A HUMAN ORGAN-SPECIFIC NEOANTIGEN DEFINED AS A MYCOPLASMA 40 KD PROTEIN (P40)

by

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November 1991

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

ABSTRACT

Human organ-specific-neoantigens (OSN) are tumor molecules found in crude cancer extracts to which patients with cancer of the same organ and histogenesis respond in the in vitro assay of leukocyte adherence inhibition (LAI). P40 is a molecule isolated from spent medium of lung and colon cancer cells, which exhibits OSN properties. Individual peptides of p40 exhibited organ-specificity. Distribution studies on a variety of cultured cell lines, fresh, and fetal tissues showed that p40 is present only on some cultured cells. Using the peptide sequences of p40 a collaborating laboratory isolated and sequenced p40 cDNA from a colon cancer cell line (LS-180). P40 was a Mycoplasma hyorhinis transport protein. Moreover, a series of experiments with anti-mycoplasma reagents showed that p40 is expressed by cells infected with mycoplasma. By the LAI assay mycoplasma proteins stimulated positive cellular responses with cancer patients leukocytes but not with leukocytes from normal controls. A non-infected lung cancer cell line expressed OSN activity distinct from p40.

ABREGE

Les néoantigenes spécifiques d'organes humains (NSO) sont des molécules tumorales retrouvées dans les extraits de cancer non traités auxquels les patients atteint de cancer du même organe et de la même histogénèse répondront à l'analyse in vitro du test d'inhibition de l'adhérence leucocytaire (IAL). P40 est une molécule isolée à partir du milieu épuisé de cellules cancéreuses provenant du poumon et du colon ayant des propriétés manifestant le NSO. Des peptides particuliers de p40 démontraient une spécificité d'organes. Des études seriés d'une gamme de lignées cellulaires, de tissus frais et de tissus foetal ont montré que p40 est présente seulement sur quelques cellules en culture. En collaboration avec le laboratoir de Dr. Stanners et utilisant les séquences peptidiques de p40, nous avons isolé et séquencé le cDNA de p40 à partir d'une lignée cellulaire du colon (LS-180). On a montré que p40 est une protéine de transport du Mycoplasme hyorhinis. De plus, une série d'épreuves avec des réactifs anti-mycoplasme ont montré que p40 est exprimée par cet cellules contaminées par le mycoplasme. Le test de l'IAL, nous a permis de demontrer que les proteines mycoplasmiques peuvent activer une réponse cellulaire positive en présence de leucocytes de patients cancéreux mais aucune réponse positive n'a été observée en présence de leucocytes de personnes normales. Une lignée cellulaire pulmonaire non-contaminée a démontré une activité NSO distincte de p40.

PREFACE

Most of the experiments described herein have been performed by the author. However, certain procedures have taken place in other laboratories and/or by other investigators and are as follows:

All p40 peptides were synthesized in Bio-Rad Laboratories, Hercules, California.

P40 cleavage by CnBr and sequencing of the resultant peptides were performed in Bio-Rad Laboratories, Hercules, California.

Trypsin-digestion of p40 was performed in our laboratory, and sequencing of the resultant peptides was performed at the Shriners Hospital, Montreal, Quebec.

P40 gene cloning was performed at Cancer Center, McGill University, Montreal, Quebec.

ACKNOWLEDGMENTS

I would like to express my appreciation to all those who contributed to the completion of this thesis:

Dr. D.M.P. Thomson, my supervisor, for providing me with a scientific and stimulating environment, and for his guidance on a most interesting and challenging subject and,

Dr. Rauch, my academic advisor, for he valuable help she has given me throughout my study.

I also gratefully acknowledge:

Drs. M. Newkirk and A. Fuks for helpful discussions,

Dr. J.L. Bourque for translating the abstract,

Dr. M. Labateya for performing the LAI assay,

Ms. V. Nguyen for performing column chromatography and butanol extractions,

Ms. M. Sutherland for performing HPLC and tryptic digestion of lung and colon p40,

Ms. N. Morissette for performing inverted microscope photography,

and

my husband Claude for his constant understanding and support throughout the course of my study.

To Claude

LIST OF ABBREVIATIONS

a-	Anti-
aa	Amino Acid
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AFP	α-Fetoprotein
AIDS	Acquired Immune Deficiency Syndrome
AMLTC	Autologous Mixed Lymphocyte Tumor Culture
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
Ax300-HPLC	Anion Exchange High Pressure Liquid Chromatography
β2m	β2-Microglobulin
BCG	Bacillus Calmette-Guérin
BM-Cycline	Anti-Mycoplasma Reagent
BSA	Bovine Serum Albumin
c-onc	Cellular Oncogene
C5a des ar	Fifth Component of Complement
Ca^{++}	Calcium Ion
CBP	Cancer Basic Protein
CD3	A Peptidic Complex Associated with the T Cell Receptor Involved in Signal Transmission
cDNA	Copy DNA
CEA	Carcinoembryonic Antigen
CH	Chymotrypsin
CH_3CN	Acetonitrile
CM	Carboxymethylated
CnBr	Cyanogen Bromide
CNS	Central Nervous System
CTL	Cytolytic T Cells
2D	Two-Dimensional
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid

DNCB	Dinitrochlorobenzene
DTH	Delayed Type Hypersensitivity
DTT	Dithiothreitol
EAE	Experimental Allergic Encephalitis
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
ER	Endoplasmic Reticulum
ETYA	Eicosatetraynoic Acid
FBS	Fetal Bovine Serum
FMLP	N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine
Gal NAc	Galactose-N-Acetylglucosamine
GTP	Guanosine Triphosphate
HEPES	(4-(2-Hydroxyethyl)-1-Piperazine-Ethanosulfonic Acid
HIC-HPLC Pressure Liquid	Hydrophobic Interaction Column High Chromatography
H-2D	Gene Encoding MHC-Class I Molecules in Mice
HIV	Human Immunodeficiency Virus
HLA	Major Histocompatibility Complex in Man
HLA-A,-B,-C	Genes Coding for Class I Type Antigens
HLA-DR	Gene Coding for Class II Type Antigen
HPRT	Hypoxanthine Phosphoribosyl Transferase
HTf	Human Transferrin
IFN-γ	Interferon-γ
Ig	Immunoglobulin
Igh	Immunoglobulin Heavy Chain
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
kD	Kilodalton
KLH	Keyhole Limpet Hemocyanin
LAI	Leukocyte Adherence Inhibition
LAIF	Leukocyte Adherence Inhibition Factor

LAK	Lymphokine-Activated Killer Cells
LMI	Leukocyte Migration Inhibition
LPS	Lipopolysaccharide
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
mAb	Monoclonal Antibody
MBP	Myelin Basic Protein
MC	Methycholanthrene
MHC	Major Histocompatibility Complex
MMI	Macrophage Migration Inhibition
6-MPDR	6-Methyl-Deoxyriboside
MPL+TDM	Trehalose Dimycolate + Monophosphoryl Lipid A
mRNA	Messenger RNA
MuLV	Murine Leukemia Virus
MW	Molecular Weight
NAI	Non Adherence Index
NaN_3	Sodium Azide
NC	Nitrocellulose Paper
NDGA	Nordihydroguaiaretic Acid
NK	Natural Killer Cells
NP-40	Nonidet-P40
OD	Optical Density
OSN	Organ Specific Neoantigens
p-onc	Proto-Oncogene
pAb	Polyclonal Antibody
PAF	Platelet-Activating Factor
PAGE	Polyacrylamide Gel Electrophoresis
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline
PC	Peritoneal Cells
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PMA	Phorbol Myristate Acetate
PMSF	Phenyl-Methyl-Sulfonyl Fluoride
PPD	Purified Protein Derivative

RB	Retinoblastoma
RBC	Red Blood Cells
RNA	Ribonucleic acid
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
RT	Room Temperature
SA	Staphylococcus Aureus
SDS	Sodium Dodecyl Sulfate
SV-40	Simian Virus-40
T,Tn	Thomsen-Friendereich antigen
TAA	Tumor Associated Antigens
TBS	Tris Buffered Saline
TCR	T Cell Receptor
TEMED	Tetramethyl-Ethylenediamine
TFA	Trifluoroacetic Acid
Th	Helper T Cells
Thy-l	Murine T Cell Differentiation Antigen
TNFa	Tumor Necrosis Factor
TSTA	Tumor Specific Transplantation Antigens
TTS	Tris Tween Saline

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CHAPTER I

INTRODUCTION

1. General Tumor Immunology

One of the oldest and most important issues, representing actually the central question of human cancer immunology, is whether malignancy is associated with antigenic changes which can be recognized as foreign by the tumor host; that is, whether neoplasms express tumor specific antigens. The existence of such molecules would make it possible to qualitatively distinguish between cancer and normal cells (140, 243, 244). This distinction would be useful in tumor diagnosis and its potential prevention and/or therapy.

2. Animal Tumor Immunology - Tumor Specific Transplantation Antigens (TSTA)

Since the beginning of this century tumor transplantation experiments were performed in animals with the aim of immunization of the host against the tumor (140,244). These early experiments successfully resulted in tumor transplant rejection. However, transplants from non-malignant tissues were also rejected suggesting lack of specificity in the rejection process. With the discovery of the individually unique Major Histocompatibility Complex (MHC) molecules, it was realized that the rejection was directed against any tissue bearing foreign MHC products (254). It was not until 1943 that immunization against transplanted tumors was demonstrated in syngeneic mice (99). More complete experimentation after 1950 (61,141,199,217) using inbred animals provided clear evidence that tumor specific antigens were responsible for the transplantation rejection response. Prehn and Main (217) provided the most definitive demonstration of the existence of Tumor Specific Transplantation Antigens (TSTA). They showed that an animal immunized against a tumor could accept skin grafts from the syngeneic animal in which the tumor was produced. Hence, tumor specific transplantation antigens have been defined as molecules capable of inducing rejection responses in genetically compatible hosts (140,141).

Immunization of animals against experimentally transplanted tumors to demonstrate TSTA may be done by any one of the following procedures: 1) Injection of a very small number of tumor cells which is not capable of sustaining tumor growth (176). 2) Injection of either irradiated or cytotoxic drug-treated cancer cells rendered incapable of replication (222). 3) Injection of cancer cell extracts obtained by treatment with butanol (97,156,248,253), 3 M - KCl (97,156,205) and NP - 40 (238). 4) Surgical excision or strangulation of the growing tumor (141). 5) Inoculation with a mixture consisting of tumor target cells and specifically immune lymphocytes at various ratios (Winn assay) (194).

Animal TSTA are most clearly associated with chemical and physical mutagen-and virus-induced tumors. A large category of animal TSTA are expressed by tumors induced by chemical agents. The first and most widely used chemical carcinogen for TSTA induction is methylcholanthrene (MC) (209,217,258,259). MC induces sarcomas and mammary carcinomas in animals (140). However, TSTA have also been found to be expressed on sarcomas induced by other aromatic hydrocarbons and hepatomas induced by azo-dyes (140). Tumor immunogenicity appears to be influenced by the individual carcinogen: MC-induced sarcomas appear to be more immunogenic than those induced by dibenzopyrene (258).

Cross-reactivity testing between different tumors induced by the same chemical carcinogen have revealed a remarkable antigenic individuality. It was demonstrated that cancers induced by physical or chemical carcinogens express TSTA which are individually specific for a particular tumor. This was true even for tumors of the same histogenesis induced by the same carcinogens in the same host genotype or for independently induced primary tumors in the same original host (140). However, recent studies suggest that the individually distinct TSTA of chemically induced sarcomas belong to a newly recognized family of cell-surface molecules called gp 96 (258). Whether the TSTA of other tumors are related to gp 96 is unknown.

Recent studies suggest that mutagen-induced TSTA often result from a point mutation, and the subtly altered protein is responsible for tumor rejection by eliciting cytotoxic cell responses (106). Although TSTA have been identified by their elicited rejection response, the lack of appropriate techniques has hindered the definition of their structure and origin. TSTA expression generated the hypothesis of immune surveillance of cancer (32). This concept proposes that the immune system evolved to eliminate abnormal cells, such as cancer cells as well as foreign pathogens. Thus, TSTA expressed on cancer cells are recognized and elicit immune reactivity against the cells expressing them, which are finally eliminated. It is proposed that eventual tumor growth results from a breakdown of immune surveillance. Subsequent to the development of this theory, it was demonstrated that, in general, immunodepressed animals did not have a higher occurrence in tumors than the normal animals (106,140), leading to a loss of credibility of the theory of immune surveillance.

During the last decade studies on in vitro mutagen treatment of murine tumor P815 mastocytoma cells have generated a variety of stable immunogenic tumors which are rejected in syngeneic mice (22). These tumors have the tum$^-$ as opposed to the tum$^+$ phenotype of the parental tumor. Tum$^-$ molecules are as diverse as TSTA. Cloning of three of tum$^-$ molecules showed that each one was the result of a point mutation with no homology to any sequence presently known or to each other. Each tum$^-$ gene product exhibited the ability to be recognized by cytotoxic T cells whereas the original tum$^+$ normal counterpart did not. These findings showed that there are T cells capable of recognizing the mutationally generated antigens and eliminating them. Thus the T cells exert an immune surveillance of the integrity of the mammalian genome.

The validity of the experimentally induced cancer model has occasionally been doubted because the experimental tumors express TSTA which may not be expressed under natural induction of the tumor phenotype (121). Studies which support this school of thought have shown that spontaneous tumors are rarely immunogenic in transplantation experiments (243). However the lack of immunogenicity of spontaneously arising tumors represents a more complicated phenomenon. Since the spontaneous tumors used in those transplantation experiments had been serially transplanted they may have been immunoselected for antigen loss variants (121). Occasionally, some spontaneous tumors do express TSTA although their levels of immunogenicity in transplantation experiments seem to be much lower than those of experimentally induced tumors (36). Again studies with tum$^-$ variants generated from tum$^+$ tumors entirely devoid of immunogenicity showed that immunization with tum$^-$ tumors conferred specific immunoprotection against the parental tum$^+$ non-immunogenic tumor (22,154). This indicated that the addition of a tum$^-$ antigen on a non-immunogenic tumor enables the tumor to elicit an immune response to TSTA which alone would be too few or too weak to generate an immune response.

