determined by inhibition of soluble antigen, immunoprecipitation and tissue

distribution (4). Both antibodies were kindly donated by Dr. W. Bodmer of the Imperial Cancer Research Fund, England.

d) Antibody to carcinoembryonic antigen and 50K fragment of the same molecule (immunologically cross reactive with carcinoembryonic antigen) were raised in horse and rabbit, respectively, these antibodies were characterized by immunodiffusion and radioimmunoassay and were kindly donated by Dr. M.J. Krantz.

Immunoprecipitation of [^{125}I]-labeled urine isolate was performed according to Kessler (5). Briefly, antisera were diluted 1:100 in NET buffer (0.15 M NaCl, 5mM EDTA, 5 mM Tris HCl supplemented with 1 mg/ml ovalbumin). The radiolabeled protein (≈20,000 cpm) was added to the tubes and incubated at room temperature for 20 min. A blank was run each time consisting of the appropriate normal serum diluted 1:100 in NET buffer. After the incubation period 1 ml of NET buffer was added to each tube and 50 µl of a second antibody (against rabbit or mouse IgG) was added and incubated further for 20 min at room temperature.

Supernatant and precipitate were collected after centrifugation at 8,000 g (Sorval HS_4 Rotor) for 10 min. Supernatant and precipitate were counted.

The percentage of binding is calculated according to equation

$$\% \text{ Bound} = \frac{B-N}{T-N}$$

where

B = counts bound

T = total counts

N = counts bound in absence of immune serum

RESULTS

Immunoprecipitation of [^{125}I] Labeled Urinary Isolated Hydroxapatite-Unbound Fraction

From its last stage of purification from the hydroxyapatite column the LAI active urinary isolate consisted of 3 major protein bands ranging in molecular weight from 31,000 to 25,000 daltons. Since 77% of lung cancer patients generally show high levels of carcinoembryonic antigen (CEA), we determined whether the isolate was contaminated with CEA or with smaller molecular weight molecules that were immunologically cross-reactive with CEA. The intact CEA molecule might be excluded from consideration because of the molecular weight of the protein in question. However, the availability of a radioimmunoassay to CEA and a smaller fragment of CEA (50,000 dalton) cross-reactive with it, prompted this investigation. The experiments that were performed with the [^{125}I] labeled LAI active protein and antisera to CEA and 50K are presented in Table 6. The results show that antibodies to CEA and 50K did not bind the protein of the LAI active fraction since the amount of labeled LAI active protein that was precipitated was not in excess of that precipitated by normal horse and rabbit serum.

Other non-specific lung cancer markers such as lactoferrin and $\alpha_1$anti-chymotrypsin were also found to be absent from the LAI active fraction as judged by the inability of antisera to these proteins to precispitate them (Table 6).

TABLE 6

Immunprecipitation of [^{125}I] Labeled LAI Active Protein (Hydroxyapatite-unbound Fraction)

Anti Serum	% Precipitated	% Difference from Control
Normal horse serum	5	0
Anti CEA	3.5	-1.5
Anti 50K	8	3
Normal rabbit serum	10	0
Anti β_2 microglobulin	6	-4
Anti normal urine protein	5	-5
Anti lactoferrin	10	0
Anti albumin	8	-2
Anti α_1 antichymotrypsin	7	-3
Anti free light chain	15	5
Normal mouse serum	10	0
Anti HLA-A,B heavy chain	10	0
Monclonal antibody -W6/32	8	-2
Monoclonal antibody DA-2	9	-1

The absence of β_2-microglobulin was apparent from SDS PAGE studies (Fig. 10, Chapter III), as no protein band was evident at the appropriate molecular weight of β_2-microglobulin. Likewise in immunoprecipitation experiments with anti β_2-microglobulin antiserum, the amount of the [^{125}I] labeled LAI active urinary protein precipitated was similar to the control normal rabbit serum (Table 6).

To study the relationship between lung tumor OSN and HLA-A,B coded molecules, two experiments with two different sources of antibody against HLA molecules were performed. Both antibodies are directed to a common site on the HLA-A,B coded molecules. Table 6 shows the immunoprecipitation of [^{125}I] labeled LAI active protein, although the two antibody populations were obtained by different methods (xenoimmunization and monoclonal antibodies), there is no evidence of specific immunoprecipitation obtained with either one since both did not precipitate the labeled LAI active protein more than normal rabbit or mouse serum. A similar result was obtained with monoclonal antibody directed against the common epitope of a molecule coded for by the DR locus.

Determination of Glycoprotein

LAI active protein is a membrane protein, requiring solubilization with papain. The glycosylated nature of the urinary LAI active protein from the hydroxyapatite column was disclosed by the ability of these proteins to bind specifically to concanavalin-A (Con-A). Figure 14 shows specific binding of

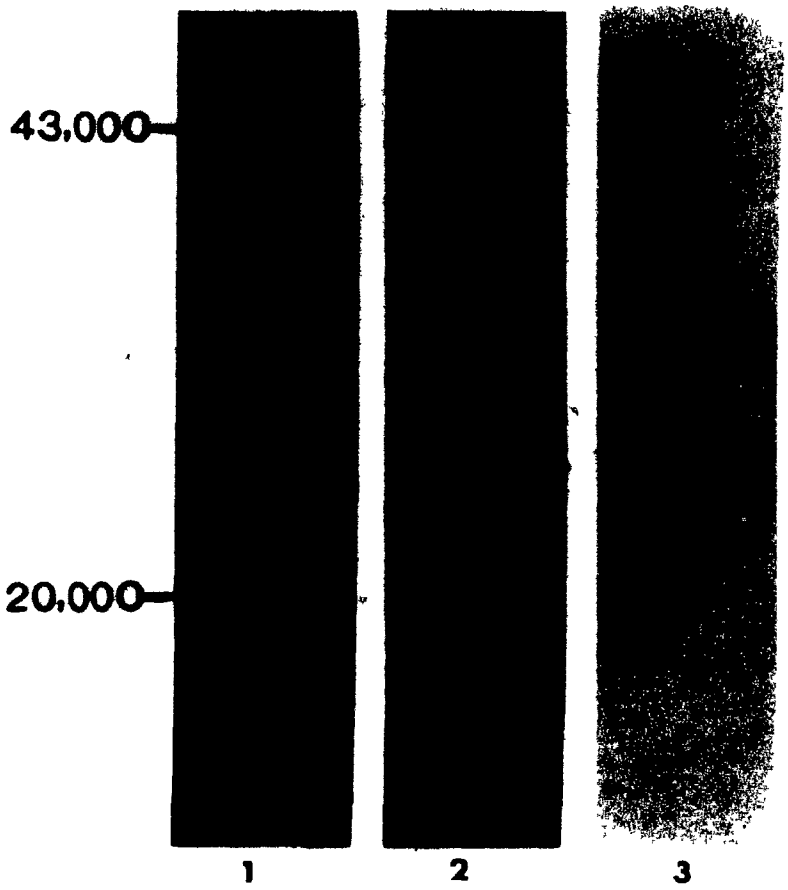

Figure 14

Binding experiments of [^{125}I] labeled Con-A to electrophoresed LAI active fraction. Lane 1 - [^{125}I] labeled Con-A is bound to all proteins. Present in the hydroxyapatite LAI active fraction. Lane 2 - standard protein markers, ovalbumin (43,000 dalton) and chymotripsinogen (25,000 dalton) are binding labeled Con-A. Lane 3 - α methyl manoside present in incubation mixture with [^{125}I] Con-A (as in Lane 1) - no binding of [^{125}I] Con-A occurs.

[^{125}I]-Con-A to the previously SDS-PAGE electrophoresed active urinary proteins. Lane No. 1 shows the binding of the [^{125}I]-Con-A to all proteins present in the LAI active fraction from the hydroxyapatite adsorption chromatography. Specificity of this binding was determined by a parallel experiment in which the incubation mixture of the [^{125}I]-Con-A also contained the specific inhibitor, α methyl manoside that inhibited binding.

In Lane No. 2 protein standards were run (Fig. 14). Only glycosylated proteins were apparent after the binding experiments.

Peptide Mapping by Limited Proteolysis

First, it was determined whether the 28,000 and the 25,000 dalton proteins were related to or were break-down products of the 31,000 dalton protein. To help answer this question, proteolytic fragments were compared on SDS gels.

Limited proteolysis of the materials was carried out with V8 protease and trypsin. Figure 15 shows the SDS PGE of the 31,000, 21,000 and 25,000 dalton proteins before and after proteolysis with V8 protease. Lanes 1, 3, 5 are proteins before proteolysis while lanes 2, 4, 6 are the same proteins after proteolysis. The proteins of 28,000 and 25,000 daltons were more resistant to V8 protease than the 31,000 dalton molecule since total proteolysis of the 28,000 and 25,000 molecules was not achieved while the 31,000 dalton molecule was totally digested. The fragments of the three polypeptides were different, indicating that they were probably different proteins.

The same experiment was carried out with trypsin. From Figure 16 is evident that the three proteins under investigation were different since different fragments were generated from them after partial proteolysis with trypsin, as well as their susceptibility to proteolysis was different.

Flat bed isoelectric focusing

The LAI active protein off the hydroxyapatite adsorption chromatography column was subjected to flat bed isoelectric focusing. This experiment

Figure 15

Proteolysis with V8 protease of the 31,000, 28,000 and 25,000 dalton proteins. Lane 1 - 30-31,000 dalton protein. Lane 2 - same protein after limited proteolysis - total digestion with generation of small molecular weight fragments. Lane 3 - 28,000 dalton protein. Lane 4 - the same protein after limited proteolysis - incomplete digestion. Lane 5 - 25,000 dalton protein. Lane 6 - same protein after limited proteolysis - incomplete degestion with different fragments as compared to Lanes 2 and 4.

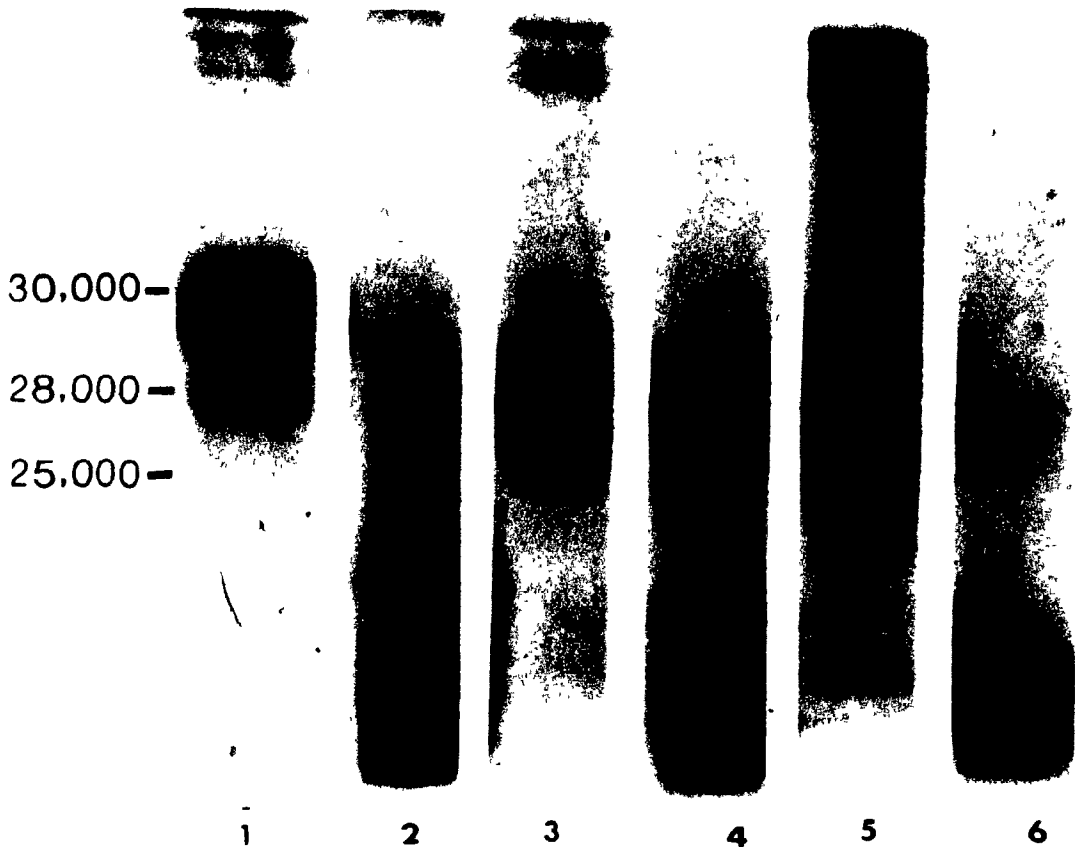

Figure 16

Proteolysis with trypsin of the 31,000, 28,000 and 25,000 dalton proteins. Lane 1 - 30-31,000 dalton proteins. Lane 2 - same protein after limited proteolysis - total digestion with generation of small molecular weight fragments. Lane 3 - 28,000 dalton protein. Lane 4 - the same protein after limited proteolysis - incomplete digestion. Lane 5 - 25,000 dalton protein. Lane 6 - same protein after limited proteolysis - incomplete degestion with different fragments as compared to Lanes 2 and 4.

was carried out for two purposes: 1) to further isolate the putative tumor antigen; 2) to determine the PI of the putative tumor antigen.