One explanation for the vast variety of the chemically (or physically) induced TSTA, is that they represent either modified pre-existing transplantation antigens (140), or a family of polymorphic molecules expressed on normal cells before transformation. When transformation occurs individual cells carrying a certain set of these molecules are selected for clonal expansion (32). As an extension of this theory is the suggestion that tumor antigens may represent alterations in molecules involved in cell-cell recognition; the dysfunction of these molecules in spontaneous tumors might result in abnormal or invasive growth (26), whereas the loss of such molecules, which might also result in a malignant phenotype, might render the tumor non-immunogenic (106).

Conversely, the endless variability of TSTA cannot be adequately explained either by amplification or activation of pre-existing genes on the normal phenotype (209). Since every carcinogen is a mutagen (3), the diversity can be better explained either by the mutagenic action of the carcinogen on the cells during transformation from normal to malignant phenotype (3) or by the genetic instability of the transformed cells during tumor progression (195).

Other possibilities suggested for the identity and extensive polymorphism of the TSTA are re-expression of silent embryonic or fetal antigens (42), derepression or modification of MHC genes (161,180,204,209); expression of gene products linked to the heavy chain (Igh) locus (214), expression of antigens related to recombinant MuLV (159,258), occurrence of epigenetic errors in the assembly of the cell membrane (26), alteration of growth factor receptors (215), and expression of heat shock proteins (133). However, only cloning of the genes encoding TSTA can provide definitive answers regarding the origin of these antigens (106).

3. Human Tumor Immunology

Unlike the success of demonstrating anti-tumor immunity in animals, attempts to induce specific anti-tumor immunity in cancer patients have been generally inconclusive (318). It remains therefore a matter of speculation whether progressive malignant tumors are immunogenic and, if they are, which types of immune cells are involved in responding (250). This was mainly due to the fact that most spontaneous human tumors appear to be non-immunogenic in the autochthonous host. Furthermore, immunization and challenge trials which would provide the evidence for expression of TSTA in human tumors are not possible: 1) because they are unethical and 2) because of the lack of syngeneic hosts. However, there have been a vast variety of molecules which have been constantly identified by means of monoclonal antibodies (mAbs) to tumor tissues with comparative specificity for the tumor, but not for normal tissues. These molecules have been termed tumor associated antigens (TAA) (77,128,129,131,170,181,234,235,303,306). In general it is not established whether TAA also act as TSTA in vivo in humans. Since tumor transplantation experiments are not possible in humans it is not possible to prove that the antigens detected by mAbs or other in vitro assays are reactive in vivo. The presence of human TSTA and possible immunoreactivity against them has been suggested by two indirect observations at the clinical level. First, cancers occasionally regress spontaneously. Spontaneous regression has been observed in neuroblastoma tumors and melanomas (176,179). Although the regression might have been due to the tumor host immunity, other factors such as hormonal induction of the regression (176) or even spontaneous maturation to benign forms could also be responsible (223). Second, human solid tumors are often infiltrated by mononuclear cells and the degree of infiltration often correlates with prognosis (250,305). These latter observations suggest that there is a local immune reaction which may play a role in the tumor control. Nevertheless, most evidence for immune responses directed against human tumors was originally obtained from in vitro studies where peripheral blood leukocytes (PBL), from patients with tumors such as neuroblastoma, sarcoma, melanoma, colon, breast, testis, endometrium and ovary were cytotoxic against autologous and allogeneic cultured tumor cells of the same histology (118). Initially, it was thought that the target structures were TAA's on the surface of cancer cells eliciting histologically specific cell-mediated immunity. However, later it was recognized that the observed cell-mediated cytotoxicity was not histologically specific. The principal effector cells involved belonged to the natural killer type with no real histological specificity in their cytolytic activity (223).

Although it is not known whether human cancers express immunoprotective antigens in vivo, accumulating experimental evidence in vitro suggests that specific molecules present on human cancer cells are being recognized by the immune system of the tumor host. To-date, three substances have been recognized and immunologically defined as immunogenic in human cancer: organ-specific neoantigens (OSN), myelin basic protein (MBP) and T and Tn Thomsen-Friedenreich antigen.

3.1 OSN

One type of human tumor antigens is called the organ-specific neoantigen since it is expressed on cancers of the same organ and histogenesis (92,150,284). All colon cancers from different individuals express a common antigen as do all lung cancers from different patients. However, the colon and lung common antigens do not cross-react with each other. OSN are tumor specific antigens which are not expressed on normal or inflamed tissues (284). Experimental data have shown that there is no in vitro reactivity of leukocytes of normal individuals or patients with inflammatory diseases to cancer extracts; nor is there reactivity to normal or inflamed tissues extracts in patients carrying tumor of the same organ (102,165,272,290). OSN are expressed by fetal organs from the 13th to 19th weeks of gestation (104). Moreover, during the 14th to 28th weeks of gestation pregnant women are sensitized to OSN expressed by cancer and fetal organs. OSN have been detected in crude tumor extracts (284), in spent medium of tissue cultured cancer cell lines (6,55,279) and butanol extracts of tissue cultured cancer cell lines (146). OSN is a protein solubilized by limited papain digestion (292) which co-isolates with β_2m (291,292) and is detected in the serum (101) and urine (166,288,289). Although it is not known whether OSN are immunoprotective as are animal TSTA, OSN share the animal TSTA property to be solubilized with 2.5% butanol. Cellular recognition of OSN in vitro can be detected by the leukocyte adherence inhibition (LAI) assay and is mediated by several immune mechanisms: Ab-dependent monocytes (174), CD8$^+$ T cells (148), and CD4$^+$ T cells (146,275). OSN recognition by any of these mechanisms results in the generation of soluble mediators by the reactive cells. Pharmacological studies, indicate the mediators are similar to leukotrienes and exert an effect on the bystander cells causing them to lose their property of adherence to glass.

3.1.1 P40 OSN

3.1.1.1 In Spent Medium

A soluble isolate from spent culture medium of a tissue cultured lung cell line, NCI-H69, was found to exhibit OSN activity in the LAI assay. After physicochemical enrichment, the OSN active material was used to produce a mouse mAb which in turn was used for affinity-purification of the individual molecule that carried the OSN epitope. A molecule of 40 kD MW (p40) was isolated with OSN activity (279).

The same mAb was used to affinity purify a similar molecule with OSN properties from a human colon cancer cell line, HCT-15 (6). The two p40 molecules from lung and colon tested in LAI did not cross-react suggesting that the immunogenic epitope recognized by the human leukocytes is distinct from the common epitope recognized by the mouse immune system. Subsequently, studies were undertaken for sequencing and molecular cloning of the gene encoding p40.

3.1.1.2 In Butanol Extracts

Immunoprotective TSTA of animal tumors can be released among other methods by solubilization with n-butanol (97,155,156,238). The possibility that TSTA and OSN might share physicochemical similarities was examined in our laboratory. The colon and lung cancer cell lines expressing p40 were extracted with butanol (146). The butanol extracts of both cell lines exhibited OSN activity indicating that OSN share with the immunoprotective animal TSTA the physicochemical property of being extracted with butanol. Moreover, subsequent testing showed that p40 was detected in butanol extracts of the two cell lines. By histochemical methods it was determined that butanol had extracted 40-80% of p40 from the cell surface (146).

3.2 Thomsen-Friedenreich Antigen

In addition to the immune reactivity against OSN, immune reactivity in tumor hosts can be detected to molecules present in a cryptic form in normal tissues. These include the Thomsen-Friedenreich T and Tn carbohydrate molecules of red blood cells (RBC) membranes which constitute the precursor of the MN blood group antigens (256,257).

Although present in most tissues, they are masked by covering sugars and are unrecognized by the immune system (256). The T antigenic form is due to exposure of the disaccharide β-Gal (1-3)-α-Gal NAc by removal of the terminal sialic acid residues after treatment with neuraminidase. The Tn antigenic structure is Gal NAc linked α to the hydroxyl group of serine (Ser) or threonine (Thr) in the aminoterminal region of the active glycoprotein. Subsequent action of β-galactosyltransferase adds Gal from Gal nucleotide and this results in Gal-β-1 to 3-GalNAc-α-O-Ser/Thr which comprise the T immunodeterminants (256,277).

There is evidence that T and Tn are stage-specific differentiation antigens expressed transiently on human fetuses arising before tolerogenic developmental phases (256). Appearance of the immunoreactive T and Tn antigens on the external surface membranes is associated with most primary carcinomas and metastases (236). In 90% of human cancers the T-antigen is expressed in the non-cryptic, immunogenic form which stimulates in vitro and in vivo immunoreactivity (256).

Tumors of the central nervous system (CNS), mesenchymal tumors, benign tumors, normal tissues, or inflammatory diseases do not cause expression of the antigenic epitopes (277). The immune responses elicited upon exposure of the T-antigenic epitope include both humoral and cellular reactivity. The humoral anti-T response is mediated by a heterogeneous mixture of low affinity Abs (mainly IgM) acting against RBC-T antigen. All human sera contain the a-T IgM whose level falls with cancer often before the cancer is detected (256,257). After curative removal of the tumor a-T IgM returns to its normal level (277). The cellular anti-T immunity is mediated by the effector cells of Delayed-Type-Hypersensitivity Reactions (DTHR). DTHR to T antigen was found to be sensitive and specific (256). Patients with ductal breast carcinoma and lung cancer exhibited positive DTHR to T antigen. In contrast normal individuals and patients with benign diseases who have IgM Abs to T antigen exhibit no DTHR to T antigen (277). Similar immune reactivity of the T antigen was observed by the LAI assay in patients with lung, breast and colon cancer and the responses were not organ related (274). On the other hand, normal subjects did not exhibit immune reactivity to T antigen (274).

3.3 Myelin Basic Protein (MBP)

The third substance to which lymphocytes of cancer patients are sensitized is MBP. MBP is a major component of the normal myelin sheath. It has an 18 kD MW and was defined because of its principal role in inducing experimental allergic encephalitis (EAE)

(94). The tertiary structure is not important in defining the determinants responsible for its immunogenic activity. The encephalitogenic determinant of human MBP is a nine residue peptide (94). Both, animals bearing cancers (315,316) as well as cancer patients (71,72,125,145) are sensitized to MBP. MBP is physicochemically very similar to a protein extracted from tumor tissues, designated cancer basic protein (CBP). CBP has been isolated from breast, bronchus, stomach carcinomas (37). The two substances, MBP extracted from CNS and CBP from cancers were tested in vitro with sensitized leukocytes. CBP was reactive to a higher degree than MBP with cells from cancer patients. On the other hand, MBP exhibited higher reactivity with leukocytes from patients with degenerative diseases of the CNS (37). In addition, mice with MC-induced tumors were found to be reactive to human MBP, although normal mice did not react to it (94). A variety of cell-mediated assays such as DTHR (94), macrophage migration inhibition (MMI) (71) leukocyte migration inhibition (LMI) (125) and leukocyte adherence inhibition (LAI) (94,276) were used to test the immune response against MBP. In humans MBP is recognized by the $CD4^+$ T cells in association with Class II MHC molecules on monocytes (276). The recognition results in release of leukotriene-like mediators which inhibit the adherence of bystander cells in the LAI assay (285).

The events that trigger the reactivity in vivo are unknown. Sensitization to MBP may be due to exposure of the very similar CBP. Also, autoreactive leukocytes can be found in normal humans and animals, which might be derepressed and upregulated as part of the overall response to the cancer (277).

4. Virus-induced Tumors

TSTA are also expressed in animals infected with oncogenic DNA or RNA viruses. Virally transformed cells are very useful in tumor development studies because the transforming agent is known and the changes occurring in the infected cell can be studied in the context of the inserted viral genome and its products (212).

4.1 DNA Tumor Virus-induced TSTA

The first observation that virally induced tumors expressed TSTA was made on animal tumors induced by SV-40 and polyoma DNA viruses (116). The expression of virally encoded TSTA appears to be obligatory for the neoplastic transformation in those two systems (116). Whether similar virus-induced TSTA exist in humans is not clear (116).

The TSTA induced by the same type of oncogenic DNA virus, unlike the chemically induced TSTA, are immunologically cross-reactive (140). Immunological cross-reactivity of TSTA induced by the same virus is observed even if the tumors are induced in hosts of different species. The common tumor cell membrane viral TSTA are not related to virion antigens (116,140,205). On the other hand, TSTA induced by different viruses are not corss-reactive (116,140). The oncogenic DNA viruses carry their own transforming gene which becomes incorporated into the host's genome during the transformation process. This gene codes for the virus specific TSTA and the tumors expressing them are strongly antigenic (139,140). Immunization against the DNA virus-induced tumors elicits T cell mediated specific transplantation resistance (116).

Three classes of DNA viruses are known to cause tumors in animals and possibly in humans: herpes viruses, papilloma viruses and adenoviruses. They differ markedly in size, characteristics and transforming abilities (212). In all three, the protein with the transforming abilities is common and related to a nuclear antigen called the T antigen (273), which, in the SV40 and polyoma virus models is coded for by genes coding for TSTA (116,219). The TSTA of polyoma virus-induced tumors are coded for by the genes encoding two antigens the large T and the middle T antigens. The TSTA of SV40 induced tumors is coded for by the gene encoding large T antigen (212).

Although it is uncertain whether similar TSTA exist in humans, there are a few examples that may be considered. These include the Epstein-Barr nuclear antigen or

EBNA, expressed in Burkitt's lymphoma and another alleged antigen expressed in cervical carcinomas associated with human papilloma viruses (67,116).

4.2 RNA Tumor Virus-induced TSTA

Oncogenic RNA viruses or oncornaviruses belong to the retrovirus class. Retroviruses use reverse transcriptase to transcribe their RNA to DNA, which becomes incorporated into the host cell DNA. There are major differences between DNA and RNA viruses involving their interactions with their host cells and their ability to transform them (67,226). One major difference lies in the fact that the oncogenic DNA viruses irreversibly damage the host cell when they start to replicate. In contrast, the RNA tumor viruses can multiply in their host cells without impairing the host's ability to proliferate and grow. Retroviral genetic material can directly transform the appropriate host cell upon infection or, indirectly, after long periods of latency and activation by radiation or chemical carcinogens (67,260). The viral genetic material contains repeat genetic sequences, designated long terminal repeats (LTR) that are present at both ends of the transcribed viral DNA and are integrated into the host's genomic DNA. LTR function as initiators and terminators of the respective RNA synthesis and appear to be required for the direct transforming event in the infected cells (31). However, these regulatory inserts do not code for a virus related protein but affect only the expression of cellular genes (31, 223). In general, the retroviral genome encodes three major essential genes: gag, encoding the viral core protein; pol, coding for reverse transcriptase; and env, encoding envelope glycoprotein (212). These viral proteins may be expressed and this is a disadvantageous event for subsequent oncogene activation (see Section 4.2.1), because they become a potential source of antigen recognizable by the host immune system (139). Even replication-defective viruses can induce envelope viral protein expression with immunosuppressive and anti-inflammatory activity (157). RNA viruses include the directly oncogenic viruses, which owe their transforming ability to a particular class of genes termed oncogenes (oncs). RNA viruses also include those which do not contain oncs, but which can cause tumors in experimental animals (58). The non-onc containing viruses include the exogenous type which are typically expressed upon infection of the cell and the endogenous viruses which are typically latent (58). Endogenous viruses are integrated as proviruses into the germ line of most vertebrates including man (58). The tumorigenic potential of latent endogenous retroviruses has been shown for murine leukemia viruses (MuLV), as well as for mammary tumor viruses (97,157). MuLV activation often occurs during tumorigenesis in mice and MuLV antigens are often expressed on chemically induced cross-reacting sarcomas acting as TSTA (258,260). The MuLV antigen responsible for the TSTA activity of some chemically-induced sarcomas, appears to be the viral envelope glycoprotein gp70. This protein, exhibiting extensive polymorphism, apparently can be expressed by chemically induced activation of endogenous latent MuLV (260).

4.2.1 Oncogenes

Tumorigenesis in animals and humans is a complex multistep cumulative process (64,79,95,113,138,307). Normal cell differentiation and proliferation involve the expression of genes strictly regulated by the external environment (79). Cancer results from changes which disrupt the balanced functions of normal cells. Both the acquisition and the loss of genes appear to have significant consequences in tumor development and progress (307). Point mutations (18,35), deletions (18,79), amplifications (86) and rearrangements of DNA (45,46) are the four major forms of genetic alteration involved in the two major features of malignancy the uncontrolled cell division and loss of differentiation. Point mutation, rearrangement and amplification lead to the activation of normal cellular genes which acquire transforming properties. The normal gene that becomes transformed is called proto-oncogene (p-onc) and the transformed form of it is called c-onc. DNA segments homologous to c-oncs have been detected in mammalian viruses. These segments were found to have been inserted into the viral DNA because of rare recombinant accidents between retroviruses and p-oncs (57,79). The excised mammalian DNA (p-onc) over time has developed into another form of oncogene, the viral transforming oncogene (v-onc). P-oncs, c-oncs and v-oncs are all homologous to each other. However, c-oncs and v-oncs contain specific differences in DNA sequences since they have evolved through different genetic pathways. Thus, they are likely to transform cells by different biochemical mechanisms (57). Three mechanisms appear to mediate oncogene action: protein phosphorylation, metabolic regulation via GTP and participation in the replication or transcription of DNA (79). Oncogene activation can induce expression of tumor antigens in two ways; first, proteins encoded by oncogenes may serve as TSTA (116); and second, oncogene-induced neoplastic transformation can induce a variety of tumor specific antigens as secondary events (116). Most oncogenes do not show any real cancer specificity. The proteins they encode are expressed at high levels in cancer cells and in trace amounts in normal tissues. Exception to this generality are the ras and the abl oncs (116).

Perhaps the most interesting of all oncogenes is the ras oncogene family because its oncogenic form has been found in a wide variety of human and animals tumors, both in solid tumors such as colon (35,65), melanoma, pancreas (23), mammary (79), lung (232) and in leukemias (35). The three members of the ras gene family are designated Harvey (H-ras), Kirsten (K-ras) and N-ras. They encode a group of related proteins with an approximate MW of 21 kD, which are collectively referred to as p21. The oncogenic, also termed activated, ras protein comprises a tumor specific antigen, or TSA, on cancers, which is recognized by mAbs and distinct from its normal non-activated counterpart, differing from it by a single point mutation (23,35). The activated ras can confer tumorigenicity in animals and humans (307). However, there has been substantial controversy about the significance of single point mutations of the p-ras in the development of cancer and particularly of human cancer. In the animal model c-ras had to collaborate with myc or the adenovirus early region 1A (EIA) oncogene, in order to transform rat embryo fibroblasts or baby rat kidney cells respectively (307). In human cancer, it is unclear whether the mutant ras gene acts as a single tumorigenic determinant or it is also accompanied by other factors, especially in view of the finding that in human tumors the normal human ras allele is lost (139).

Nonetheless, sensitive biochemical methods have shown that 30-40% of all human colon carcinomas carry mutations at either codon 12 or 61 in the K-ras gene (79) indicative of the importance of point mutations in the activation of this oncogene.

In addition to activation by point mutation, oncogenes may be activated by gene amplification, as seen in human small cell lung carcinoma (79), breast cancer (79) and colon cancer (65), as well as in human neuroblastomas where amplification of the oncogene n-myc induces down regulation of MHC Class I molecules rendering them less susceptible to cellular immune control mechanisms (79) . Furthermore, oncogenes are activated by non-random gene rearrangement particularly found in hematopoetic malignancies such as chronic myelogenous leukemia, promyelocytic leukemia, etc. (45,46).

RNA virus gene expression and oncogene activation followed by subsequent expression of the faulty proteins in the cancer cells elicit immune responses in the tumor host (31,116). The immune responses against the virally transformed cells (either containing oncs or not containing oncs) are mainly mediated by cytotoxic T cells (16,116). However, in some systems the T helper phenotype plays a key role (116,167). Furthermore, Abs have been observed to confer immmunoprotection against oncogene induced tumors (16).

4.3 Anti-Oncogenes

Tumor formation resulting in the production and expression of abnormal proteins or protein expression is a mutational process with three distinct stages: initiation, promotion and progression (65). The mutational events affect two types of genes, those which when mutated lead to cell transformation and tumor growth and those which upon mutation lose their property to inhibit tumorigenesis. In the first group, oncogenes are identified by their positive role in the transformation of appropriate host cells. Tumor "suppressor" genes or anti-oncogenes are the second type of genes which have a negative effect on transformation, thus driving cells towards normality. Mutations causing the loss or inactivation of these inhibitors of malignancy are oncogenic (65,79). Loss or mutational inactivation of one allele of the diploid gene pair plays an essential role in the genesis of retinoblastoma (RB), osteosarcoma and Wilm's tumor (79). This event has been regarded as evidence that the affected regions contain tumor suppressor genes. In cells these genes act recessively indicating that the normal alleles can suppress neoplasia in the respective tissue and both copies must be inactivated in order for the suppressor function to be eliminated. The loss or mutation of both alleles of the same gene comprises the "two-hit" theory proposed by Knudson on the RB gene model (142).

A very much studied protein affecting tumorigenesis as a tumor suppressor is the nuclear protein p53 (232). The mutant p53 is overexpressed in tumor cells and associates with SV40 viral T antigen in SV40 transformed cells suggesting a positive role in tumor formation. On the other hand, the wild type p53 plays a potential role against transformation by Friend erythroleukemia virus and suppresses transformation induced by the mutant p53 in ras-transfected cells (232).

Analogous to the dual activation theory for oncogene function, it has been suggested that in some cases both the inactivation of a suppressor gene and the activation of an oncogene are necessary to achieve neoplastic transformation in animals (79) and in humans (232).

Colorectal neoplasia is a well studied tumor model in which mutations and loss of specific chromatosomal locuses have been observed. It has been suggested that a combination of ras oncogene activation and loss of putative suppressor genes in chromosomes 17 and 18 through mutations and chromosomal losses occur in the cells leading to malignancy (64); however, the accumulation of the events rather than the order of the events is most important for tumor formation (65). The colorectal model of tumorigenesis is supported by studies of other human tumors such as lung, bladder, breast, astrocytoma (65) and malignant melanoma (232). It thus provides a very attractive tumor model in which oncogene activation and anti-oncogene inactivation collaborate to create the full malignant phenotype.

5. Effectors of Specific Immunity against Tumors

5.1 T Cell-mediated Immunity

5.1.1 T Cell Antigen Recognition

Tumor rejection responses against experimentally transplanted tumors are largely mediated by cytolytic T cells. T cell recognition of antigen is mediated by the T cell receptor (TCR) which recognizes peptides of partially cleaved proteins in association with either Class I ($CD8^+$ T cells) or Class II ($CD4^+$ T cells) MHC molecules (21,106,112). Class I molecules consist of a highly polymorphic integral membrane glycoprotein (α-chain) which is non-covalently bound to β_2m. Class II molecules consist of two noncovalently bound, highly polymorphic integral membrane glycoproteins. Like other cell surface proteins, MHC molecules are cotranslationally inserted into the endoplasmic reticulum (ER) and following chain assembly are transported to the Golgi complex. Soon after their biosynthesis, Class II molecules become non-covalently associated with a molecule lacking polymorphism, hence designated the invariant chain (44,112). The invariant chain, unlike the β2m of the Class I molecules, dissociates from Class II molecules, when the Class II molecules enter the endosomes where Class II molecules associate with antigen (103). The association of antigen with the two different classes of molecules of the MHC complex depends on the origin of the antigen. Endogenously derived peptides from viral or transformed proteins associate with Class I MHC molecules in the endoplasmic reticulum, are bound to the groove present on Class I molecules and are transported to the cell-surface for presentation to the $CD8^+$ subset of T cells (28,164,185). Exogenously derived proteins are endocytosed and degraded in the endosomes where they associate with and bind to a groove on the MHC Class II molecules. The antigen - Class II complex is then transported from the endosomes to the cell-surface of antigen presenting cells (APC) and is presented to the $CD4^+$ subset of T cells (208,314).

5.1.2 Anti-Tumor T Cell Responses

5.1.2.1 Animal Studies

Resistance to grafts of chemically-induced tumors implanted in syngeneic hosts has been passively transferred by means of immunocompetent cells from spleen, lymph nodes, or peritoneal exudates (205). Since cellular rather than humoral immunity appears to mediate the destruction of solid tumors (106,205), it has been the focus of much experimental attention. Using mAbs the effector cells responsible for in situ anti-tumor response have been shown to be T lymphocytes and macrophages (304). Cytotoxic T lymphocytes (CTL) and cytolytic macrophages have been isolated from malignant tissue and had the capacity to kill autologous tumor cells while the natural killer cell population did not seem to play such an important role in in situ anti-tumor responses (304).

There is controversy over the subpopulations of T lymphocytes responsible for tumor-specific rejection in vivo. Helper T-cells (Th) and cytolytic T cells have both been implicated in tumor rejection in vivo. Although the mechanism by which $CD4^+$ and $CD8^+$ T cells recognize antigen has and is being extensively studied, little information is available on T cell recognition and responsiveness to tumor specific antigens. T cells exert an immune surveillance of the integrity of the mammalian genome, recognizing antigenic peptides produced by gene mutations (22,106). Since tumor-specific antigens are endogenously synthesized, it is possible that they are presented in the context of Class I MHC molecules to $CD8^+$ T cells. Certain studies support this viewpoint (245). However, although the $CD8^+$ T cells are the cytolytic effector cells they must collaborate with other cells to induce cytolysis. Thus, in some cases activation of $CD8^+$ T cells required factors secreted by Th cells (IL-2), although not necessarily the presence of the Th cells. In other studies APC were essential for in vitro tumor-specific $CD8^+$ CTL generation suggesting that APC presented the TSTA to $CD4^+$ cells which in turn induced the CTL clone generation (136). On the other hand, CD4$^+$ T cells directly mediate anti-tumor responses, particularly of weakly immunogenic murine tumors (39,202). It has been shown that immunity against tumors can be transferred by T cells bearing the markers of helper/Delayed Type Hypersensitivity (DTH) subset (Th) (68). The transfer of Th cells in the mouse and rat is effective in prevention or even therapy of chemically and virally induced sarcomas (68), virally induced leukemia (96), spontaneous plasmacytoma (81) and spontaneous thymic lymphoma (191). Similar results have been obtained in allograft rejection experiments (293). In vitro response of tumor specific CD4$^+$ T cells depends on tumor-antigen processing and presentation by APC expressing Class II molecules Apparently, in vivo activation of T helper cells may also depend on tumor antigen processing and presentation by APC. Support of this view was provided by in vivo administration of anti-Ia Abs which inhibited DTH response to an Ia-fibrosarcoma and inhibited tumor rejection (207). Although Th can also lyse cells, their primary function is to lend help to B cells and other T cells by means of lymphokine secretion (191). The two different functions of the Th cells are carried out by two different subpopulations (189): The Th1 subset secretes interleukin-2 (IL-2) a lymphokine which enhances specific T cell responses and interferon-γ (IFN-γ) a cytokine with antiviral, mitogenic, cytostatic and activating properties. Th1 cells are believed to induce DTH responses and macrophage activation. The other subset, Th2 produces interleukin-4 (IL-4) and interleukin-5 (IL-5), molecules with B cell stimulating properties but does not secrete IL-2. Th2 cells are believed to be major contributors in humoral responses by providing help to B cells to carry out their immune functions. The role of lymphokines involved in the induction of tumor-specific T cell proliferation is still unclear. However, it was shown that different T cell subsets may require the presence of different lymphokines in order to respond to various types of tumors, suggesting that an effective manipulation of lymphokine activated effector cells could be applicable in immunotherapy in humans (135,245).