Figure 17 demonstrates the different isoelectric points of the [^{125}I] labeled proteins which constitute the LAI active hydroxyapatite fraction. Two peaks were evident. The smaller peaked focused at a pH of 7.2 and the larger focused at pH,5.8.

Figure 18 shows the pattern on isoelectric focusing of the [^{125}I] labeled 31,000 dalton protein. The solitary peak had a pH of 5,12 and 5,02. The isoelectric focusing of the 28,000 dalton and 25,000 dalton proteins is presented in Figure 19 and Figure 20, respectively. The 28,000 dalton protein displayed a PI of 5,16 to 4,92. A different pattern was manifested by the [^{125}I] labeled 25,000 dalton protein. Two peaks were observed: the larger had its PI of 5.50 to 5.33 and the smaller peak focused at a PI of 5.22 to 5.14.

Two Dimensional Gel Electrophoresis

Since the 31,000 dalton protein might be the LAI active protein, its pattern was determined by two dimensional gel electrophoresis in the presence of 8 molar urea (Fig. 21). The 31,000 dalton protein, in the presence of urea, resolved into multiple fragments with different PI point ranging between pH of 7.2 to 6.2.

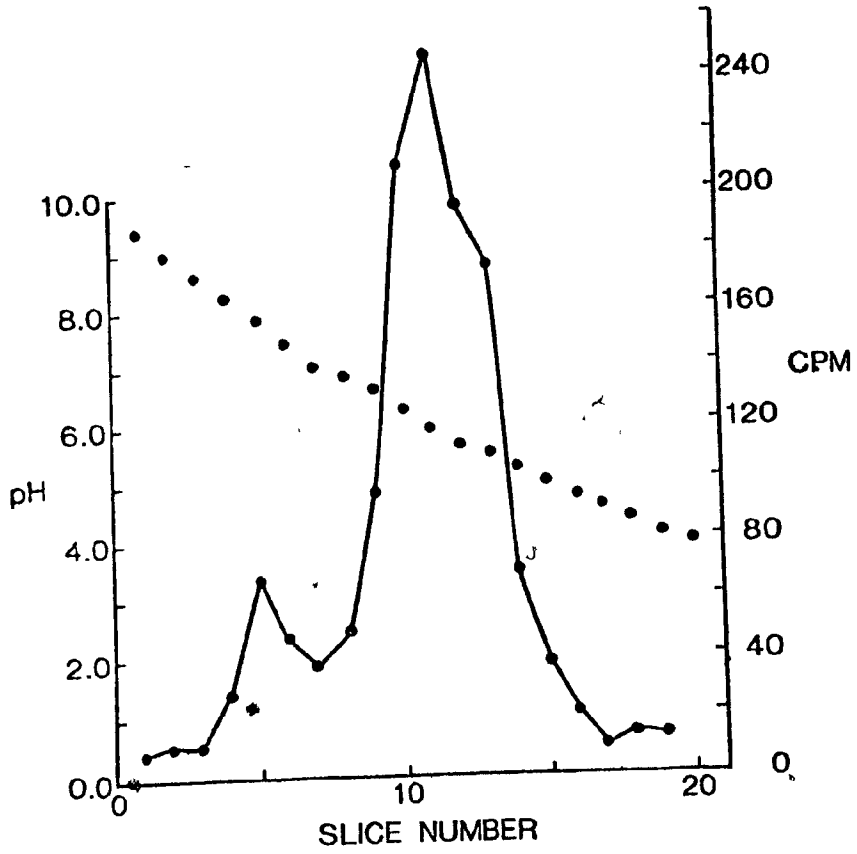

Figure 17

Flat bed isoelectric focusing of [^{125}I] labeled LAI active urinary isolate (unbound fraction from adsorption chromatography). Two peaks are shown. The smaller focused at pH of 7.2 and the larger peak, focused at pH 5.8.

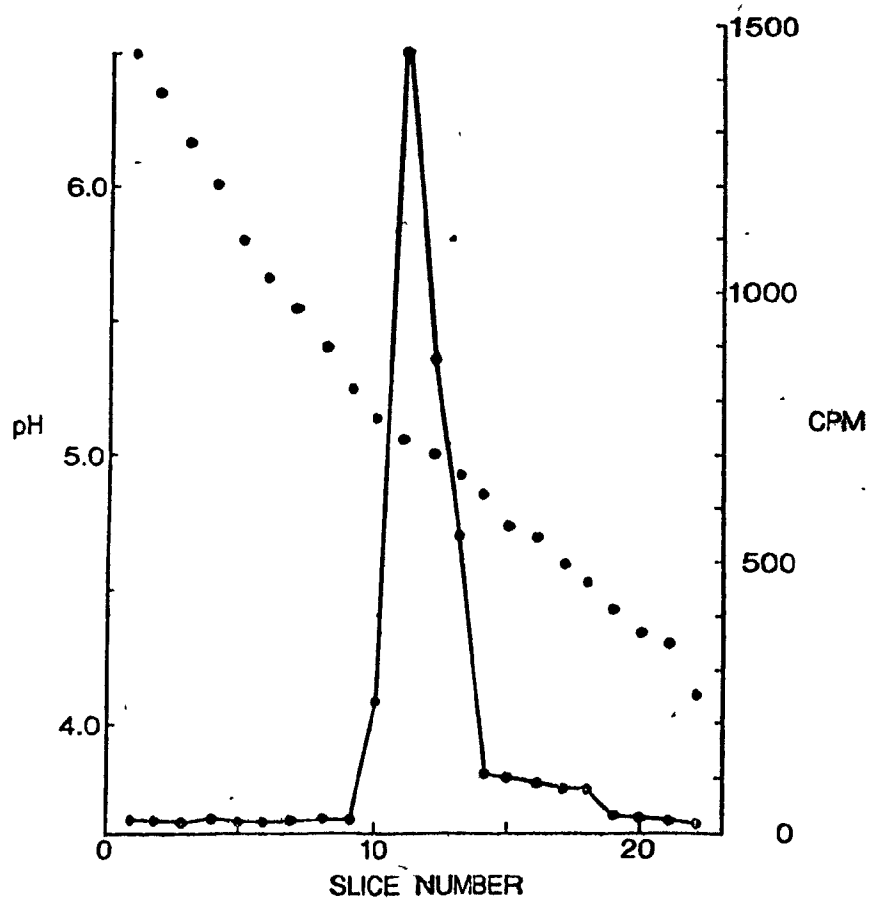

Figure 18

Flat bed isoelectric focusing of [^{125}I] 31,000 dalton protein. One main peak is being focused at pH of 5.12 to 5.02.

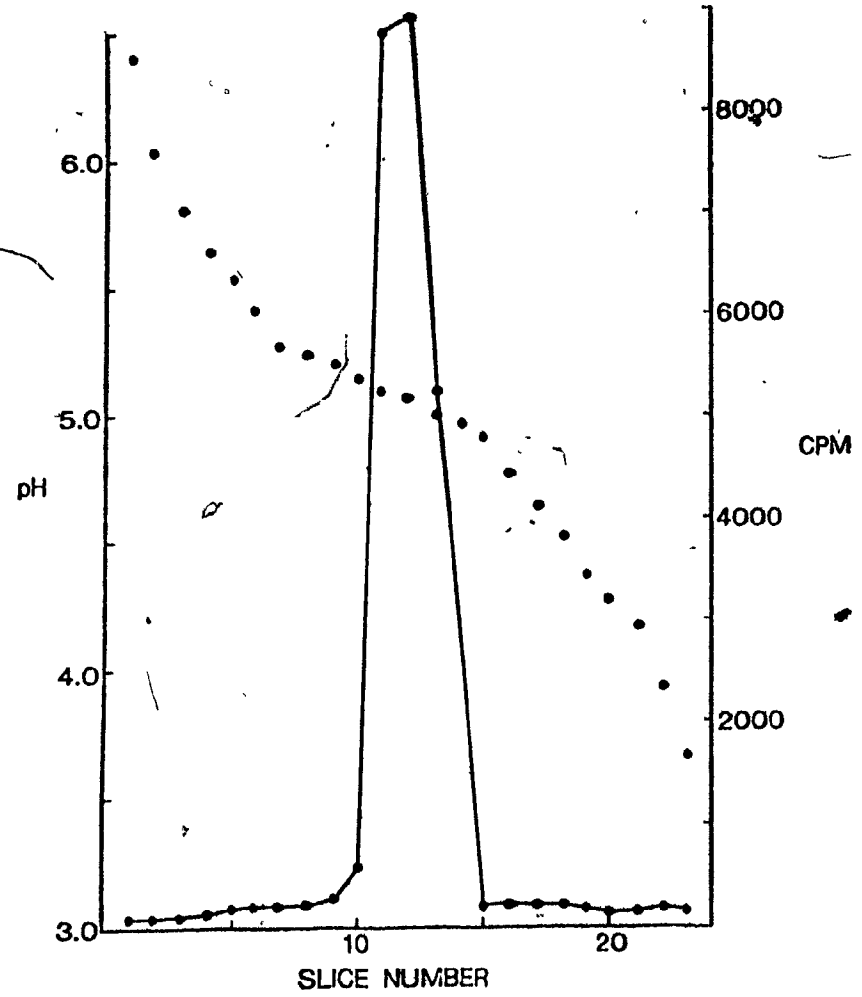

Figure 19

Flat bed isoelectric focusing of [^{125}I] labeled 28,000 dalton protein. One peak is focused at pH of 5.16 to 4.92.