5.1.2.2 Human Studies

The role of T cells as central elements of the immune response has been recognized. When lymphocytes are exposed to the relevant antigen both $CD4^+$ and $CD8^+$ T cell subsets become activated and proliferate (283). Immune reactivity against tumor antigens can be demonstrated by both in vitro assays and in vivo tumor transplantation experiments in animals. By contrast in humans, in vivo demonstration of anti-tumor reactivity is not possible for obvious ethical reasons. Thus specific anti-tumor responses mediated by T cells, have to be defined by using a variety of in vitro tests. Cytotoxicity and ^3H-Thymidine uptake in autologous mixed lymphocyte tumor culture (AMLTC), leukocyte migration inhibition (LMI) and leukocyte adherence inhibition (LAI) are in vitro tests utilizing the potential of T lymphocytes to recognize tumor antigen. The only in vivo test used is the DTHR to tumor extracts.

5.1.2.2.1 Anti-Tumor Cytolytic T Cell Assays

AMLTC is an assay in which in vitro PBL proliferation is induced in the presence of autologous non-proliferating tumor cells. Briefly, PBL from tumor patients are mixed and incubated with extracts from autologous tumor in the presence of ^3H-Thymidine (299). The tumor cells have been treated with mitomycin C or irradiated so that they cannot proliferate. The T cells which have been sensitized in vivo by the tumor antigen undergo blastogenesis and proliferation and can effect cytotoxic functions. By contrast the cytotoxic functions and blast formation do not take place after mixing with autologous normal cells (299).

The cytotoxic functions are determined by measuring the difference between target cell and control cell lysis detected by radioisotope release. AMLTC has been tested with soft tissue sarcoma, thyroid cancer, kidney, colon, ovarian carcinoma, malignant melanoma and lung cancer (299). The assay exhibits specificity and shows a correlation between post-surgical clinical course, suggesting that in vitro activities may reflect in vivo immune control of tumor growth (299).

The LMI assay measures cellular immunity of sensitized leukocytes by determining their migration from microcapillary tubes. Briefly, PBL from cancer patients are mixed with extracts of various tumors. Normally, leukocytes migrate out of the tubes. However, if the T cell population and probably other cells in the assay react with the tumor antigen they release a factor designated leukocyte migration inhibition factor which reduces the migration of the leukocytes. Leukocytes from cancer patients are not reactive with extracts of normal tissues but react with tumor extracts (319). The assay is highly specific and the response against tumor antigens declines three months after removal of the tumor (319).

The LAI assay measures cellular immunity of tumor sensitized leukocytes by measuring the inhibition of adherence to glass. In this assay leukocytes from cancer patients are incubated with extract of cancer of the same organ and histogenesis. Normally, the leukocytes adhere to glass. However the presensitized T cells and macrophages upon recognition of the sensitizing antigen (OSN) in vitro release mediators which induce loss of adherence of bystander cells. Since the development of the assay, LAI has attracted a great deal of interest. It has been used to detect immune reactivity to various forms of cancer and to other antigens, including viral, bacterial, fungal and rheumatoid synovium in man (284). The LAI assay and the mechanism mediating the non-adherence phenomenon are described in detail in section 10 of this chapter.

DTHR is an in vivo assay measuring presensitization of a person (or animal) to a particular antigen by administering this substance subcutaneously. The presensitized subject reacts by an inflammatory response at the site of the injection. Microscopically the reaction shows infiltration with polymorphonuclear cells at the early stages and infiltration with mononuclear cells at the late stages. DTHR has been demonstrated in cancer patients injected with extracts of their own tumor (277). Melanoma patients exhibited a correlation of DTHR with tumor burden. Early stages of the disease correlated with more positive responses than late stages and large tumor burdens (218).

5.1.2.2.2 Human Tumor Antigens Defined by Autoreactive T Cells

T lymphocytes with potential cytolytic activity against autologous tumor cells can be isolated from cancer patients. Tumor specific CTL can be cloned and thus provide highly specific probes for TSTA which consequently can be used to define whether tumor antigen is a TSTA (243). Recently, cytotoxic T cell clones have been isolated that are strictly reactive with autologous melanoma tumors (120) and mammary carcinoma cells (198).

Malignant melanoma is one of the most extensively studied human tumors with regard to the host's immune response. Both primary tumors and metastasis can spontaneously disappear. The reactive area is characterized by dense lymphocyte infiltration around the melanoma cells that undergo degeneration and disintegration (179). Because of the evidence for immune reactivity against melanoma, active immunotherapy has been tried with patients receiving preparations of whole killed melanoma cells or cell lysates; as a result, some patients have experienced a remission of the disease (184). It therefore appears that human melanoma cells may express molecules with TSTA-like properties. In fact, at least four tumor antigens are selectively expressed on human melanomas (167,230). Two of them, AMF-6 and AMF-7, were initially identified by means of mAbs (230). The two antigens were shown to be involved in vitro in the adhesion process to endothelium and in the fibronectin-induced motility of human melanoma cells (50). More importantly, they were also recognized in vitro by T cell clones with cytolytic anti-melanoma activity, suggesting an interaction of the melanoma specific antigens and specific anti-melanoma T cell receptor (51). Similarly, a human mammary cancer antigen defined by mAb 3A2 has been described. The antigen was expressed on autologous and allogeneic breast cancer cells but not on autologous normal mammary gland or other neoplasms. A $CD8^+$ T cell clone established from the same patient recognized and lysed the cells expressing the antigen in vitro suggesting potential in vivo reactions (198).

A significant number of human tumors can also express Class II MHC molecules. In the same melanoma tumor model, one of the tumors expressed HLA-DR determinants which are not normally expressed by normal melanocytes (230). The expression of Class II molecules probably results from the malignant transformation; consequently melanoma tumors can process and present exogenous antigen. The expression of the HLA-DR antigens on human melanoma cells induces activation of autologous lymphocytes (76,230). In some cases these activated lymphocytes exerted cytotoxic activity against the melanoma cells expressing Class II molecules (76). Whether this phenomenon could relate to TSTA presentation and subsequent tumor elimination in this, or other, tumor model has not been defined yet. If so, it places the Th1 subset of Th cells in a central role in the human tumor rejection process.

5.2 Antibody-Mediated Immunity

Specific immunity against tumors is mediated by complex humoral and cellular effector mechanisms. Mainly, specific-immunity against tumors is mediated by T cells in association with Class I and Class II MHC molecules. However, tumor antigen expression also results in B cell production of anti-tumor Abs (88,302). Anti-tumor Abs can "arm" cells expressing Fc receptors and effect anti-tumor functions. Nonetheless, due to the fact that transplanted tumor rejection is mainly mediated by T cells, Ab-mediated anti-tumor immunity appears to be of lesser importance. Moreover, this notion has been supported by findings of Ab-stimulation of tumor growth (216), of blockage of specific anti-tumor Ab by shed antigen (302), or masking of antigenic determinants on cancer cells occurring by Ab specific for the neoplastic cells (117). Yet, there are instances where the humoral immune response predominates. For example, the growth of B cell lymphomas, expressing specific TSTA defined as idiotypic determinants on their surface immunoglobulin is controlled by humoral effector mechanisms (85). Additionally the presence of anti-tumor Ab in the serum of animals and humans with solid tumors has been demonstrated (5). There are also several reports which show that anti-TSTA Ab is bound on the tumor cells of mice with virally or chemically induced tumors. The Ab presence was parallel to tumor growth suggesting the involvement of humoral immune responses (5).

The demonstration of cytotoxic Ab in the sera of animals bearing chemically induced tumors using conventional cytodestructive assays is not easy, mainly due to the weak affinity of the Abs (205). However, to-date, two classical pathways by which the anti-tumor Abs exert their effects have been defined: 1) Fixation of complement. It has been shown that serum from virally infected mice with progressively growing tumors and from mice where tumors have regressed possess complement dependent cytotoxic Abs which can be detected in vitro (205). 2) Cooperation with cells of the lymphoreticular network for tumor cell destruction in vitro. Tumor cytolysis involving the cooperation of humoral and cellular networks can be classified as: i) Antibody-dependent lymphocyte-mediated cytolysis (ADCC) (201), and ii) Macrophage-cytophilic Ab-mediated cytolysis (24, 211).

In humans anti-tumor Abs have been observed in the sera of patients with melanoma (221), ovarian carcinoma, lung carcinoma, breast carcinoma, squamous cell carcinomas and colon tumors (5). These Abs bound to tumor cells in tumor tissues, dissociated cells, or tissue cultured tumor cell lines. A well studied human cancer antigen which appears to elicit Ab-mediated immune reaction is the FD antigen (221). The FD antigen is a glycoprotein of ≈90 kD MW which was detected on melanoma cells. A specific anti-FD Ab was found in the serum of the patient expressing the FD antigen. Mouse mAbs raised against the FD antigen showed that the antigen was expressed on other tumors and some normal tissues. However, the epitope detected by the human Ab in the patient's serum was strictly expressed on the patient's melanoma cells, suggesting that FD is a common molecule expressing a unique determinant upon transformation. Similar to this, six more antigens have been serologically defined (221). Serologically recognized human tumor antigens have been classified into three major categories: Class 1 cancer antigens are restricted to the autologous tumor only, and are not expressed by normal or other malignant cells; Class 2 cancer antigens are found on the autologous tumor and also on a proportion of allogeneic tumors and on a limited range of normal cells and some are found to exhibit autoantigen properties (221); and Class 3 cancer antigens are present on normal and malignant cell types and do not have any differentiation or tumor restriction pattern. The three classes of cell surface antigens have been defined by studying patients with melanoma, astrocytoma, renal cancer and acute leukemia (221). Gene cloning will be required to assess whether the serologically defined antigens are related structurally.

5.3 Non-T Cell-mediated Immunity

5.3.1 IL-2 and Lymphokine-activated Killer (LAK) Cells

The production of a substance with T cell growth factor properties was first observed in 1976 by Morgan, Ruscetti and Gallo (187). Purification and characterization of this compound called IL-2 showed that it is a polypeptide whose growth promoting action was due to interaction with a specific receptor on T cells (225). It is produced by Th1 cells upon activation with either a mitogen or an antigen presented by a macrophage. The interaction of T cell-macrophage results in release of IL-1 by the macrophage; in turn, IL-1 induces the expression of IL-2 receptor and IL-2 synthesis by the T cells (200). Release and binding of IL-2 to its receptor results in proliferation of activated cytotoxic T cells, immunoglobulin production by B cells, growth and activation of natural killer (NK) cells, and increased cytotoxicity by monocytes (144).

Incubation of PBL with interleukin-2 can induce lysis of fresh tumor cells, tumor cell lines and virally infected cells in vitro. The phenomenon of the IL-2 induced non-MHC restricted cytotoxicity is designated the lymphokine activated killer, or LAK, phenomenon (114). LAK cells appear to have characteristic ultra-structure similar to that of activated CTL and NK cells by light microscope, but they appear different by scanning and transmission EM. They also appear to destroy tumor cells including NK resistant cell lines by mechanisms similar to those of other cytotoxic effectors having as their initial target the tumor cell membrane (98). Administration of IL-2, either alone or in combination with LAK cells, was found to significantly reduce preliminary or hepatic metastasis in mice (144). In clinical trials treatment of melanoma patients with activated in vitro LAK cells caused tumor regression in approximately 23% of these patients (227. However, not all cancers respond to the IL-2-LAK treatment; tumor burden and the individual patient's immunological responses are factors which may have an undefined role in the ability to attain meaningful responses (144). In addition, although the cytotoxic effect of LAK cells on tumor regression is quite impressive, it is non-specific and weak compared with the effect of tumor infiltrating cytotoxic T cells (144).

5.3.2 Macrophages

Monocytes mediate tumor lysis after priming with IFN-γ and triggering with molecules such as bacterial lipopolysaccharide (LPS) (1,63,167). Activated macrophages are able to selectively recognize and kill tumor cells in a non-phagocytic process. During the killing, they induce a characteristic pattern of changes in the tumor cells, such as inhibition of DNA synthesis , inhibition of mitochrondrial respiration and loss of intracellular iron (54). During the interaction with the tumor cells, macrophages secrete factors such as Tumor Necrosis Factor (TNFa) and L-arginine-derived reactive nitrogen intermediates which can be toxic to the tumor cells (134,304) . It has been suggested that the mechanism mediating the killing is determined by the tumor cell type and the pathway of macrophage activation .

5.3.3 Natural Killer (NK) Cells

Natural killer cells are represented by lymphoid cells displaying the "large granular lymphocyte" phenotype and are able to lyse tumor cells without previous sensitization and with no requirement for MHC restriction on target cells (167). Although NK cells do not appear to play an important role in anti-tumor responses in humans in certain animal models NK activity is important in the control of tumor growth (167).

The NK cells are $CD3^-/TCR^-$ cells which commonly express CD2, CD7, CD16 and NKH1 surface molecules. Since they lack both surface Ig and CD3-TCR complex, it is generally thought that they are unable to recognize whole antigens or peptides of the antigens presented in association with Class I or Class II MHC molecules (186). However

NK cells are capable of recognizing alloantigens on the surface of normal PHA-induced blasts as target cells (40) . Whether there is a relationship between the recognition of normal cells and the selective lysis of tumors by NK cells remains to be clarified. There are two mutually comparable theories concerning the structures recognized by the NK cells. One claims that there is one specific "receptor" recognizing target cell structures. This "receptor" is not necessarily coded for by a gene undergoing rearrangement. The second theory states that there is a balanced interaction between a number of monomorphic receptors present on NK cells and their ligand molecules expressed on target cells (186).

Recently a new molecule GL 183 which is selectively expressed by a subset of NK cells was detected (186). Monoclonal Abs to GL183 or CD16 can mediate an inhibitory effect on the NK cell activation. This suggests that, as with T cells, NK cells surface molecules can also mediate activation initiation and that mechanisms exist that regulate their function.