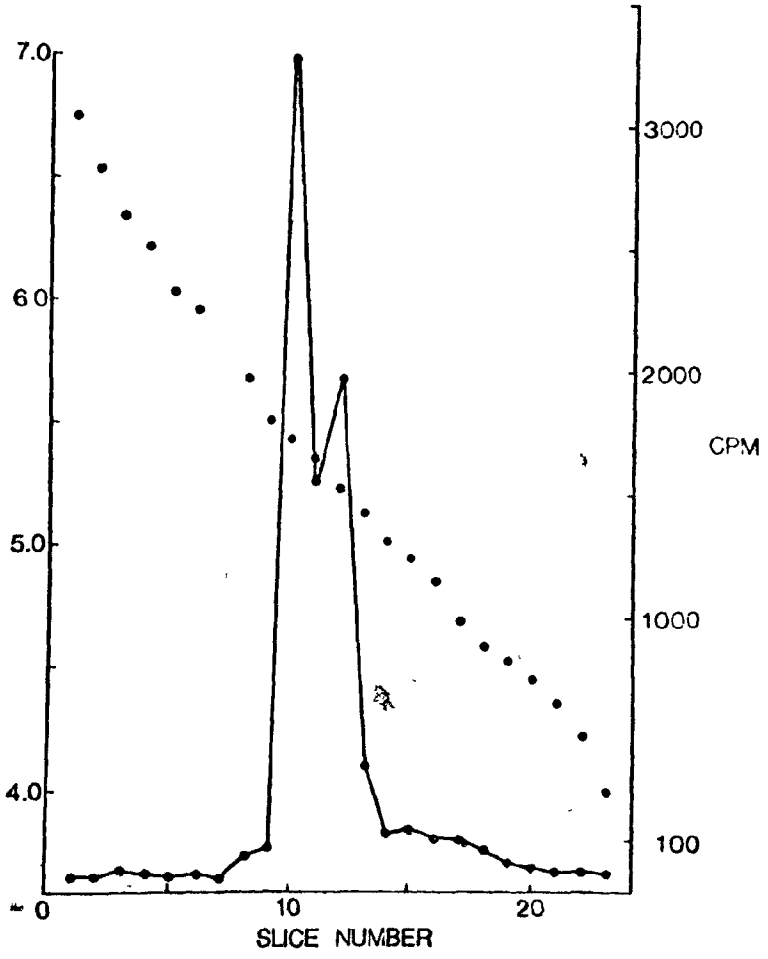

Figure 20

Flat bed isoelectric focusing of [^{125}I] labeled 25,000 dalton protein. Two peaks are shown. The larger focused at pH 5.5 to 5.33 and the smaller peak focused at pH of 5.22 to 5.14.

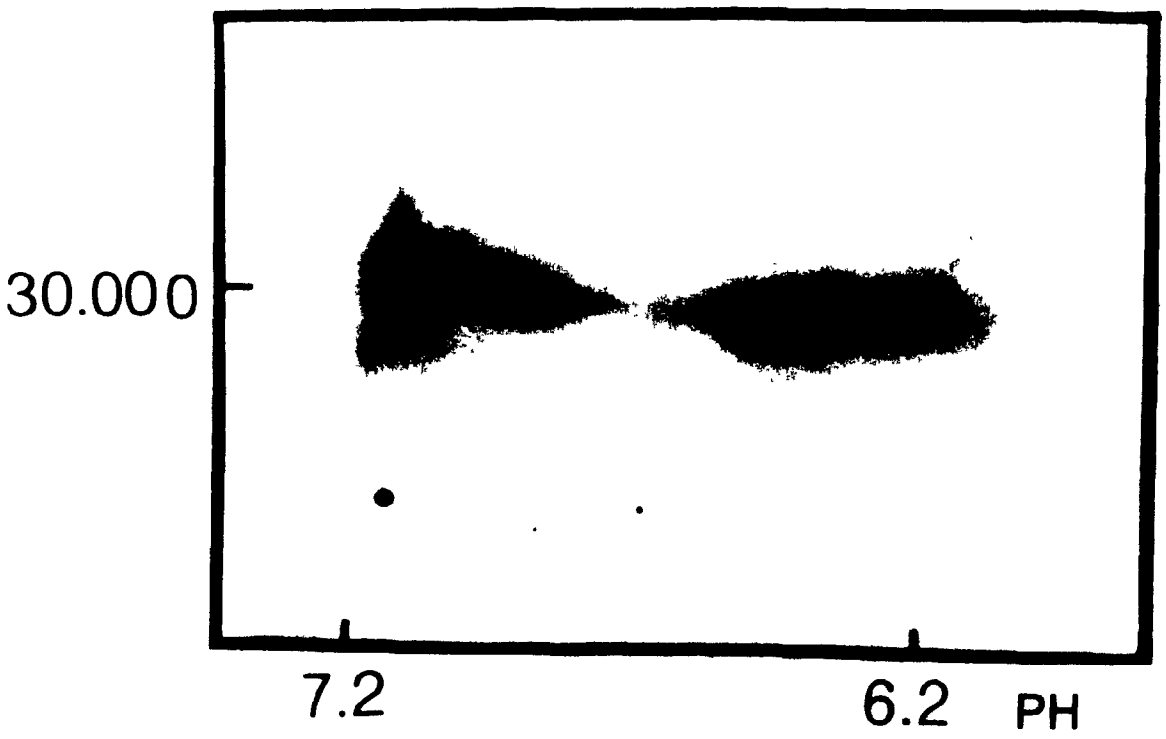

Figure 21

Two dimensional gel electrophoresis (autoradiography) [^{125}I] labeled 31,000 dalton protein electrophoresed in two dimensions in the presence of 8 molar urea. The 31,000 dalton protein resolved into multiple fragments with different PI points ranging between PH 6.2 - 7.2

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CHAPTER VI

Isolation and Purification of Lung Tumor Organ-Specific Neoantigen - Alternate Pathway

The purification scheme as presented in Chapter III, Table 2, was felt to be, in some ways, inefficient in terms of overall yield. Furthermore, it was noted that light chains constitute a major contaminant, and the anti human light chain affinity column had low efficiency in removing light chains and bleeding from it contributed yet more contaminates It was decided to bypass this step by using preparative isoelectric focusing. Likewise, to cut down antigen losses we modified the conditions for protein binding at both the ion-exchange and adsorption chromatography steps

In this chapter, I present the results for an alternative scheme for isolating a markedly enriched lung tumor OSN.

Material and Methods

Patients: Five patients having advanced lung cancer were asked to collect daily urine output. All patients had squamous cell carcinoma and were males. Their mean age was 53 years. During urine collection, the patients did not receive chemotherapy or radiotherapy. In all, 90 litres of urine were collected.

Urine collection and ammonium sulfate precipitation of concentrated urine was performed according to the same method described in Chapter III.

Ion exchange chromatography

The concentrated and precipitated urinary protein was dialyzed and equilibrated with 0.003 M Tris HCl pH,7.8, + 0.05 M NaCL. DEAE Sephadex A-50® (Pharmacia, Uppsala-Sweden) was swollen and equilibrated with the same buffer. The gel was poured into a column 5.0 x 60 cm resulting in a bed volume of 500 cc, and the column had a flow rate of 80 ml/hr. After the gel had settled, the sample was applied and eluted in two fractions.

1 Unbound fractions eluted with 0.003 M Tris pH,7 8, + 0.05 M NaCl

2 Bound fraction eluted with 0 003 M Tris pH,7.8, + 0.5 M NaCl

Each fraction was collected separately (3 times bed volume) and concentrated to 5 ml in an Amicon ultrafiltration using a PM-10 membrane.

Adsorption chromatography

Adsorption chromatography was achieved using hydroxyapatite (LKB, Sweden). The preswollen gel was layered in a column 5 0x60 cm to create a bed volume of 300 cc and a flow rate of 100 ml/hour. The gel and sample were equilibrated with 0.005 M Tris HCl pH,6.8 + 0.02 M potassium phosphate The sample from the ion-exchange chromatography was well dialyzed against the starting buffer and applied to the column. Two fractions were eluted from the column:

1. Unbound fraction eluted with starting buffer

2 Bound fraction eluted with 0.005 M Tris HCl pH,6.8 + 0.2 M potassium phosphate.

Each fraction was collected in 1200 ml and concentrated in an Amicon mounted with PM-10 membrane.

High performance liquid chromatography - Ax-300 ion-exchange

Protein after dialysis against 0.003 M Tris HCl pH,8.0 was subjected to high performance liquid chromatography (HPLC) on an Ax-300 exchange column, which is a 10 micron macroporous spherical silica with a bounded polymeric layer of amino. Elution protocols are similar to those on traditional DEAE column (1).

The HPLC pumping system was purchased from Waters Associates, Milford, Mass., U.S.A. Elution of protein was monitored at 280 μm by Waters 450 variable Detector, Milford, Mass., U.S.A., and recorded by Omniscribe Recorder Houston Instruments, U.S.A.

Columns. a) Ion exchange column, Anspec Synchropak AX-300 (25 cm x 1.4 mm i.d)

b) Guard Column - filled with Synchropak ASC (5 cm x 2 mm i.d)

c) Precolumn - filled with Synchrosorb Ax (25 cm x 1.4 mm i.d)

Buffers: Buffer A = 0.003 M Tris acetate pH,8.0; buffer B = 0.003 M Tris Acetate + 0.1 M sodium acetate pH,8.0; buffer C = 0,0 M Tris acetate + 0.5 M sodium acetate pH,8.0

Gradient was generated automatically from 100% A to 100% B over 62 min according to the following program and the flow rate was 1 ml/min. A linear gradient was obtained by following the outlined program.

Time/min	%A	%B	%C	Flow/ml/min
Initial	100	0	0	1
2	100	0	0	1
62	0	100	0	1
63	0	100	0	1
93	0	0	100	1
94	0	0	0	1

RESULTS

Isolation and Purification of Lung Tumor OSN from Urine of Lung Cancer Patients - Alternate Pathway

Three grams of protein were recovered after ammonium sulfate precipitation and were purified according to the scheme presented in Table 7. The amount of protein recovered at each purification is presented in Table 8.

A) DEAE A50 Ion Exchange Chromatography

Since in previous experiments we determined that the putative tumor antigen eluted with low ionic strength buffers. To enhance recovery of antigen, elution was performed with 0.003 M Tris HCl pH,7.8 + 0.05 M NaCl as starting buffer on 5.0 cm x 60 cm column creating a bed volume of 500 ml. The unbound fraction was collected and concentrated. Protein in the amount of 234 mg was recovered (Table 8) and constituted 7.8% of the 3 gm applied.

B) Adsorption Chromatography - Hydroxyapatite

Further purification of the LAI active protein was achieved by subjecting it to adsorption chromatography on hydroxyapatite. The LAI active urinary protein was applied to a 5.0 cm x 600 cm column and eluted with 0.005 M Tris HCl pH,6.8 and to 0.02M potassium phosphate. With this buffer the LAI active protein was not retained by the column. As shown in Table 8, 145 mg of protein was eluted in the unbound fraction, constituting 1.3% of the initial amount of urinary protein processed.

TABLE 7

Purification of lung tumor organ specific neoantigen alternate pathway

1. Urine protein precipitated by 80% ammonium sulfate. Redissolved precipitate in 0.003 M Tris pH,7.8 + 0.05 M NaCl.
2. Ion exchange column: DEAE-A50 Collect fraction eluted by 0.005 M Tris pH,7.8 + 0.05 M NaCl.
3. Adsorption chromatography: hydroxyapatite collect unbound fraction eluted by 0.005 M Tris HCl pH,6.8 + 0.02 M potassium phosphate buffer pH,6.8.
4. Preparative isoelectric focusing collect protein in pH,5 to pH,6.
5. Molecular sieve chromatography: G-75 superfine collect P44 fraction.
6. High performance liquid chromatography - ion exchange - Ax-300. Collect separate fractions.

TABLE 8

Yield of lung tumor OSN purification from urine of patients with metastatic lung cancer - alternate pathway

Purification step	Total protein (mg)	µg/unit*	lung OSN units recovered	% recovery	Sp.Ac. u/mg	enrichment (fold)
Urine protein precipitation	3000					
Ion exchange chromatography	234			7.8		
DEAE-A50-eluted with 0.003 M Tris pH,7.8 + 0.05 M NaCl						
Adsorption chromatography hydroxyapatite - eluted with 0.005 M Tris pH,6.8 + 0.02M K_2PO_4	145			1.3		
Preparative isoelectric focusing fractions focused at pH,5.6	9			0.3		
Molecular sieve chromatography P44 fraction	2,2	2	1100	0.07	550	
High performance liquid chromatography - 2.2 min	0.4	0.1	4000		10,000	40
- 8.3 min	0.2		50		250	
- 14.5 min	0.1					

*The minimal amount of protein required to specifically block the LAI response plus one unit of activity is defined as the amount of material which will negate the LAI response.

C) Preparative Flat Bed Isoelectric Focusing

It was demonstrated (Chapter V) that the LAI active urinary isolate focused in two main peaks. OSN activity was found in the larger peak which focused at pH 5.0 to pH 5.8. Hence, previously lyophylised LAI active urinary protein was subjected to flat bed isoelectric focusing on agarose. Proteins that focused at pH,5, to pH,6.0, were eluted and pooled. Protein in the amount of 9 mg was recovered (Table 8).

D) Molecular Sieve Chromatography - Sephadex G75 Superfine

Further purification of the material recovered from the previous step was achieved by molecular sieve chromatography on Sepharose G-75 superfine. The elution profile of this column was monitored at 280 nm and is presented in Figure 22. Four main peaks were evident, corresponding to elution volume of gamma globulin, bovine serum albumin (BSA), ovalbumin and chymotrypsinogen. With this order of the purification, proteins were found at the elution volume of gamma globulin and BSA. However, the peaks corresponding to the elution volume of ovalbumin (P44) and chymotrypsinogen (P25) were similar to these presented in Figure 5 (Chapter III). Proteins eluted in these peaks were 2.2 mg and 3.0 mg, respectively and were separately pooled. Their activity in the LAI assay was determined.

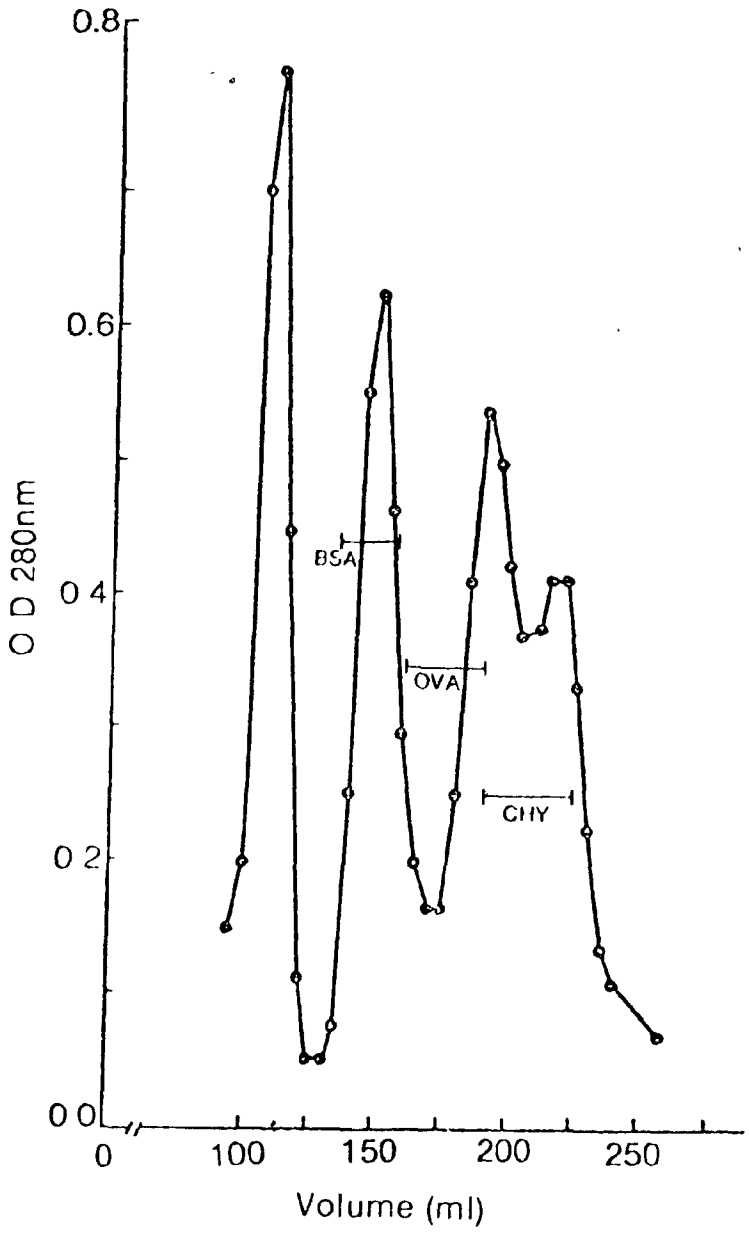

Figure 22

Elution profile of urinary protein isolate on molecular sieve chromatography-Sephadex G-75 superfine. Elution recorded at 280 nm. Peaks corresponding to elution, volume of ovalbumin (P44) and chymotrypsinogen (P25) collected separately and concentrated.

Blocking LAI experiments with urinary protein isolates - P44 from the molecular sieve chromatography

Since the fractions taken in this scheme were slightly wider than similar fractions taken previously and shown to contain LAI active protein, only at the last step of molecular sieve chromatography was the material checked for LAI activity. Figure 23 shows the results. Urinary isolate P44 (eluted at the elution volume of ovalbumin) demonstrated activity in the blocking of the LAI assay at protein concentration from 30 µg to 2 µg but no activity was demonstrated with lower protein concentration. Specificity was determined since the urinary isolate was unable to abrogate the LAI response of leukocytes derived from colon cancer patients. No activity was demonstrated when P25 (eluted at the elution volume of chymotrypsinogen) was tested in the blocking assay.

SDS PAGE

Figure 24 shows the patterns of the various isolates on SDS gel-stained with Coomassie blue. A faint protein band with an apparent molecular weight of 31,000 dalton is seen in lanes 2 to 5. The P25 fraction from the molecular sieve column, which was devoid of activity in the blocking assay, did not show a band at 31,000 dalton on SDS PAGE.

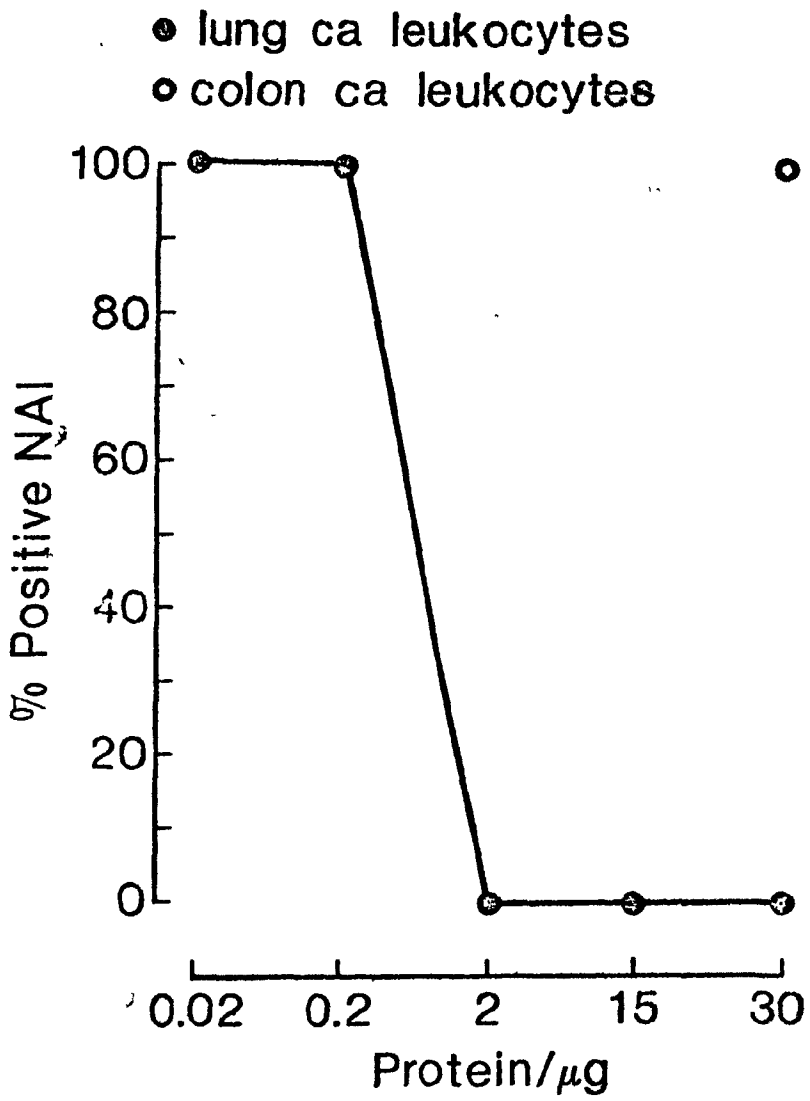

Figure 23

Titration of the activity of protein urinary isolate after molecular sieve chromatography. ●— blocking with PBL derived from lung cancer patients activity in the blocking assay is demonstrated in protein concentration ranging from 30 μg to 2 μg. ○— blocking with PBL derived from colon cancer patients. No activity is demontrated at maximal concentration (30 μg).

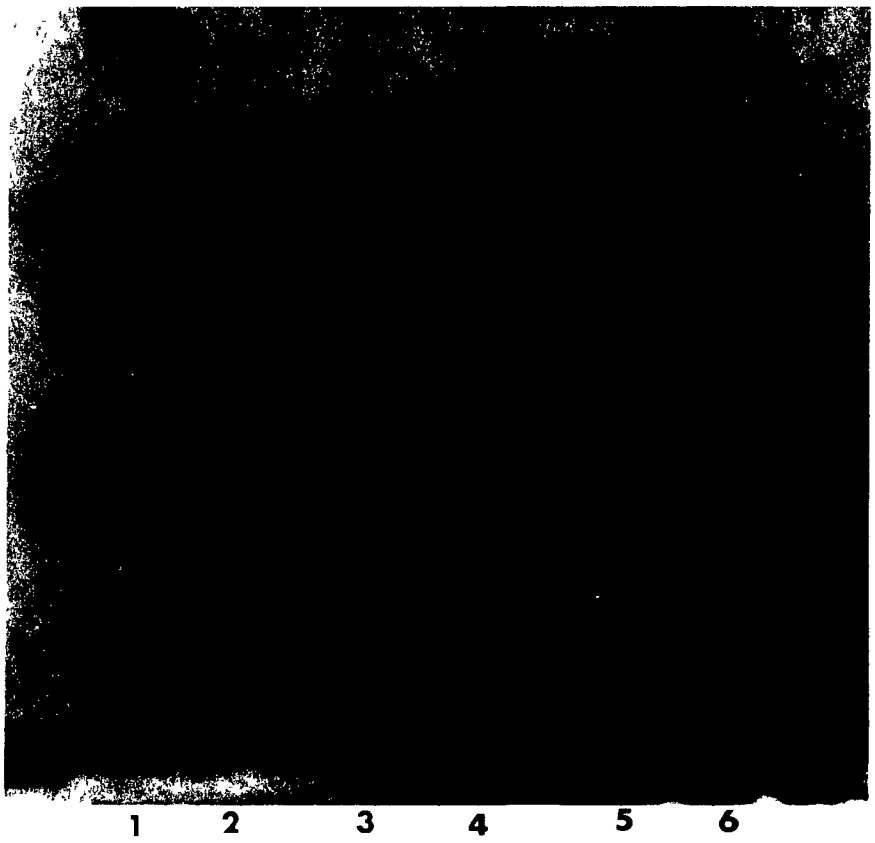

Figure 24

SDS analysis (Comassie blue stain). Various purification steps according to alternate scheme.