6. Tumor Markers

The term tumor marker has been used to designate biological substances that are produced uniquely or in excessive amounts by malignant neoplasms (277). The tumor markers are physiologically normal substances which appear in the circulation and are also produced by normal differentiated tissues. Tumor markers are often called tumor antigens. This term originated from the immunological methods (xenoimmunization) used for the detection of these molecules. However, they have not been demonstrated to be antigenic for the autochthonous tumor host. Tumor markers are clinically important as indicators for the presence of the disease and the disease progress. Some of the most important tumor markers are:

6.1 Carcinoembryonic Antigen (CEA)

CEA is a 180 kD glycoprotein originally observed in tumors of the gastrointestinal tract (277). CEA was discovered by Gold and Freedman by immunization of animals with tissue extract from colon cancer (91). The antisera obtained detected "tumor specific" antigens on colon tumor tissues from different patients. Subsequently, CEA was discovered in a large number of tumors and some normal and fetal tissues (277). Circulating CEA is found in the serum of patients with cancer . Patients with greater bulk of metastatic disease express higher serum levels of CEA (78). CEA titers generally increase with overt disease and often the increase precedes recurrence. The function and significance of CEA to malignant transformation are thus far unknown. CEA is not an autoantigen because no Abs against it have been observed in the serum of cancer patients (277). Moreover, no evidence for cell-mediated immune reactivity against CEA was observed with lymphocytes of patients with gastrointestinal cancer (158). A great number of mAbs have been prepared against at least six epitopes on CEA. Most of the antigenic epitopes appear to depend on the protein tertiary structure and are not carbohydrate related (10). The amino acid sequence of CEA shows that it is related to the immunoglobulin superfamily (15) which besides the immunoglobulin molecules includes T cell receptors, growth factor receptors and intracellular adhesion molecules. Recently, CEA was demonstrated to act as an intercellular adhesion molecule in vitro and in vivo (15).

6.2 α-Fetoprotein (AFP)

AFP is synthesized by yolk sac and liver cells in human fetuses (137) and appears to be a group of cross-reacting microheterogeneous species. The members of the AFP family are associated with hepatomas and germ cell tumors of the testis, ovary and organs that contain yolk sac elements (277). AFP also occurs in neoplasms of the uterus and melanomas (137). AFP is a useful marker for monitoring AFP-producing malignant neoplasms. The specificity of the AFP measurements for malignant neoplasms can be enhanced by the use of two high-affinity mAbs raised against two separate epitopes of the molecule (137). AFP together with chorionic gonadotropin are useful for evaluating germ cell neoplasms (277).

6.3 CA 125 and CA 19-9

CA 125 and CA 19-9 represent the first examples of commercially developed tumor markers using mouse mAbs. CA 125 represents a determinant on an established human cystadenocarcinoma cell line. It is expressed on more than 80% of epithelial ovarian carcinomas and it can be elevated before overt ovarian carcinoma (317). Like CEA, CA 125 may be observed in sera of patients with non-malignant diseases (277).

CA 19-9 is a colon carcinoma cell surface antigen defined by mAb 19-9 which was raised against the colon carcinoma cell line SW 1116 (151). CA 19-9 may be co-expressed with other antigens on cancer cells other than colon. Thus it may be co-expressed with CA 125 in ovarian cancers (317). In a test comparing the sensitivity and specificity of CEA and CA 19-9 for malignant neoplasms, CA 19-9 was found to be more sensitive for pancreatic and bile duct carcinomas; CEA was more sensitive for colon, breast and gastric carcinomas (137).

Both CA 125 and 19-9 share common biochemical characteristics of large MW, high carbohydrate content and microheterogeneity (151).

7. Fetal Antigens

The concept that tumor antigens are products of genes normally expressed in embryonic life and for which there was not self-tolerance was formulated by Boyse in 1968. He reasoned that if a fetal antigen is completely suppressed during adult life but reappears during malignancy then this fetal antigen might elicit an immune response with its reappearance in tumors (25). With regard to this concept, a series of observations were made in chemically-induced tumors in experimental animals to suggest that they expressed fetal antigens (42). Virally-induced tumors also possess embryonic and fetal antigens as shown by experiments using fetal cells to immunize the animals against subsequent SV40 and adenovirus tumor induction (89). Protection against MC induced sarcoma tumors has been observed in female guinea pigs and mice and in male guinea pigs and mice immunized with x-irradiated guinea pig fetus (42). Lymph node cells of normal pregnant mice are cytotoxic for several non-cross reactive sarcoma TSTA induced in the same strain by MC (29). However, the same cells were not cytotoxic for normal fibroblasts. These data suggest that fetal cells and mouse sarcoma cells possess cross-reactive antigens, which can elicit cell-mediated immune responses in syngeneic mothers. On the other hand, mouse fetal homogenates and human kidney fetal cells gave cross-protection against SV-40 tumor induction in hamsters (223). Moreover, pregnant hamsters had generated cytotoxic cells against several DNA virus-induced tumors in vivo and in vitro (42).

In summary, fetal antigens can protect against chemical or viral tumor induction. The protection provided by fetal antigens resembles that of TSTA. However, it has been suggested that TSTA exist independently of fetal antigens; that is, a cancer cell may carry both fetal antigens and TSTA (294). Similar observations concerning immune reactivity to fetal antigens expressed on cancer tissues have been made in humans. Initially, immune interactions between fetus and mother had been confirmed by the appearance of specific Abs in the blood of pregnant women (42). The Abs enter into a specific reaction with antigen of the tissues of the fetuses at particular stages of development. However these Abs do not disturb the natural tolerance between mother and fetus. The immune reactivity of pregnant women against fetal antigens expressed on tumors has been demonstrated in vitro by the LAI assay (104). Leukocytes from pregnant women at 14-28 weeks of gestation reacted with lung, colon, breast cancer extracts, but not normal tissues. On the other hand, leukocytes from cancer patients reacted against an antigen(s) expressed on fetal organs with specificity for the organ, acting as an OSN. The fetal OSN was expressed on fetal lung and colon organs which were 13-19 and 14-19 weeks old respectively. The OSN activity was not detected on fetal organs of older fetuses. Patients with other cancers and normal subjects did not react to fetal lung or colon extracts. It is still unclear why fetal antigens re-expressed in individuals with progressive cancer do not elicit effective immune responses. If the tumor-embryo parallel is correct, then the role of fetal antigens in the survival of the fetus as a homograft may be also effective for the survival of the neoplastic cells (42).

8. Tumor Escape Mechanisms

Immunoreactivity and protection against some malignancies has been found in experimental animals by the resistance shown by these animals to tumor challenge following immunization with tumor cells or extracts. In the absence of previous immunization, however, successful immunity is rarely seen in tumor-bearing animals, even in the presence of strong tumor-specific rejection antigens (206). The preimmunization requirement for inhibition of tumor induction and growth has been demonstrated in many experimental models suggesting that tumor bearing hosts are often unable to combat mechanisms facilitating tumor escape (140). In humans tumor progression is similar to that of non-immunized animals with only a few exceptions of spontaneous tumor regression (176,179). However, the existence of TSTA in animal tumors and OSN in human tumors raises the question of how antigenic cancer cells are able to grow and eventually kill the host despite the immune response against them. It has been suggested that tumors escape the immune reaction in different ways in different systems.

8.1 Induction of Suppressor Cells

Progressively growing tumors have been found to induce suppressor cell functions which counteract the immune response in vivo (194). Early excision of the tumor mass results in loss of suppressor cells whereas when excision is delayed the effect is not easily reversible. This phenomenon could have implications in immunotherapy, where after excision of a large tumor an immune response could be rendered ineffective by suppression.

8.2 Antigen Loss

Tumors present a heterogeneous population of cells. The appearance of tumor cell variants which do not express tumor rejection antigens has been demonstrated as a rejection escape mechanism (223). The most prevalent tumor rejection mechanism is mediated by T cells. The direct cytolytic phenomenon is mediated by the CD8$^+$ T cell subset in association with Class I MHC molecules. The expression or lack of expression of Class I molecules on tumor cells appears in some cases to influence tumor growth, in vivo. Experimental evidence obtained with spontaneously or chemically transformed mouse cells has shown that induction of expression of Class I molecules by transfection into tumor cells confers protection against metastases in mice (7). Along these lines, virally transformed mouse lymphocytes which conferred resistance to lymphoma progression in mice, exhibited high levels of H-2D antigens. On the other hand, cells that progressed to frank lymphoma were negative for H-2D antigens.

In humans there is a good indication from work on colorectal tumors that Class I molecules expression may have clinical importance. In 7 of 9 mucinous tumors, the majority of tumor cells was Class I negative, whereas normal colorectal epithelium expressed normal levels of the antigen (298). It has been postulated that loss of MHC Class I expression is due to a selective secondary mutation and allows the tumors to escape from CTL surveillance. However, low levels of MHC antigens appear to be associated with an enhanced NK cell activity in some instances suggesting that the NK mechanism of tumor cell killing may be particularly important for destruction of MHC Class I - deficient tumors where T cell involvement is absent (7).

8.3 Blocking Factors

There are blocking factors observed in vitro and in vivo. In vitro, serum from tumor bearing animals was found to block the cytotoxic activity of immune lymphocytes (223). Immune complexes of tumor specific Ab and tumor soluble antigen were thought to be the main reason for the abrogation of cytotoxicity. Others have reported that anti-idiotypic Abs generated against the anti-TSTA Ab binding site can also block cytotoxicity (223).

A second category of blocking factors has been observed in in vivo assays and may involve tumor antigen shedding (302). With cell activation during growth or mitogen-stimulation of normal cells there is an increase in synthesis, processing and eventual insertion of certain membrane glycoproteins. Shedding of cell surface proteins is coupled to their synthesis and membrane insertion and is an important aspect of normal protein turnover in the cell membrane (277). Transformed cancer cells have altered growth behavior. In general, transformed cells can be considered to be activated cells and therefore shedding of tumor cell surface components follows this state of activation (277). Cell surface tumor antigens are released from cancer cells along with other molecules. This release may decrease the amount of tumor antigen on the cell surface, rendering the cell incapable of recognition by the immune effectors. Tumor antigen shedding may also cause binding and saturation of immune effectors especially in the tumor microenvironment where the antigen concentration is likely to be the highest rendering reactive immune cells or Ab ineffective (302). Thus the lack of immunogenicity of spontaneous tumors might result from masking of humoral response effectors by shed antigen.

9. Mycoplasmas

Mycoplasmas are minute prokaryotic organisms with the simplest ultrastructure. They contain no cell wall but only a single plasma membrane. Their genome consists of a single, double stranded DNA molecule rich in adenine and thymidine which contains information for the synthesis of cell proteins. They lack metabolic pathways found in other known bacteria and for this reason they require more complex growth media to provide essential nutrients (220).

They all belong to the class Molicutes which contain one order, Mycoplasmatales with one family Mycoplasmatacae. The family includes several species: Mycoplasma, which require cholesterol for their metabolism; Acholeplasma which do not require cholesterol; Ureaplasma which hydrolyze urea and Spiroplasma which have a helical shape (106). Mycoplasmas are implicated in plant, animal and human diseases and are common contaminants of cell cultures (11,38,84,115,130,263,271,301,312).

Mycoplasma contamination of cell cultures was first reported in 1956 (224). They reside mainly in the extracellular environment in intimate association with the host cell membrane, which appears to express molecules acting as specific receptors for mycoplasmas. Both protein and carbohydrate receptors have been described. The receptors vary with both mycoplasma species and cell line (66,83,123,172,173,255). Under the electron microscope, the borders of infected human amnion cells were characteristically projected in the form of microvilli in the neighborhood of the microorganisms. The plasma membrane of the mycoplasma may become continuous with the plasma membrane of the mammalian cell at the tips or along the sides of the microvilli. However, even the non-villated regions of the cell border show attachment (30,59). The attachment is generally by direct apposition of plasma membranes but often appear to be through plasma membrane extensions. Although mycoplasmas reside extracellularly, EM studies on cancer cell lines infected with mycoplasma show that they can also exist intracellularly after fusion with the cell membrane (246). On other occasions, fusion has not been observed. Instead, there is "capping" of the mycoplasmas on the surface of infected lymphocytes as well as other cell types (262). Subsequently, the mycoplasma "cap" is shed and contains the host membrane receptors to which the mycoplasmas attach, as well as other bystander proteins.

Mycoplasmas are closely associated with the host cell membrane. In fact, kinetic studies show that the organisms at low rates of mycoplasma multiplication are attached to the cell membrane and few if any are present in the surrounding culture medium. At high rates of multiplicity such as during the course of adaptation of mycoplasmas to cell culture, the number of the organisms capable of associating with the cell surface increases, suggesting an alteration of the human cell membrane to accommodate more microorganisms (311).

Interaction between mycoplasmas and cell membranes may lead to alterations in activity and function of contaminated cell cultures and thereby markedly affect the results of studies (11,162,261,270,301,312). HeLa cells and other cells in established cultures derived from human tumors fail to grow in suspension in the presence of mycoplasma infection but still grow well in monolayers under the same conditions (246). Some mycoplasmas alter the antigenicity of the cell membrane causing the production of autoantibodies as was shown for the I blood group antigen which was altered after mycoplasma infection and induced anti-I agglutinin Ab production (11). Likewise mycoplasmas can induce Class II MHC expression in a myelomonocyte cell line and Class I and II expression in primary bone marrow macrophage cultures independent of the presence of lymphokines (269). Mycoplasmas can also induce tumoricidal activity to macrophages <u>in vitro</u> (163).

The species Mycoplasma hyorhinis has been shown to selectively remove and incorporate Thy-1.1 differentiation antigen, a non-transmembrane protein (34) from the surface of T cells in their membrane (310,312). This removal may alter the function of the infected cell and it was proposed that it may represent a mechanism by which mycoplasmas avoid or alter the host's immune response (311) . Some other effects that mycoplasmas have upon cells in culture include alterations of host cell metabolism (11,115) alteration of ribosomal RNA profiles (242) and alteration of autocrine substance secretion (47). They can also cause either inhibition or enhancement of virus yields (162,301), haemagglutination (11), a decrease in interferon activity and induction and inhibition of the hybridization process in cell fusion experiments (252). Many of the host cell changes brought about by mycoplasma contamination are linked to depletion of the culture medium of arginine (296). Lack of arginine is associated with stable and unstable chromosomal rearrangements, chromosomal breakage, formation of ring chromosomes and changes in chromosome number in mammalian cell cultures (193,261). These changes may possibly lead to gene mutations which in turn may cause cell transformation. Moreover, chromosomal abnormalities and lymphocyte transformation have been reported to occur by ureaplasmas and by two other fermentative mycoplasmas, that use glucose as an energy source and do not utilize arginine (178). This suggests the presence of a transformation mechanism other than arginine depletion. Furthermore arginine depletion does not always cause alterations to the cells, especially when the medium is changed regularly (12) and contains antibiotics (33).

The presence of mycoplasmas in cell cultures often leads to deleterious changes in the growth potential of the infected cells. However the effects that mycoplasmas exert on host cell growth are varied and can span from stimulation to complete destruction of the cells (261). Morphologic changes such as numerous areas of condensed chromatin designated the "leopard effect" and segregation of nuclear components into large aggregated masses reminiscent of a similar phenomenon induced by viruses have also been observed in cells contaminated with mycoplasmas (115,264). On the other hand, some mycoplasmas, including the arginine users, do not necessarily alter cell morphology or the initial cytopathic effects may be inapparent (270). Moreover, all species of mycoplasmas cause overt or occult cytopathic effects on animal cells, and mycoplasmas sometimes escape detection by the usual agar plate cultivation technique (252). Particularly noteworthy are the "non-cultivable" strains of Mycoplasma hyorhinis which require the presence of animal cells to be cultured (296). Infection of mammalian cell lines with most Mycoplasma hyorhinis strains usually do not produce apparent or gross changes in cell morphology or growth. However, in some cell types certain phenotypic alterations such as transfer of HPRT activity to HPRT deficient cell lines (252,297), inhibition of ^{3}H-Thymidine incorporation (252) and inhibition of tumorgenicity in nude mice (75,297) can occur.

Properties possessed by mycoplasmas and shared with viruses are size, filterability (87,233), morphology under the electron microscope (30), sensitivity to ether, ability to haemagglutinate (87) lack of inhibition by antibiotics, cytopathic effects in cell culture (126) and plaque formation (11). Occasionally mycoplasma and viruses are found to co-exist in blood of patients with leukemia (115,190,241) or AIDS (233).

A variety of reports in the 1960's showed association between mycoplasmas and tumor tissues in animals and humans (115). Most reports could not exclude the possibility of a secondary contamination of the infected material when placed in tissue culture although direct isolation from leukemic tissues was also achieved (13,115,190,210). At that time, although this association was very intriguing it was generally dismissed as an opportunistic event, and mycoplasmas were felt to be present in the cancer tissues for the same reasons that other infectious agents such as bacteria and fungi are found in immunocompromised hosts (115). However, the long known association of mycoplasmas and animal autoimmune conditions (11,271) and recent new findings associating mycoplasmas and AIDS disease (233) have augmented the interest in these microorganisms.

10. Leukocyte Adherence Inhibition (LAI) Assay

10.1 Historical Review

The phenomenon of LAI was discovered by Halliday and Miller in 1972. The LAI assay detects cell-mediated immunity to defined antigens such as dinitrochlorobenzene (DNCB) and purified protein derivative (PPD) (107,213). LAI also detects cell-mediated immunity to tumor antigens in animals (107) and in humans (70,92,100,110), to fungal, bacterial and viral antigens (284), in a fashion similar but of shorter duration than that of macrophage migration inhibition (111). LAI is characterized by the decreased ability of leukocytes to adhere to glass surface after exposure to specific antigen, reflecting in vitro the in vivo immune cell-reactivity to the antigen (111,122,124,284).

LAI was first described for peritoneal cells (PC) from mice tested for their ability to adhere to glass and to exhibit tumor antigen-induced adherence inhibition during a one hour incubation period with the sensitizing tumor extract (111). The original procedure employed a haemocytometer as the cell adherent surface because of the convenience of the marked squares for cell counting. Subsequently, two more LAI assay variations were developed, the tube (122) and the microplate (124) LAI assays. Both variations are based on the principle that leukocytes from an immunologically sensitized host lose their adherence to glass surfaces after encountering the sensitizing antigen in vitro. A landmark in the history of the assay LAI was the First International Workshop on Leukocyte Adherence Inhibition held at the Roswell Park Memorial Institute, Buffalo in 1978 where the three variations of the LAI assay were successfully demonstrated with coded samples of tumors (92).

The assay was originally developed in an effort to reduce the inaccuracy of the capillary-tube migration of macrophages in the macrophage migration inhibition (MMI) assay. The observed adherence inhibition of tumor-sensitized mouse leukocytes on glass surfaces was thought to reflect an early change in leukocyte behavior that was also responsible for the phenomenon of MMI. The LAI assay showed the same specificity as in vivo tumor rejection or in vitro cytotoxicity when responses to animal tumor antigens were assayed (111).

Because of the similarity between LAI and MMI, the non-adherence was expected to be mediated by a soluble lymphokine. In the case of the haemocytometer and microplate assay, data from experiments involving tumor (107,109,110) as well as defined antigens (107,124) confirmed that a lymphokine-like soluble factor mediated LAI. The mediator released by antigen-stimulated cells was termed leukocyte adherence inhibition factor (LAIF) (124).

In mice the cells responsible for the production of LAIF were studied using defined antigens, such as bacterial flagellin, contact sensitizing haptens, such as DNCB, and tumor extracts (107,213). T cells, which required the presence of macrophages as accessory cells, seemed to be the source of LAIF (107,213). In studies using human leukocytes, LAIF was also detected. Human LAIF was found to be species non-specific because it acted on mouse and guinea pig peritoneal cells (107). The cells responsible for the LAIF production in human leukocyte populations in response to sensitizing antigens DNCB and PPD, were the Tγ cells possessing Fc receptors for IgG (107,213). However, in these studies, the presence of macrophages as accessory cells was not necessary (107).

Although LAIF production was found by both the haemocytometer (107) and the microplate assay (124), LAIF production was not initially detected by the tube LAI assay (102,122,174,213).

The tube version of the LAI assay was introduced in 1974 (122) to measure the antigen-induced inhibition of adherence of presensitized leukocytes to glass surfaces. Although the basic principle of the three versions of the assay is the same, experimental data from the different assays revealed an array of different factors that contributed to the LAI phenomenon.

In the original tube LAI assay, where peritoneal cells from antigen-sensitized rats were used, the principal cells exhibiting inhibition of adherence were monocytes/macrophages. No mediator was identified (122). Instead, the mechanism of LAI seemed to be due to direct interaction between a receptor present on the PC cell membrane and the sensitizing antigen that induced the changes in adherence behavior. However, it was not determined whether the receptor was a structural part of the membrane or a serum factor bound to the PC surface (122).

Subsequent studies in the human leukocyte-human tumor antigen system by tube LAI showed that, depending on the experimental protocol, the reactive cells in this system were monocytes armed with cytophilic anti-tumor Ab (100,174) and T cells (148,249). A mediator released by the reactive cells was detected (285). Pharmacologic evidence suggested that the mediator was related to leukotrienes, the mediator being derived from the leukocyte's membrane arachidonic acid and metabolized through the lipoxygenase pathway (285).

With the microplate version of LAI, a soluble factor (LAIF) was found to mediate the loss of adherence of bacillus calmette-guérin (BCG)-sensitized mouse peritoneal leukocytes after incubation with PPD (124,284). The cell reacting with the antigen was shown to be a T lymphocyte (124). A LAIF with similar properties was also observed in supernatants of Con-A stimulated human leukocytes (124).

10.2 The LAI Response to Human Cancer

10.2.1 Principle

In the tube LAI assay, the leukocytes and the specific or unrelated (control) cancer extracts are mixed and incubated for two hours at 37°C. The total protein concentration of the cancer extract used is approximately 100 µg/tube. Titration experiments have shown that the LAI concentration-response curve is bell shaped with diminished responses at low and high antigen concentrations. Maximal response occurs only over a narrow range of antigen concentration (102,274). The concentration of antigen chosen is based on titration curves showing that the greatest difference in non-adherence between specific and control cancer extracts is exhibited at about 100 µg protein (175). When the presensitized leukocytes encounter the sensitizing tumor antigen in the added tumor extract, about 40-65% of the total population of added leukocytes lose their adherence to glass compared to about 15-40% when the same leukocytes are incubated with an unrelated cancer extract (102).

10.2.2 Features of the Tube LAI Response to Human Tumors

The phenomenon of the LAI response depends on three consecutive events: Immunological recognition of the antigen by the presensitized leukocytes, generation and release of mediators by the reactive cells, and mediator-inhibition of adherence of the bystander cells. Immunological recognition of the tumor antigen is the critical step in the cascade of events and takes place in different ways depending on the antigen involved. The form and nature of the recognized antigen define the types of the reactive cells that participate in the response.

10.2.2.1 Tumor Antigen Recognition by Monocytes

Initially, the tube LAI assay was perfomed in our laboratory with PBL from melanoma and breast cancer patients. The PBL were depleted or enriched for various cell populations. Particulate tumor extracts contained tumor antigens recognized by monocytes (100). Antigen recognition is mediated by monocytes "armed" with cytophilic IgG Ab bound to receptors on the monocyte's cell surface (174). The term "cytophilic Ab" refers to the property of the particular Ab to bind to Fc receptors of monocytes before interaction with the antigen (24). Subsequent studies with monocytes and a monocytic cultured cell-line U937 (which responds chemotactically to several chemoattractants, exhibits ADCC and has receptors for IgG and complement) showed that "armed" monocytes released a mediator(s) which inhibited leukocyte adherence to glass. The mediator(s) production and action were negated, as assayed by the LAI response, by drugs such as FPL55712, eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid (NDGA) which block the lipoxygenase pathway of arachidonic acid metabolism. Indomethacin, an inhibitor of the cycloxygenase pathway, did not negate the LAI response (285). On the other hand, authentic leukotrienes such as LTC_4 and LTD_4, which are products of the lipoxygenase metabolic pathway of arachidonic acid induced LAI (285). The induction of adherence inhibition by leukotrienes is similar to that induced by tumor antigen. Moreover, after preincubation with leukotrienes, the leukocytes were no longer able to exhibit the LAI phenomenon when challenged with the sensitizing OSN (285). The lack of non-adherence suggested the leukotrienes (LTC_4, LTD_4) had already triggered the response, and the cells could not respond to the tumor antigen.

10.2.2.2 Tumor Antigen Recognition by T Cells

The recognition of antigen by T cells is mediated by the clonotypic T cell receptor in the context of MHC molecules and in association with the CD4 or CD8 differentiation molecules and the CD3 molecule which is involved in signalling after antigen binding (127). When LAI reactive PBL are depleted of monocytes and enriched for T cells by passage through a nylon wool column, the enriched T cell population does not react with extracts of heterologous cancer (100). However, pure T lymphocytes, incubated with autologous cancer extract show positive LAI responses as well as changes in their transmembrane signalling (247,249) When the same pure T cells were incubated with autologous normal tissue extracts of the same organ, they did not exhibit LAI reactivity or transmembrane potential change, indicating that the T cells recognized an antigen specific to the cancer tissue (249). Moreover, the autologous pure T cell response to autologous tumor extracts was negated by mAbs directed against tumor target HLA,-A,-B,-C molecules, β_2m or the T cell CD8 or CD3 complex (148,247). In contrast, a-HLA-DR (a-Class II) or a-CD4 antiserum coating of target tumor and T cells respectively did not affect the pure T cell reactivity to autologous tumor, suggesting that the CD4$^+$ subset of T cells in the absence of monocytes was unable to mediate LAI responses to the autologous cancer extract (148,247). Furthermore, enriched CD8$^+$ T cells responded to autologous cancer but enriched CD4$^+$ T cells did not (148). On the other hand, a-Class II, a-Class I mAbs did not impair monocyte Ab-dependent recognition of heterologous tumor antigen (247). Overall, the studies indicated that in the absence of monocytes, the response to autologous particulate tumor antigen is mediated by the CD8$^+$ T cells in the context of Class I MHC molecules in the tumor extracts. The crude cancer extracts consist of both cancer cell membrane fragments and soluble material. Thus, a tumor antigen associated with Class I MHC molecules on the cancer cell membrane can be recognized by CD8$^+$ T cells. The CD4$^+$ T cells do not respond to either autologous (148) or allogeneic (247) cancer antigen in the absence of monocytes as antigen presenting cells.

LAI induced by pure T cells to autologous cancer was inhibited by ETYA and FPL 55712, which are antagonists of the lipoxygenase pathway of arachidonic acid metabolism, indicating that arachidonic acid metabolites of the lipoxygenase pathway are important for LAI responses of pure T cells to autologous cancer. The T cell LAI response was not inhibited by indomethacin an antagonist of the cycloxygenase pathway (249). Moreover, authentic leukotriene LTB$_4$ induce an LAI response to pure CD8$^+$ and CD4$^+$ T cells and the response is not negated by either a-CD8 or a-CD4 mAbs (148).

CD4$^+$ T cells recognize antigen in association with Class II MHC products on the surface of antigen presenting cells, and in some instances, tumor cells express Class II MHC antigens and seem to present antigens (192). So far, in all of the experiments with

PBS-extracted autologous (148,249) or allogeneic (100) cancer antigen where pure T cells were used, the only reactive subset was the CD8$^+$ in the autologous situation. The CD4$^+$ subset did not appear to participate, either because the residual tumor cell membranes in the extracts did not express Class II molecules, or the CD4$^+$ T cells needed the presence of Class II MHC molecules on APC's in order to respond. However, in two different experiments, the CD4$^+$ subset were found to be the reactive cells. The first experiment was done to examine the reactivity of MBP in the LAI assay as a cancer antigen. In these studies, pure MBP was used as a soluble antigen and triggered LAI positive reactivity with leukocytes from cancer patients. The MBP-induced LAI was abrogated by a-CD4, a-CD3 or a-HLA-DR mAbs indicating that monocytes presented MBP in association with Class II molecules to the CD4$^+$ subset of T cells. LAI induced by MBP was negated by drugs antagonizing the lipoxygenase of arachidonic acid metabolism (276).