Lane 1 - molecular weight standards - (top to bottom) phosphorylase (90,000) albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), chymotrypsinogen (25,000) and cytochrom C (12,400)

Lane 2 - protein eluted at low ionic strength from the ion exchange chromatography DEAE A50.

Lane 3 - protein eluted at low ionic strength from adsorption chromatography (Hydroxyapatite)

Lane 4 - protein eluted from flatbed isoelectric focusing at pH 5-6.

Lane 5,6 - protein eluted off molecular sieve chromatography - P44 and P25 fractions.

E) High Performance Liquid Chromatography Ion Exchange - Ax-300

Ax-300 ion exchange column designed for use in high performance liquid chromatography (HPLC) was the last step of purification because this method was rapid, reproducible and seemed to have superior ability to resolve proteins. By this technique, we hoped to obtain a homogeneous LAI active protein without denaturation, thus overcoming the problems of equivocal specificity encountered after elution of protein from SDS PAGE.

The LAI active protein from the G-75 molecular sieve chromatography, in the amount of 0,7 mg; was applied to the column. The elution of the various peaks was monitored at 280 nm and collected manually. The elution profile is shown in Figure 25. There were three main peaks at elution times of 2.2 (PK 2.2), 8.5 (PK 8.5), and 14.5 (PK 14.5) minutes and each was collected separately.

SDS PAGE

To show the differences in the polypeptide chains which in turn might be the LAI active polypeptide, the protein from the 2.2 minute and 14.5 minute peaks were [^{125}I] labeled. The same number of radioactive counts from each peaks were analyzed by SDS PAGE. Figure 26 shows the results. Lane 1 shows the proteins in PK 2.2 indicating the presence of 31,000 and 28,000 dalton proteins. Conversely, Lane 2 shows the proteins in PK 14.5; the 31,000 dalton protein is not seen while the 28,000 dalton is apparent.

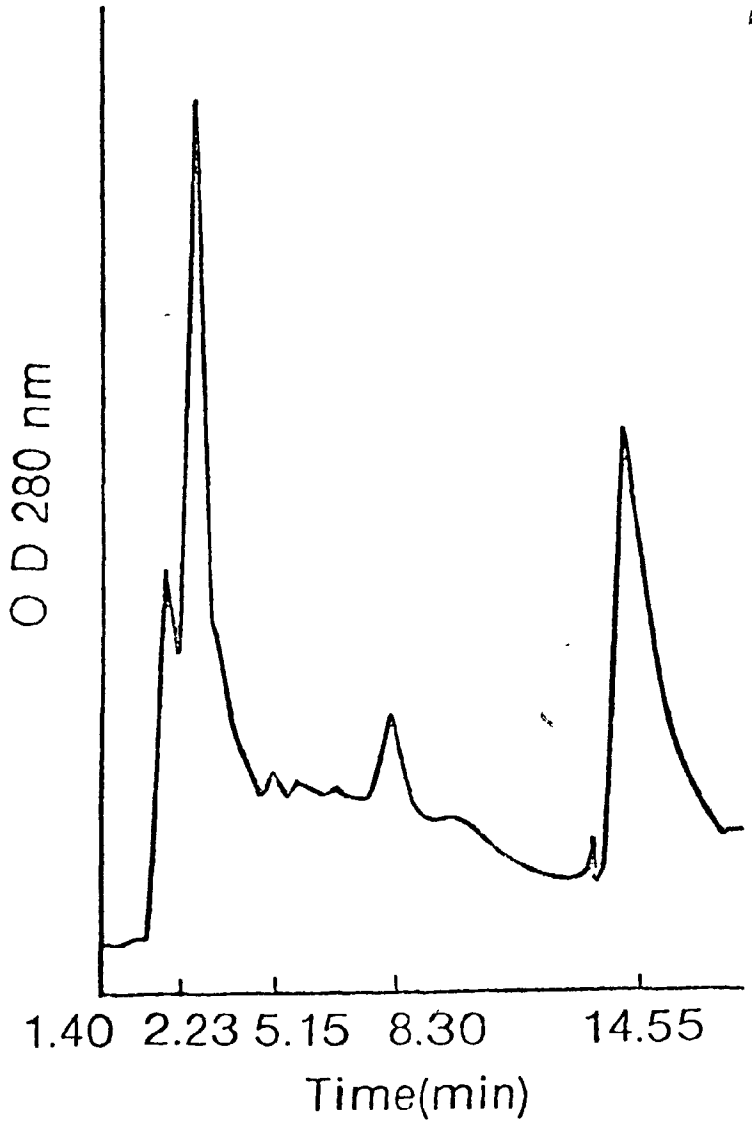

Figure 25

Elution profile of urinary protein isolate from high performance liquid chromatography (HPLC) - ion exchange. Peaks eluted at different time elapsed since application on to the column.

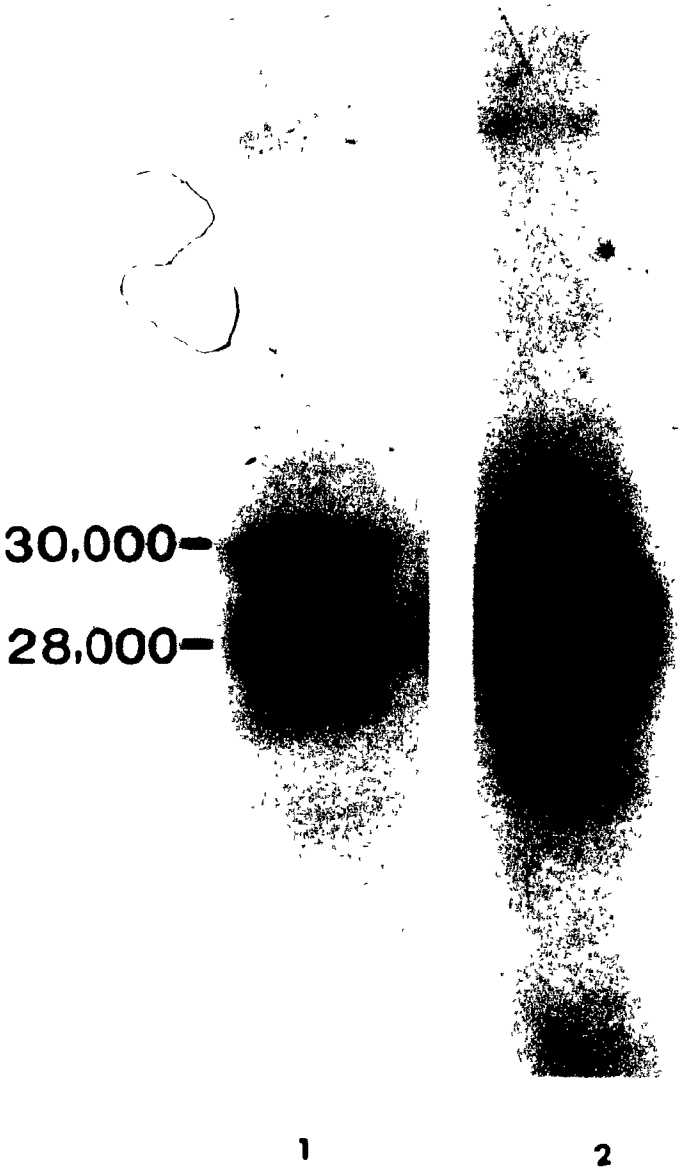

Figure 26

SDS PAGE analysis (autoradiography) of [^{125}I] labeled isolate, PK 2.2 and PK 14.5 of the HPLC ion exchange column. LANE 1 - protein eluted at PK 2.2 to note the evident 30-31,000 dalton protein. LANE 2 - protein eluted at PK 14.5 - no evidence for the presence of the 31,000 dalton protein band.

HPLC ion exchange-Ax-300 fractionation of combined LAI active protein

The LAI active protein derived from the last stage of purification from both pathways was combined, namely: 1) Adsorption chromatography-unbound fraction from the first pathway; 2) Molecular sieve chromatography - P44 fraction from the alternate pathway.

After extensive dialysis and equilibration with the starting buffer, a total of 2 mg protein consisting of 0.9 mg and 1.1 mg, respectively, were applied to an HPLC ion exchange Ax-300 column (25 cm x 1.4 mm). Figure 27 shows the elution profile from this column. Four peaks were seen, at different times after sample application. The first peaked at 2.53 minutes (PK 2.5) while second, third and fourth peaked at 7.00, (PK 7.0) 8·10 (PK 8.1) and 13:35 (PK 13.3) minutes.

Blocking studies with urinary isolates from HPLC ion exchange, Ax-300

Figure 28 shows the blocking assays carried out with each peak eluted from the HPLC column. PK 2.5 was the most active and showed a dose-response inhibition of the LAI response of PBL from lung cancer patients from 1 µg to 0.001 µg, whereas the same protein at the amount of 0.0001 µg failed to negate the LAI response. PK 7.0, 8.1, and PK 13.3 were also titrated in the blocking assay and were less active since they blocked the LAI response of PBL from lung cancer patients only at concentration ranging from 1 µg to 0.01 µg.

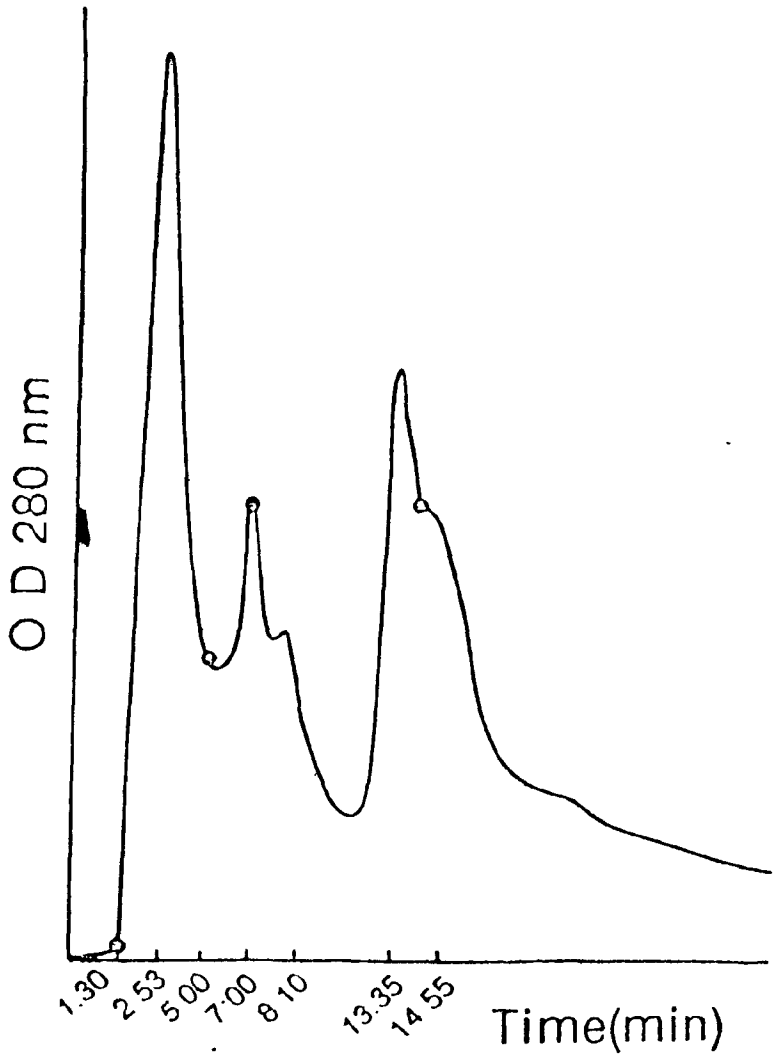

Figure 27

Elution profile from HPLC ion exchange Ax-300 column. Four peaks are present, each eluted at different time elapsed since application of protein. Peaks 1, 2, 3, and 4 peaked at 2:53, 7:00, 8:10 and 13:35 minutes, respectively.

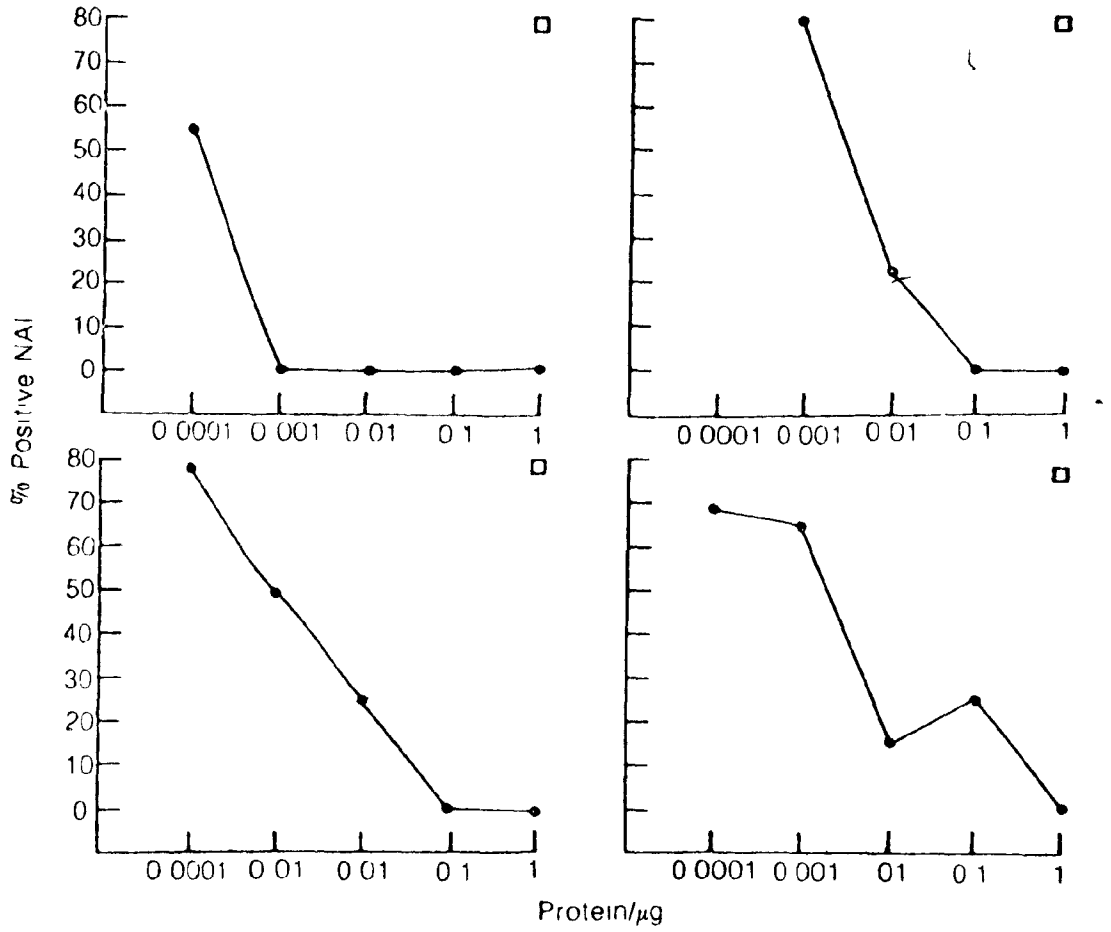

Figure 28

Titration of LAI activity of urinary protein isolate after passage through the HPLC column

PK 2.5 (top right) - managed to abrogate the LAI response of PBL from lung cancer patients at concentration ranging from 1 μg to 0.001 μg No activity demonstrated at the amount of 0.0001 μg. Specificity determined as 1 μg of this fraction failed to abrogate the LAI response of PBL from melanoma patients.

PK 7 0, 8.1, 13.3 - managed to abrogate the LAI response of PBL from lung cancer patients in protein concentration ranging from 1 μg to 0 01 μg. No activity demonstrated at lower concentration. Specificity of the blocking determined in all fractions as 1 μg of protein failed to negate the LAI response of PBL from melanoma patients.

All four blocking experiments were specific because 1 µg of protein from each peak failed to negate the positive response of PBL from melanoma patients.

SDS PAGE

Figure 29 shows the SDS PAGE pattern after autoradiography of [^{125}I] PK 2.5 protein and [^{125}I] PK 13.3 protein. Similar radioactive counts were placed in two separate wells and analyzed by SDS PAGE. The autoradiograph was intentionally over exposed to detect minor protein bands. Lane 1 shows the protein eluted in PK 2.5. One main protein band was evident at 30,000 - 31,000 dalton, minor bands at 40,000, 25,000 and 20,000 dalton were also seen. Lane 2 shows the protein eluted in PK 13.3. The most prominent protein band was seen at 30,000 - 31,000 dalton as well as 28,000 and 20,000 dalton. A faint band was also seen at about 40,000 dalton.

Two dimensional gel electrophoresis

Figure 30 and 31 show the two dimensional gel electrophoresis of [^{125}I] labeled proteins eluted in PK 2.5 and PK 13.3. Both fractions were separated into multiple discrete spots and only proteins of 30,000 - 31,000 daltons were seen, having an isoelectric point between 6.2 - 7.2.

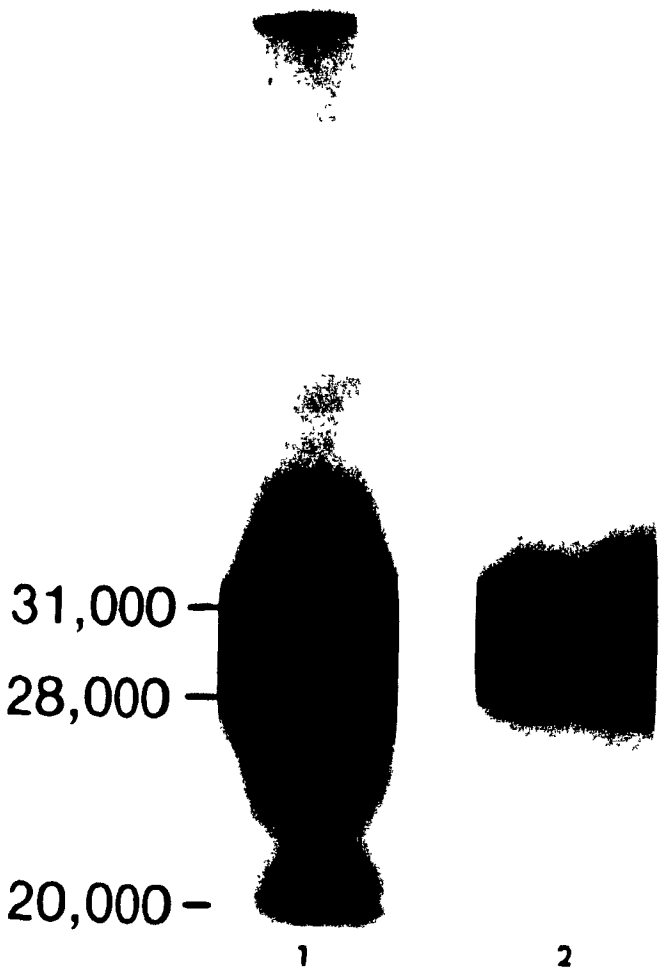

Figure 29

SDS PAGE analysis (autoradiogram) of [^{125}I] labeled urinary isolate.
Lane 1 - protein eluted in PK 2.5 from HPLC ion exchange. One main protein band is evident at 30 - 31,000 dalton, minor band are seen corresponding to molecular weight of 45,000, 25,000 and 20,000 dalton.
Lane 2 - protein eluted in PK 13.3 from HPLC ion exchange. Most prominant is the 31,000 dalton band as well as 28,000 and 20,000 dalton. Faint bands are seen at 40,000 dalton.

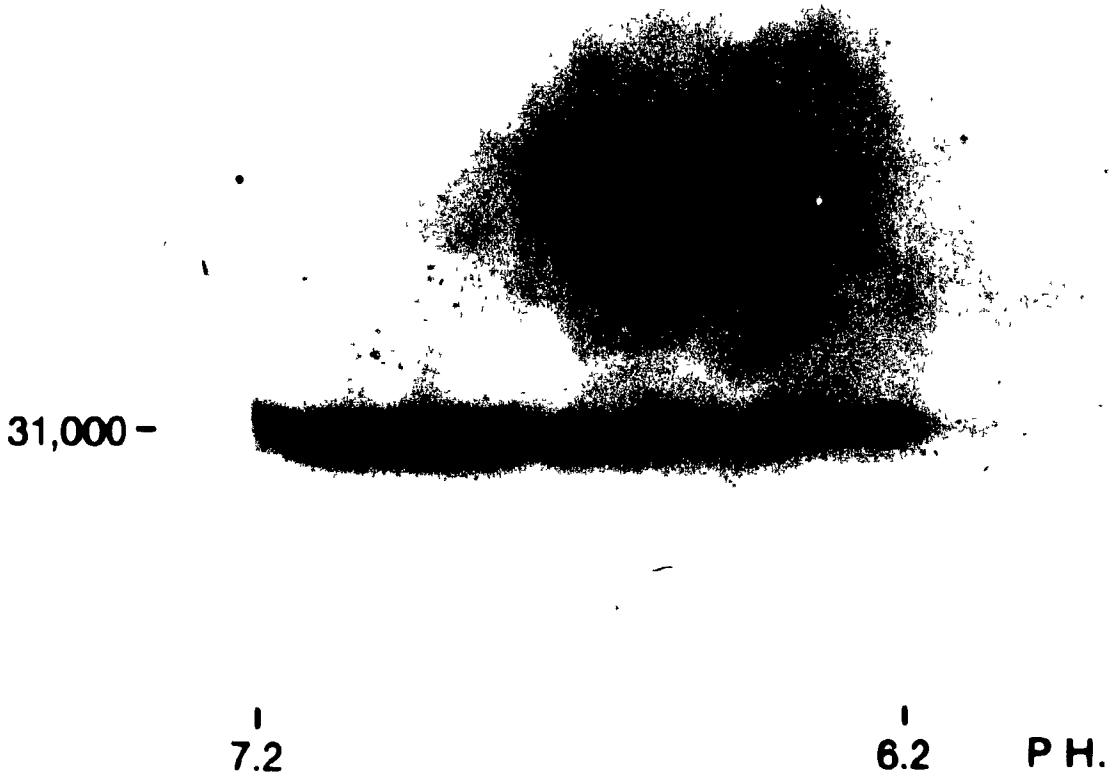

Figure 30

Two dimensional gel electrophoresis [^{125}I] labeled PK 2.5 eluted from HPLC ion exchange column. This fraction resolved in the presence of 8M urea into multiple fragments having molecular weight of 31,000 dalton and PI points ranging between pH,6.2 - 7.2.