The second experiment was done in order to test the ability of tumor OSN to be extracted with butanol as is done with the immunoprotective animal TSTA (156,238). The butanol-extracted cancer material exhibited positive LAI reactivity with either autologous or allogeneic PBL (146). When the PBL were fractionated to individual cell populations, total pure T cells responded to the autologous butanol extracted cancer antigen. The CD8$^+$ T cell subset was the effector population, as shown by negation of the response by a-CD8 and a-CD3 mAbs. By contrast, the CD4$^+$ T cell subset did not participate in the response because the use of a-CD4 mAb did not affect the LAI. However, when total mononuclear cells were used, the LAI response to autologous tumor butanol extract was not antagonized by a-CD8 mAb. However, it was partly antagonized by a-CD3 mAb, indicating that also monocytes and possibly certain T cells were mediating the LAI. On the other hand, when total mononuclear cells were used with allogeneic butanol extracted cancer antigen, LAI was abrogated by a-CD4 and a-CD3 mAbs. This suggested that allogeneic butanol extracted tumor antigen was recognized by the CD4$^+$ T cell subset and that the recognition required the presence of APC's .

In summary, in the absence of APC, pure CD8$^+$ T cells mediate crude tumor antigen-induced LAI in the context of Class I MHC complex, provided that the tumor is autologous, whereas CD4$^+$ T cells can mediate the LAI response when the antigen is soluble and does so in the context of Class II MHC complex on the surface of antigen presenting cells. On the other hand, Ab-dependent monocytes mediate LAI responses to autologous and allogeneic tumor extracts. As discussed, tumor antigen-induced LAI by monocytes, CD8+ or CD4+ T cells can be negated by inhibitors of the lipoxygenase pathway of arachidonic acid metabolism. Although different forms of antigen induce LAI by eliciting immunological responses from different cell populations, the resulting non-adherence takes place through a common pathway. The pathway involves arachidonic acid metabolites of the lipoxygenase pathway generated by the cells recognizing tumor antigen.

10.2.3 Biochemistry of the OSN-induced Tube LAI Response

Tumor antigen-induced non-adherence is an active process resulting from interaction of the antigen with antigen-sensitive cells. Interaction of tumor antigen with Ab dependent monocytes or T cells initiates a cascade of events that leads to the release of arachidonic acid metabolites (27). Leukotrienes are products of the 5-lipoxygenase metabolic pathway. Prostaglandins are products of cycloxygenase metabolic pathway of the arachidonic acid metabolism.

Cancer patients with early disease often have positive LAI responses to the sensitizing cancer extract, whereas leukocytes from patients with advanced cancer seldom [illegible] (102,147,248,281). Also, leukocytes from LAI positive patients become refractory in the assay 7-14 days after surgery (74). The use of substances such as prostaglandins PGE$_2$ and PGE$_1$ or aminophylline, which increase the intracellular nucleotides, restores the LAI response. A five minute incubation of refractory leukocytes with PGE$_2$ returns the absent LAI response to positive (147,281,286). However, prostaglandins themselves do not affect the actual non-adherence since indomethacin does not inhibit tumor antigen-induced LAI nor does PGE$_2$ trigger LAI (282,286). On the other hand, inhibition of phospholipase

A_2, which hydrolyzes the cell membrane phospholipids to arachidonic acid, negated the LAI response (286). Moreover, metabolic inhibitors of both cycloxygenase and lipoxygenase pathways, negated the LAI response. Since the cycloxygenase pathway generated prostaglandins were not involved in the LAI response, the effect of inhibiting the lipoxygenase metabolic pathway was studied. FPL55712, a specific arachidonic acid lipoxygenase pathway inhibitor, inhibited LAI (286). The products generated through this pathway include hydroxyeicosatetranoic acid and leukotrienes.

Authentic leukotrienes LTB_4, LTC_4 and LTD_4 inhibit the adherence of leukocytes to glass at low concentrations and give bell-shaped dose-response curves (148,285). LTB_4 is a very potent chemoattractant within the leukotriene family (282,283). The fact that LAI was induced by LTB_4 suggests that LAI depends on chemoattractant factors altering the adherence properties of leukocytes, possibly as part of the change for optimal locomotion (282). Other chemotactic factors, including the chemotactic fifth component of complement (C5a des arg), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), platelet-activating factor (PAF) and phorbol myristate acetate (PMA), induce LAI of human leukocytes with bell-shaped dose-response curves (282). The maximum LAI responses are induced by a narrow range of chemoattractant/antigen concentration with high concentrations enhancing adherence rather than reducing it, probably due to an effect on adherence (274). The chemoattractant-induced LAI can be abolished by thromboxane synthetase antagonists, indicating that chemoattractants stimulate the synthesis of arachidonic acid metabolites of which thromboxane in turn mediates the non-adherence of bystander leukocytes (282).

The phenomenon of chemoattractant-induced LAI leads to the hypothesis that the LAI mechanism is related to chemotaxis and by extension to the inflammatory response in vivo. To test this hypothesis, LAI, macrophage chemotaxis in vitro and macrophage inflammatory responses in vivo were compared (278). The studies were done with recombinant inbred strains of mice deriving from high and low responders for the traits of macrophage chemotaxis in vitro and inflammatory responses in vivo. The results showed a genetically controlled correlation in the magnitude of response between LAI, chemotaxis (macrophage accumulation) in vitro and macrophage inflammatory response in vivo. This suggested that the chemoattractant-induced loss of leukocyte adherence is related to the cellular events mediating macrophage chemotaxis in vitro and inflammatory responses in vivo. Furthermore, studies on cancer antigen-induced loss of adherence of leukocytes have shown that, contrary to adherence to glass, the non-adherence is an active cellular event involving changes in transmembrane potential consisting of depolarization and hyperpolarization (248,249), generation of ATP, action of microtubules and microfilaments, calcium entry and increase of intracellular free Ca^{++} (283).

10.2.4 LAI as a Diagnostic Test in Human Cancer

The LAI response of leukocytes from cancer patients is directed against an antigen(s) common to tumors derived from the same organ. Using the haemocytometer LAI, patients with different stages of cancer have approximately the same frequency of positive tests (107). The haemocytometer assay has been used to detect immune responses to hepatoma (109), colorectal adenocarcinoma (108), malignant melanoma and mammary carcinoma (110,171). The responses detected were tumor specific and normal subjects rarely exhibited responses to the tumor extracts. The microplate LAI has been used to detect immune responses to breast and pancreatic cancers and distinguish between pancreatic carcinoma from acute pancreatitis (93,231).

The computerized tube LAI assay performed in our laboratory has been used to detect immune responses to lung, colorectum, pancreas, breast, stomach cancers and malignant melanoma (8,169,175,272). In the tube LAI assay, patients with high tumor burden often exhibit no response, but this unresponsiveness can be corrected by preincubation with PGE_2 (147,175,280,286). The validity of the LAI assay as a means for detection of human immunity against tumor-organ specific neoantigens has been confirmed repeatedly in our laboratory in blind studies with coded samples, at different times and by different researchers (165,284).

11. Objective

Human organ-specific-neoantigens (OSN) are tumor molecules detected in crude cancer extracts to which patients with cancer of the same organ and histogenesis respond in the in vitro assay of LAI. P40 is a molecule isolated from spent medium of lung and colon cancer cells that exhibits OSN properties. P40 was enriched by physicochemical methods and purified by affinity chromatography using monoclonal anti-p40 (a-p40) antibody directed against a common epitope of both colon and lung p40. However, not all OSN activity is bound by the a-p40 affinity column.

Butanol extraction of tissue-cultured human lung and colon cancer cells releases molecules with OSN properties. P40 is present in butanol extracts which exhibit OSN activity as detected by LAI, and p40 elutes in a single peak from reverse-phase HPLC. As with spent medium OSN activity of butanol extracts is present in the areas outside of the p40 peak.

The original aim of this thesis was: 1) the isolation and characterization of the molecule(s) responsible for the OSN activity in butanol extracts, outside the p40 peak, and 2) the distribution of this molecule(s) and p40 in human cancer. I will show that a molecule (p25) co-isolating with OSN activity outside the p40 peak is not an OSN, and p40 is identified as a mycoplasma protein.

CHAPTER II

MATERIALS AND METHODS

1. General Methodology

1.1 Sodium Dodecyl Sulfate (SDS) Gel and Two-Dimensional (2D) Polyacrylamide Gel Electrophoresis (PAGE)

1.1.1 SDS-PAGE

SDS-PAGE were performed by the method of Laemmli (149) with a continuous 7.5 to 20% polyacrylamide gradient (running gel) and a stacking gel. The 7.5% solution contained 5.4 ml of deionized H_2O, 2 ml of 1.875M Tris-HCl (Pierce) buffer pH 8.8, 0.1 ml 10% SDS (Pierce) and 2.5 ml of an aqueous solution of 30% acrylamide (Bio Rad) and 0.8% N,N-bis-methylene-acrylamide (Bio Rad). Just before use 7 µl of N,N,N', N'-tetramethyl-ethylenediamine (TEMED, Eastman) and 35 µl of 5% ammonium persulfate (Bio Rad) were added. The 20% solution contained 1.2 ml of deionized H_2O, 2 ml of 1.875 M Tris-HCl, pH 8.8, 0.1 ml of 10% SDS, 6.7 ml of an aqueous mixture of 30% acrylamide and 0.8% N,N-bis-methylene acrylamide. Just before use 5 µl of TEMED and 20 µl of 5% ammonium persulfate were added. Seven and a half ml of each solution were placed in a gradient mixer and a continuous gradient was introduced by a Miniplus 2 (Gibson) pump into a slab gel apparatus (Bio-Rad) with plates separated by 0.75 mm spacer. The gel was left for at least two hours to solidify and then it was layered with the stacking gel. The stacking gel contained 7.8 ml of deionized H_2O, 1 ml of 1.25 M Tris-HCl, pH 6.8, 0.1 ml of 10% SDS, 1 ml of a mixture of 30% acrylamide and 1% N,N-bis-methylene-acrylamide in deionized H_2O. Again, just before use 7 µl of TEMED and 110 µl of 5% ammonium persulfate were added. The gel was laid on the top of the running gel with a plastic teeth spacer for sample groove formation in place. The gel was left to polymerize for 1 hour. The protein samples were prepared by mixing at a 1:1 ratio with 15 µl of sample buffer. Sample buffer contained Tris-HCl 1.25 M (Fisher Scientific) pH 6.8, 2-mercaptoethanol (Sigma), 10% SDS, glycerol and deionized H_2O in a ratio of 1:1:4:2:2

respectively with a trace of bromophenol blue (Fisher Scientific) as coloring agent. The samples were placed in boiling H_2O for 1.5 minutes to accelerate the action of mercaptoethanol and SDS and were applied onto the gel. Crude protein extracts were used at 10-20 µg/well, purified proteins at 1-2 µg/well.

The top (cathode) and the bottom (anode) of the gel were immersed in running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS). Electrophoresis was carried out with a current of 20 mA/slab until the bromophenol blue marker line reached the bottom of the gel. The gel was stained with Coomassie brilliant blue R250 (Bio Rad) and with silver stain (Bio Rad kit) when needed. In sequence, after staining with Coomassie the excess of the dye was removed by subsequent soaking of the gel into fixative reagents of increasing ethanol dilutions: 1) 40% methanol + 10% acetic acid for 30 minutes, 2) 10% ethanol +5% acetic acid for 15 minutes, c) 10% ethanol +5% acetic acid for 15 minutes. Then the gel was placed in the silver reagent for 20 minutes and finally in the developer until the reaction was completed changing the solution three times. The reaction was stopped by transferring the gel into a 5% acetic acid solution.

1.1.2 2D-PAGE

2D-PAGE was performed according to the method described by O'Farrell (197). The separation of the proteins in the first dimension was performed with a pH gradient of 3.5 to 10. The gel, formed in thin 5 mm diameter cylinders 125 mm long, consisted of 6 ml of 10% Nonidet-P40 (NP-40, BDH Chemicals), 16.5g urea (Bio Rad), 3.99 ml of an aqueous solution of 28.4% acrylamide and 1.5% N,N'-bis-methylene-acrylamide, 5.88 ml of deionized H_2O, 0.75 ml of ampholytes pH 3.5-10 (Bio Rad) 0.15 ml of ampholytes pH 8-10 and 0.6 ml of ampholytes pH 4-6. Just before use 45 µl of 10% ammonium persulfate and 30 µl of TEMED were added. The tubes were filled with the gel and overlaid with 8 M urea, and the gel was allowed to polymerize overnight at room temperature. Next day, 50 µl of a solution containing 9.5 urea, 2% ampholytes pH 3.5-10, 2% Nonidet-P40, 75 mM Dithiothreitol (DTT, Boehringer Mannheim) were added to the top of the gel and they were prerun for 30 minutes at 200V. The samples were incubated in lysis buffer (9.5 M urea, 2% Nonidet-P40, 2% ampholytes pH 3.5-10, 5% 2-mercaptoethanol) for 1 hour at room temperature. At the end of this period, 3 µl of a 0.1% bromophenol blue solution was added to each sample. Then the samples were placed on the prerun gels and were overlaid with an equal volume of a stock solution consisting of 4.75 M urea, 1% ampholytes pH 3.5-10, 1% Nonidet p40, 37 mM DTT. The upper reservoir was filled with 0.04 M degassed NaOH and the lower one with 0.02 M H_3PO_4, and the gels were run for 18 hours at 400 V and then for 4 hours at 600 V. After the run was finished, the gels were placed in equilibration buffer (0.625 M Tris, pH 6.8, 10% glycerol, 2% SDS, 75 mM DTT) and frozen for up to two months The second dimension was then run on a continuous 7.5-20% polyacrylamide gel.

1.2 Western Blots

After a complete run, the proteins were transferred from the gel onto a 0.45 µm pore size nitrocellulose paper (NC, Bio-Rad) following the principle of Towbin (295). The transfer buffer contained 0.025 M Tris, 0.19 M glycine, 20% v/v methanol. The transfer was done overnight at 4°C.