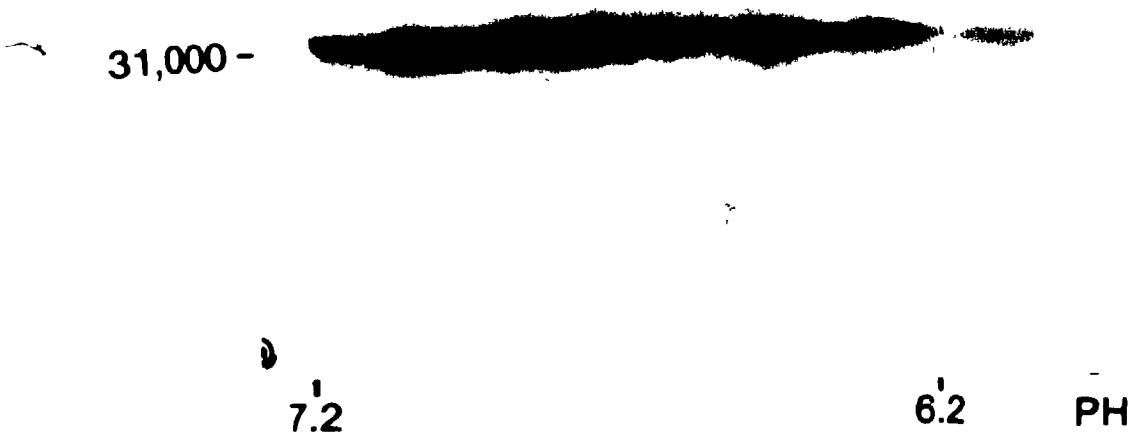

Figure 31

Two dimensional gel electrophoresis [^{125}I] labeled PK 13.3 eluted from HPLC ion exchange column. This fraction resolved in the presence of 8M urea into multiple fragments having molecular weight of 31,000 dalton and PI points ranging between pH,6.2 - 7.2.

REFERENCE

1. Majors, R.E. 1980. Recent advances in HPLC packing and columns. J. of Chromatographic Science 18, 488.

CHAPTER VII

General Discussion

The studies presented in this thesis describe the detection, isolation and characterization of lung tumor specific neoantigen. This work was based on two premises: 1) Organ specific neoantigens of human cancer exist and are recognized by the primary host's immune response. 2) The LAI assay as employed in our laboratory and elsewhere (1, 2, 3) detects, *in vitro*, the immune response of the primary host against the organ specific neoantigen (OSN).

As was previously stated, OSN are shed from the tumor cell surface during cell metabolism and probably also with cell death (4, 5). Some circulating OSN is subsequently filtered into the urine retaining its immunogenicity to the authochtonous and allogeneic tumor-bearing host when tested *in vitro*. It was demonstrated by Jehn et al (6) and Gupta et al (7) that the amount of OSN filtered through the urine is proportionally dependent on tumor mass. Upon tumor excision the amount of tumor antigen in the urine decreases greatly. We thought that the urine might serve as an excellent source of OSN for purification since urine samples were available and relatively easy to collect. Furthermore, by collecting urine from non proteinuric patients some natural purification might be expected. Although *in vitro* assays detected the presence of the OSN in the urine of cancer patients, it was well appreciated that the absolute amounts of OSN in the

total urinary protein was very low. This fact is well appreciated by examining studies done on other cell surface molecules which are also secreted in the urine. Although HLA coded antigens are present in abundance in many organs, the quantity of the HLA antigens in normal urine is very small, indeed Reisfeld et al (8, 9) and Bernier et al (10, 11), when trying to isolate HLA-A9 from urine, were obliged to collect urine from patients after kidney transplantation or from patients with cystinosis in which a great amount of protein was excreted. In order to avoid gross contamination of the OSN by serum proteins, it was necessary to exclude patients with proteinuria. Only after collecting 300 litres of urine and precipitating 10 gm protein were we able to isolate reasonable quantities of OSN. Urine was collected from two types of lung cancer, namely, oat cell carcinoma and bronchogenic carcinoma. Pooling of these two different sources was possible since it was demonstrated from earlier studies by Ayeni et al (12) that the OSN are organ specific and depend on the histogenesis of the tumor. By pooling the urine samples, we also sought to avoid arguments that the recovered OSN was present only for a particular patient rather than most patients with advanced lung carcinomas.

The necessity of monitoring each purification step by the blocking assay and the scarcity of LAI positive leukocytes from lung cancer patients

(mostly from Stage I and II) obviously slowed the purification process. Faster progress was achieved after the demonstration in our laboratory by Kaneti et al (13) that prostaglandin E_2 converts the leukocytes of advanced cancer patients from a nonresponder to a responder state and that the same leukocytes maintain their activity in the blocking assay.

To limit the number of LAI assays necessary, to speed the purification process, and to decrease losses during the purification scheme (Chapter III), an alternate purification scheme was devised (Chapter VI). Comparing both purification schemes, it is evident that the second is faster and has a much higher yield. The alternate purification scheme was based upon knowledge gained by the first purification scheme; nonetheless, the alternate scheme is now recommended since the number of blocking experiments is decreased to a minimum and the yield of OSN is greatly increased.

Three major steps were the key elements in both purification procedures: 1) DEAE ion exchange chromatography; 2) molecular sieve chromatography; 3) adsorption chromatography. These three precedures were able to purify the lung tumor antigen to a great extent since they depleted much urinary protein that was of no interest, namely: albumin, gamma globulin, and light chains. DEAE ion exchange chromatography as a first step seems logical since it removed the bulk of contaminating urinary protein. Hydroxyapatite adsorption chromatography focused the LAI active urinary protein in one fraction that was almost devoid of light chains. Light chains were a bothersome contaminant that could be removed only by repeated passages through an anti-light chain. However, the anti-light chain affinity column was associated with losses and contamination of the

sample because of bleeding protein from it. Molecular sieve chromatography served to concentrate on proteins in the range of 43,000-31,000 daltons where the LAI active protein was found.

The presence of the OSN was monitored by the blocking tube LAI assay. Inherent in this assay is the fact that blocking should be demonstrated to be specific. Specificity in this assay is achieved by the showing that the urinary protein isolate blocked the response of leukocytes derived from lung cancer patients and not the response of leukocytes from patients with other types of cancer (colon, melanoma).

The LAI phenomenon was shown by Marti et al (14) to depend on the presence of cytophylic antitumor antibodies. These antibodies "arm" blood monocytes by the virtue of the latter's Fc receptors. Only specific tumor antigens, which the antibodies are directed against, can bind to the monocytes and activate them to produce mediators which will amplify the in vitro reaction by causing the leukocytes' to be nonadherent to the glass. The preincubation in vitro of PBL from a lung cancer patient with the sensitizing cancer antigen result in antigen binding and monocyte activation. So if the urinary isolate contains tumor antigen, upon incubation with PBL previously shown to be LAI positive the cells will be actived and upon subsequent incubation with the corresponding tumor extract will not react. Conversely, if the same experiments are done with PBL derived from patients having a different type of cancer (colon, melanoma) they will not bind the antigen and be activated. Consequently, the monocyte will respond on a subsequent incubation with the specific tumor extract resulting in leukocyte mobility (nonadherence). In this fashion the specificity of the urinary isolate is shown.

The results of the experiments showed the presence of the lung cancer OSN in the urinary isolate and its specificity. Specificity was not demonstrated with one sample: partial blocking was obtained when the 31,000 dalton molecule was cut from SDS gels and was tested with PBL from colon cancer patients.

LAI activity is manifested by a protein having a molecular weight less than 43,000 dalton but not less than 30,000 dalton

Since we failed to show specificity of the homogeneous 31,000 dalton protein, only indirect evidence is available about the specific protein band that might possess the OSN epitope. Several lines of evidence suggest that the molecular weight of the LAI active protein was lower than 43,000 daltons:

1) The LAI active protein was constantly eluted from a molecular sieve chromatography with the elution volume of ovalbumin (P44).

2) SDS PAGE pattern from adsorption chromatography consisted of protein in the molecular weight range of 31,000 dalton to 20,000 dalton.

On the other hand, it seemed that LAI activity is not dependent on the presence of proteins having a lower molecular weight than 30,000 dalton, since:

1) Proteins eluted in the elution volume of chymotrypsinogen (P25) were devoid of LAI activity.

2) Normal urine processed according to the same purification scheme showed an SDS PAGE pattern devoid of proteins having a molecular weight higher than 28,000 dalton.

3) LAI active proteins resolved on HPLC ion exchange also demonstrated activity that was not dependent on the presence of molecules with a molecular weight lower than 31,000. The active fraction eluted at 2.55 minutes and consisted of a major band resolving at about 31,000 dalton although it contained some minor bands at higher and lower molecular weights. The fact that LAI activity may be demonstrated at a concentration of 0.001 µg suggested that the activity was probably dependent upon polypeptides well visualized on SDS gels. Material with less activity eluted at 13:35 minutes and consisted of a protein band at 31,000 dalton with lesser degree of intensity when visualized on autoradiography.

Physicochemical characteristics of a lung tumor OSN

The results enables us to define some features of the lung tumor OSN derived from lung cancer patients' urine.

1) Precipitated in 80% ammonium sulfate.
2) Molecular weight in the range between 40-31,000 dalton.
3) Glycoprotein
4) Acidic isoelectric point at about pH 5.0.

Lung OSN is common to all lung tumors

Our studies indicate that the lung tumor OSN was an antigen found on different histological types of lung cancer since it blocked the reactivity of sensitized PBL from patients with oat cell and squamous cell carcinoma.

Likewise, PBL from patients with adenocarcinoma, oat cell carcinoma and squamous cell carcinoma all react to a tumor antigen shared by lung tumors of different histologic types (12). McCoy et al (15) using leukocyte migration inhibition assay (LMI) also showed that there was no correlation between LMI reactivity and histological type of the lung tumor since PBL from patients with oat cell, squamous cell and adenocarcinoma were reactive against the same lung cancer extracts. Thus it is possible that the lung OSN expresses an epitope that is specific to the lung histogenesis but is unrelated to the histological appearance.

Since most studies on isolated lung tumor antigens used xenoantisera to detect the lung antigen, there is no correlation between these results and mine. However, data surveyed from the literature survey will be cited. Wolf (16) and Wolf et al (17) described a lung tumor antigen derived from a pleural effusion from patients with squamous cell carcinoma that was purified physicochemically and recognized by xenoantisera raised in rabbits to biopsy material of squamous cell carcinoma. The purified antigen had a molecular weight of 43,000 daltons and was common to all histological types of lung cancer. In another study, (18, 19), xenoantisera was raised in monkeys and rabbits against highly purified plasma membrane of an oat cell carcinoma of the lung and the specificity of the antisera was found to be common to all histological types of lung cancer. In another approach (22), xenoantisera was raised to extracts of human bronchogenic carcinoma by also passively immunizing the rabbits to normal human lung extracts at the same time as immunizing with the cancer extracts. When tested by quantitative complement fixation (21, 22), immunodiffusion (38) or enzyme linked

immunoabsorbent assay (ELISA) (20), a tumor antigen was demonstrated having a molecular weight of 100,000 daltons which shared specificities with all lung carcinomas with no distinction with regard to histological types. Kempner et al (23) also showed no histological specificity of a 32,000 dalton protein derived from pooled malignant lung tissue and recognized by xenoantiserum raised in a rabbit. Although these studies clearly show that xenoantisera recognizes all histological types of lung cancer, it is also evident that the specificity of the antisera is not absolute and cross-reacts to some degree with normal lung tissue (22) and other tumors, especially colon and breast carcinoma (17, 18, 23) and neural tissue (19, 23).

Lung tumor OSN is a fetal component

A key element in oncology is the notion that cancerous tissue resembles in some respect the corresponding fetal organ. It is postulated that gene depression may be operational and re-expression of fetal proteins will ensue. There is some evidence that these fetal proteins are immunogenic in the primary host thus fulfilling the criteria for tumor OSN.

In delayed type skin hypersensitivity, Herberman (24) showed that cancer patients respond in a positive manner to the injection of soluble tumor OSN from allogeneic as well as autologous sources. Furthermore, the same positive skin reactions were obtained by injecting intradermally the corresponding fetal organ.

Similarly Vose and Moore (25) using cell mediated cytotoxicity showed that PBL from cancer patients not only showed greater cytotoxicity when exposed to the corresponding tumor cells but also to fetal cells. Likewise,

Zoller et al (26) arrived to the same conclusion using the leukocyte migration inhibition assay.

This study was not concerned with the fetal origin of the OSN recovered from lung cancer patients. Nevertheless, recent studies performed in our laboratory have shown that in the LAI assay, lung tumor extracts are recognized by PBL derived from multiparous pregnant women up to 20 weeks of gestation. Furthermore, fetal lung extracts from fetuses between 11-19 weeks of gestation can substitute, in the standard LAI assay, for the lung tumor extract.

Other studies, employing xenoantisera raised against tumor extracts, also mention the occurrence of cross reactivity between lung tumor antigen and fetal lung. Kempner et al (23) demonstrated that the antiserum raised in xenogeneic animals cross reacts with fetal lung tissue. Likewise Sega et al (27, 39) isolated by means of xenoantiserum, raised in a rabbit against a pool of lung cancers (epidermoid and adenocarcinoma), a lung tumor antigen common to all lung cancers that recognizes the same epitopes existent in fetal lung and liver (gestation age of 10-20 weeks).

Lung OSN is not related to HLA coded molecules

Soluble HLA antigens have been detected in the urine (28). These molecules were found to have a molecular weight of 40,000 daltons and a pI at pH of 5.0

Our studies indicate that lung OSN isolated from the urine of lung cancer patients was probably not related to molecules coded for by the HLA genes. The evidence presented in this study consists of the failure to immunoprecipitate the [^{125}I] labeled LAI active protein with monoclonal antibodies specific to molecules coded for by the A, B and D alleles. Furthermore, we also failed to precipitate the same molecules with antibodies to HLA raised in rabbits that showed in previous studies by Rauch et al (29) to recognize the common epitope of HLA A,B coded molecules. These results are in contrast to those presented by Rauch et al (29) and Thomson et al (30) who isolated OSN from melanoma, hepatoma and breast cancer. LAI active protein was derived from these tumors by limited papain digestion of the tumor cell membrane. The OSN was shown by means of anti-β_2 microglobulin affinity chromatography to copurify with β_2 microglobulin and HLA molecules. HLA activity, by radioimmunoassay, resided in the same fraction which was bound to anti-β_2 microglobulin affinity chromatography. On SDS PAGE the LAI active fraction showed proteins in the molecular weight range of 40,000 to 12,000. My results clearly differ since I failed to demonstrate HLA activity within the LAI active fraction or the presence of β_2 microglobulin by either immunoprecipitation or SDS PAGE.

The results obtained in the present study are in agreement with McCabe et al (31, 32) who could not find any correlation between melanoma associated antigen and HLA antigens nor with β_2 microglobulin. Conversely, Malley et al (33) demonstrated that melanoma antigen activity as assessed by mean of the LAI assay, derived from melanoma extracts bound to anti-β_2

microglobulin affinity column. The evidence to date is conflicting on whether tumor OSN are associated with HLA antigen or are modified HLA antigens. It seems that tumor OSN obtained from solid tumors as was done by Rauch et al (29) Thomson et al (30) and Malley (33) still retained their association with β_2 microglobulin. Tumor OSN obtained from spent culture medium (33) or from urine did not display this association. It is possible that tumor OSN in culture medium, systemic circulation and urine dissociate from β_2 microglobulin, while in their native state this association may exist.

One conclusion that may be drawn from this study is that β_2 microglobulin is not essential for OSN activity in the LAI assay, indicating that the epitope which is recognized by the immune response does not contain sites involving parts of the β_2 microglobulin molecule. β_2 microglobulin is associated with molecules outside the H-2 locus such as T/t and Qa or with other genetic loci such as the H-Y system (41). It seems that $\beta_2$microglobulin is not exclusively associated with the MHC complex but may be associated as well with genetic loci that have regulatory roles during cell differentiation. Since it is possible that the OSN may function as a differentiation antigen, playing a major role in organogenesis, the possible association of the OSN with β_2 microglobulin is not surprising or contradictory.

Lung tumor OSN does not contain known lung tumor associated molecules

In 77% of patients with oat cell carcinoma of the lung one may detect by means of a radioimmunoassay elevated levels of CEA. I demonstrated the absence of this molecule in our lung tumor OSN preparation. Previously Thomson et al (34) showed that LAI active protein in urine derived from patients with colon carcinoma is devoid of CEA. Furthermore, I demonstrated

that a smaller molecular weight protein having a molecular weight of 50,000 dalton and immunologically cross reactive with CEA was also absent.

Others have used xenoantisera for detection of lung tumor associated antigen and did not detect the presence of CEA (35, 40). Another tumor marker, lactoferrin, present in patients with lung tumors was not examined because it has a high molecular weight that does not correspond to the molecular weight of the lung tumor OSN. Furthermore, by immuno-precipitation of the [^{125}I] urinary OSN isolate with anti-serum to lactoferrin, no precipitates were obtained.

Recently Braatz et al (36) and Gaffar et al (37) described a lung tumor associated antigen having a molecular weight of 42,000 dalton. This lung tumor associated antigen was purified by means of xenoantisera raised in rabbits against a pool of 17 lung cancer extracts. The tumor associated antigen was purified by affinity chromatography, gel filtration and preparative gel electrophoresis. The same authors have demonstrated (36) that their preparation of lung tumor associated antigen cross reacted with α_1 antichymotrypsin present in abundance in lung cancer tissue. Coded samples of various purification steps of our lung OSN were analyzed by Braatz's group by means of radial immunodiffusion, it was found that fractions containing the LAI active protein was devoid of α_1 antichymo-trypsin, though large amounts of this protein was found to reside in LAI negative frations. In parallel the [^{125}I] labeled LAI positive fraction off the hydroxyapatite column was tested in our laboratory by immunoprecipi-tation against a commercially available anti α_1 antichymotrypsin with comparable results.

In conclusion the lung OSN detected did not contain contaminants similar to the described lung tumor associated antigens.

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SUMMARY

In experimentally induced tumors, tumor specific transplantation antigens (TSTA) and oncofetal neoantigens are expressed. Proof for the existance of TSTA in human cancers is precluded by ethical reasons and by the lack of syngeneic donor hosts, but still there is evidence based on *in vitro* assays that there are organ specific neoantigens (OSNs) against which the primary host responds The leukocyte adherence inhibition assay (LAI) is one such assay demonstrating antitumor immunity. The assay is based on the phenomenon that leukocytes from a tumor bearing host, after *in vitro* incubation with an extract of the sensitizing cancer, lose their ability to adhere to glass surfaces. Preincubation of leukocytes that react positively in the LAI assay with tumor OSN negates their subsequent response in the assay - hence blocking. Neoantigens of human cancer and experimentally induced TSTA are shed from the tumor cell surface into the circulation Some of the shed neoantigens are filtered into the urine.

Organ specific neoantigen (OSN) shed from the tumor of patients with metastatic lung cancer and filtered into the urine was purified, in part, using physicochemical and affinity chromatography methods. OSN activity was monitored by blocking of the tube LAI assay.

Two methods for purifying the lung tumor OSN were developed. Urinary protein was precipitated by 80% saturated ammonium sulfate. In the first method, 300 liters of urine were processed. Affinity chromatography was used to remove albumin, gammaglobulin, and light chains. Physicochemical

methods of DEAE ion exchange chromatography, molecular sieve chromatography and hydroxapatite adsorption chromatography achieved further purification of the OSN. Analysis by SDS PAGE revealed that the urinary isolate from the last purification step containing the OSN activity was composed of several protein bands in the molecular weight range of 31,000 to 20,000 dalton. The specific activity of the lung tumor OSN as measured by the blocking tube LAI assay was about 20,000 μ/mg.

Physicochemical characterization of this restricted group of proteins demonstrated that they were glycosilated and had an isoelectric point at pH 5.0 - 6.0. None of the isolated and homogeneous protein bands eluted from the SDS gels showed OSN activity. Furthermore, by limited digestion with V-8 protease and trypsin the proteins visualized in this purification step were shown not to be related. The group of proteins containing OSN activity were not related to HLA antigen nor to β_2 microglobulin as determined by immunoprecipitation with anti-HLA and anti-β_2m antisera. Furthermore, lung tumor associated molecules such as CEA, lactoferrin, and α_1 antichymotrypsin were absent from the material from the last purification step.

The second method used 90 liters of urine and was developed to find a faster and more efficient way to purify the OSN. After ammonium sulfate precipitation of the urinary protein, it was processed by DEAE-ion exchange chromatography, hydroxyapatite adsorption chromatography, preparative flat bed isoelectric focusing, molecular sieve chromatography and ion exchange high performance liquid chromatography. The specific activity of the lung tumor OSN at the last step was about 10,000 u/mg. SDS PAGE analysis of the urinary isolate from this last step disclosed the same group of proteins in

the molecular weight range of 31,000 dalton to 25,000 dalton. Two dimensional gel electrophoresis of the proteins revealed many discrete spots migrating over a pH of 6.2 to 7.2 and all had a molecular weight of 31,000 dalton.

Urinary protein from normal subjects was processed in the same manner and did not show OSN activity. SDS PAGE analysis showed only proteins at the molecular weight range of 28,000 dalton and lower.

From the following studies it is suggested that the protein responsible for OSN activity in the tube LAI assay resided in the molecular weight range of 31,000 to 43,000 daltons. This protein was not associated with β_2 microglobulin nor did it seem to be a modified or altered HLA coded molecule.

OSN is a glycosilated integral membrane protein that is expressed by organs of a normal fetus up to about 19 weeks gestation. In the fetus the OSN may perform some recognition function for organogenesis.

When the OSN is re-expressed on the tumor cell surface (gene derepression), it is organ specific and is common for all tumors arising from the same organ that are of the same histogenesis. The expressed fetal component is recognized as foreign by the syngeneic host bearing a cancer that expresses the OSN molecule.

STATEMENT OF ORIGINALITY

1) Methods for the isolation of lung tumor organ specific neoantigen (OSN) from the urine of patients with metastatic lung cancer weredeveloped.

Both methods employed various physicochemical procedures and were shown to highly enrich a molecule which showed specific activity in the blocking tube LAI assay. The second method which avoided the necessity of affinity chromatography was found to be rapid and efficient. The second method used ammonium sulfate precipitation of urinary protein, DEAE ion exchange chromatography, hydroxyapatite adsorption chromatography, preparative isoelectric focusing, molecular sieve chromatography and ion exchange high performance liquid chromatography.

2) The LAI assay detects the hosts' immune response to a tumor antigen; this antigen was highly enriched and partially characterized. The uniqueness of this tumor antigen lies in its ability to behave as an immunogen in the tumor-bearing host while all lung tumor associated antigens previously described are not. Tumor associated antigens are defined and recognized by antiserum raised in xenogeneic animals. The presently described tumor antigen is a true tumor antigen - termed an organ specific neoantigen (OSN).

3) Healthy individuals urine processed in the same manner did not show any OSN activity in the blocking tube LAI assay. Furthermore, the proteins found in the normal urine isolate were different from those in an isolate

4) OSN activity was confined to proteins having a molecular weight higher than 30,000 dalton, and may be attributed to protein or proteins in the molecular weight range of 31,000 dalton.

5) Partial characterization of single protein bands at 31,000, 28,000 and 25,000 dalton disclosed that they were not inter-related although they were all glycoproteins with an acid isoelectric point.

6) OSN activity was not related to β_2 microglobulin nor to HLA coded molecules. Furthermore, OSN was not related to CEA or other lung tumor associated molecules.