After completion of the transfer the NC paper was blocked in Tris-Tween-Saline (TTS) containing 0.01 M Tris-HCl, 0.05% Tween 20 (Baker analyzed) 0.15 M NaCl, pH 7.5 and incubated with the appropriate antisera overnight at room temperature on a shaker. After extensive washing with TTS the appropriate second antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories, Inc.) was applied at 1/5000 dilution for 4 hours at room temperature. After 20 minutes of washing with four changes of TTS, 60 µl of 30% icy cold H_2O_2 were added to 20 ml of cold methanol containing 60 mg of the chromogenic agent 4-chloro-naphthol (Bio-Rad) in Tris Buffered Saline (TBS, 0.02 M Tris, 0.5 M NaCl) pH 7.5 for 15-20 minutes or until full development of the bands. The NC paper was washed and stored in humidified containers at 4°C.

1.3 ELISA

The ELISA assay was used for screening of binding activity of monoclonal and polyclonal antisera against test antigens. Polysterene Immulon II microtitration "U" plates (Dynatech Laboratories) were used. To each well 50 µl of PBS (Phosphate Buffered Saline, 0.14M NaCl, 0.1M Na phosphate) pH 7.3 containing 500 ng of protein (50 ng in case of affinity purified p40) were added and incubated overnight at 4°C. Then, the plates were washed five times with PBS containing 0.05% (v/v) Tween 20. This solution was used for washing between the steps of the assay. The non-specific sites were blocked with 5% (w/v) bovine serum albumin (BSA, fraction V, Sigma) in PBS for 1 hour at room temperature (RT). At the end of the blocking period the plates were washed five times. Then the appropriate anti-sera were added to the plates at 50 µl/well in duplicate and incubated for 1 hour at RT. Monoclonal antisera were used neat and polyclonal antisera were diluted in PBS in the range of 1/100 to 1/100,000. The plates were washed again eight times and the second Ab was added. Fifty microliters of sheep anti-mouse or goat anti-rabbit IgG- peroxidase conjugate diluted 1/5000 in PBS containing 0.05% (v/v) Tween 20 and 0.01% (w/v) BSA were added to the wells. Incubation at RT was done for 1 hour after which the plates were washed eight times, and the wells were developed with 100 µl of 0.018% (v/v) H_2O_2 in 0.1M citrate buffer pH 4.5 containing 1 mg/ml 0-phenylenediamine (Fisher Scientific) for 30 minutes at RT in the dark. The absorbance (O.D.) was read at 410 nm on a microelisa Minireader II (Dynatech Labs.). The background value was determined by replacing the sera with PBS.

1.4 Antibody Generation

A number of monoclonal and polyclonal Abs generated at various points in time were used in ELISA and western blots for detection of their respective epitope. Monoclonal Ab a-P40 is directed against a common framework determinant of lung and colon cancer cell surface molecules (279,6). Monoclonal Ab derived from a hybridoma producing IgG not binding to P40 was used as a control (279).

Monoclonal Ab anti-P_4C_2 (a-p25) is directed against a 25 kD molecule common to lung and colon cancer cells. It was produced by immunization of mice with LAI positive NCI-H69 lung cell butanol extract derived from reverse phase high pressure liquid chromatography (RP-HPLC) column with retention times 35.50 to 38.25 minutes. Briefly, the butanol material was linked to keyhole limpet hemocyanin (KLH) at 1:1 ratio with the coupling agent 1-ethyl-33-dimenthylaminopropyl carbodiimide. The complex was emulsified in Freund's complete adjuvant and was injected at day zero and ten intraperitoneally into female Balb/c mice more than eight weeks old. Each were again injected with antigen conjugate in Freund's incomplete adjuvant. At six weeks the mice had a bleed test. Serum from non-immunized mice was used as a control. Hybridomas were produced with spleen cells from the immunized mice (143).

Polyclonal Ab anti-p40 (a-p40) was produced by immunizing a rabbit with 800 µg of lung affinity purified p40 linked to KLH at a 1:1 ratio in 800 µl of complete Freund's adjuvant. Four weeks later 250 µg of the same antigen were used in MPL+TDM (trehalose dimycolate + monophosphoryl lipid A, Ribi Immunochem Research) emulsion to boost the animals which were finally bled 3 weeks later.

Polyclonal Ab a-peptide #2 was raised against a synthetic peptide identical to a 15 amino acid (aa) hydrophobic stretch (aa 284-301) of a larger CnBr-cleaved, LAI-positive 54 amino acid segment (CN-5, aa 279-333) of lung affinity purified p40. The Ab was produced by immunizing a rabbit with 1 mg synthetic peptide #2 linked to KLH at a 1:1 ratio in complete Freund's adjuvant. Three weeks later the animal was boosted with 1 mg of antigen linked to KLH in incomplete Freund's adjuvant and three weeks later it was bled from the ear.

A mAb to lung peptide #2 was produced by fusion of myeloma NS-1 cells with spleen cells of immunized Balb/c female 13 weeks old mouse. Briefly, the mouse was injected with 100 µg of synthetic peptide #2 linked to KLH at a ratio 1:9 in 200 µl incomplete Freund's adjuvant (Gibco). The animal was boosted four weeks later with 50 μg of antigen in 400 μl of PBS, and fusion took place two days later.

Polyclonal Ab a-peptide #1 was raised in a rabbit against a synthetic peptide, copy of the segment between aa 310-324 of the same LAI-positive CnBr fragment CN-5 of lung affinity purified p40. The immunization of the animal was done with 1 mg of synthetic peptide #1 linked to KLH at a 1:1 ratio in complete Freund's adjuvant. Three weeks later the animal was given 1 mg of antigen linked to KLH in complete Freund's adjuvant and four weeks later the animal was bled.

Polyclonal Ab a-C1D was raised against a synthetic peptide identical to a 13aa LAI positive tryptic digest (aa 107-119) from colon affinity purified p40. It was produced by immunizing a rabbit with 1 mg of synthetic C1D peptide linked to KLH at a 1:1 ratio in complete Freund's adjuvant. Three weeks later the animal was boosted with 250 μg antigen linked to KLH in MPL+TDM emulsion. Three weeks later the animal was bled.

2. Generation of OSN Active Material

2.1 OSN from Solid Tumors

Cancer tissues obtained from liver metastases were dissected free of necrotic and normal tissue and homogenized in a vessel placed in ice by Virtis 45 homogenizer. The homogenate was spun at 13,000 xg in a Sorvall superspeed RC2-13 refrigerated centrifuge (Ivan Sorvall Inc.) for 1 hour. The supernatant was recovered and its protein content determined by the method of Lowry (168). The supernatant was then enriched for OSN by sequential applications to Blue Sepharose (Pharmacia), Orange A (Amicon) and Phenyl Sepharose (Pharmacia) columns. Before application to each column, the OSN active material was dialyzed 200x the column volume with 0.1M Na phosphate (Fisher Scientific, buffer for the Blue Sepharose and 20 mM Tris-HCl, pH 8.0 for the Orange A column. The unbound and bound fractions were eluted with 0.1 M Na phosphate, pH 7.0 and 3M KSCN (A & C American Chemicals), respectively, from the Blue Sepharose column and 20 mM Tris-HCl, pH 8.0 and 2 M NaCl, respectively, from the Orange A column. Before application onto the Phenyl Sepharose column, the sample was dialyzed in 1.0 M $(NH_4)_2SO_4$ (Anachemia) in 0.02 K phosphate (Fisher Scientific) and was eluted with 1.0 M $(NH_4)_2SO_4$ first and subsequently with 0.02 M K phosphate pH 7.0.

The bound and unbound material from each column were tested for OSN activity.

2.2 OSN from Tissue Cultured Cells

2.2.1 NCI-H69 Lung and Colon Cancer Cell Lines and Culture Methods

A small cell type lung carcinoma NCI-H69 grows adherent to glass roller bottles in culture medium RPMI 1640 (Gibco) containing 0.013 M HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid (Boehringer Mannheim), 0.2% sodium bicarbonate (Fisher Scientific) 3 x 10^{-8} M sodium selenite (Anachemia) 10^{-8} M hydrocortisone (Sigma) 10^{-8} M 17-β - estradiol (Sigma), 5μg/ml bovine insulin (Sigma) 1 mg/ml galactose (Gibco) and 2.5 μg/ml human transferrin (HTf) (Sigma). HTf was iron loaded before use by adding to 50 mg of HTf dissolved in 4.5 ml of distilled H_2O 225 μl of 5 mM $FeCl_2$ in 0.1M sodium citrate; 250 mg of sodium bicarbonate were added and the volume was adjusted to 5 ml. The solution was incubated for 3 hours at 25°C and then overnight at 4°C. Antibiotics were used routinely: 67,000 IU/l penicillin (Flow), 67,000 μg/l streptomycin (Flow), 500 μg/l fungizone (Flow) and 30 μg/l gentamycin (Schering).

The serum-free medium was changed approximately every 2-3 days. With each change cells were shaken loose in the roller bottles. Phenyl-methyl-sulfonyl fluoride (PMSF, Sigma) and 0.02% NaN_3 (Fisher Scientific) were added to the harvested supernatant. Cells and cellular debris were isolated by centrifugation at 7,000 xg for 20 minutes. at 4°C. The pellets were pooled and stored at -40°C until used.

HCT-15 colon cancer cell line like the lung cell line, grows adherent to glass roller bottles in the same type of culture medium supplemented with 10% NU-Serum. Once the cells were confluent, NU-Serum was decreased to 1%. The confluent cells were alternated between 2 days of RPMI 1640 medium containing 1% NU-Serum and 5 days of serum-free RPMI-1640 medium. Under these conditions the cells maintained constant growth.

Only spent medium during the serum-free cycle was harvested for preparation of the OSN. The colon OSN was separated in an identical fashion to the lung OSN.

2.2.2 OSN - Butanol Extraction

The collected lung or colon cancer cell pellets were thawed and extracted at a ratio 1:10 with 2.5% (v/v) 1-Butanol (Fisher Scientific) in PBS pH 7.3 containing 10 μM colchicine/ml for 5 minutes. The insoluble material was removed by centrifugation at 20,000 xg for 20 minutes. The supernatant was then dialyzed against 60 liters of PBS at 4°C and ultracentrifuged at 160,000 g for 60 minutes. The supernatant was concentrated by ultrafiltration in an Amicon-stirred cell (Amicon) with a PM10 membrane (Amicon) tested for protein content and stored at -40°C. Each butanol extract preparation contained from 0.4 to 1.0 mg of protein.

2.2.3 LAI-Monitoring of OSN Activity

The antitumor activity of in vivo sensitized leukocytes was tested in vitro in the standard LAI assay as described by Grosser and Thomson (102). The procedure is as outlined below.

2.2.3.1 Leukocyte Preparation

Heparinized venous blood was obtained from cancer patients (or patients with benign disease serving as controls, depending on the test), in 10 ml Vacutainer tubes (Becton, Dickinson and Co.). The tubes set at a slight angle were incubated for 45 minutes at 37°C. From the leukocyte-rich plasma fraction a leukocyte suspension was isolated after centrifugation at 1400 rpm and lysis of the precipitated red blood cells (RBC) with Tris-Buffered isotonic ammonium chloride (ACT) pH 7.2. The leukocyte fraction was adjusted to a concentration of 10^7 cells/ml. All leukocytes were preincubated with 1.5×10^{-6} M prostaglandin E_2(PGE_2) for 5 minutes before testing (284).

2.2.3.2 LAI Assay

The assay was done in triplicate sets in 15 x 150 mm 20 ml glass test tubes. Each set of tubes received 0.3 ml of medium 199, 0.1 ml crude tumor extract containing 100 μg protein based on titration curves (102) and 0.1 ml leukocyte suspension The mixture was well agitated and laid horizontally at 37°C in a humidified incubator fcr 2 hours. At the end of the incubation period the tubes were set upright. The suspension of the non-adherent leukocytes was well agitated and a sample from each tube was placed on a haemocytometer and counted with a 10 x objective lens by computerized image analysis (Omnicon 1100). The counting was performed automatically in four fields of the haemacytometer. Then the computer (PC IBM) calculated and printed the mean number of cells, the standard deviation and expressed the results as Non-Adherence Index (NAI).

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

where A = the number of non-adherent cells in the sample from the tube that received the specific cancer extract and B = the number of non-adherent leukocytes in the sample from the tube that received the non-specific cancer extract.

An NAI value of 30 corresponds to the cut-off point for negative and positive values. This arbitrary cut-off value was chosen because 95% of control subjects fall below this value, whereas about 80% of patients with cancer had value greater than 30 (284). When OSN activity of isolates was tested the LAI assay was slightly modified. For each sample tested the appropriate crude cancer extract was tested as a positive control. The two sets of tubes A and B received 0.1 ml of FBS (Fetal Bovine Serum, 1% dilution) and tubes A received 10 µl of the sample to be tested. Medium 199 and leukocytes were added as in the standard LAI assay. The FBS was added as a protein source which helps the mobilization of the non-adherent cells.

The LAI-inhibition assay was also used on certain occasions. It is based on the phenomenon that when two antigens are added together at concentrations shown to give maximum LAI responses separately, the positive LAI response is negated (274). In this case the two antigens were added in tube A and a control cancer extract in tube B. If a sample inhibited the LAI response of leukocytes from patients with the specific type of cancer to the respective crude cancer extract by a statistically significant amount, the sample was regarded as having OSN activity. In parallel, the same test sample should not affect the LAI response of leukocytes from patients with a different cancer.

When samples were tested coded, the coding was done by impartial individuals. The LAI values of the studies reported here represent the mean ± standard error of the mean.

2.2.4 Chromatographic Procedures for OSN Isolation

The following procedures were used for OSN enrichment after butanol extraction.

2.2.4.1 Phenyl Sepharose

Phenyl Sepharose CL-4B (Pharmacia) is a hydrophobic interaction column separating proteins according to their hydrophobic properties. The absorbed proteins are eluted in decreasing order of their hydrophobicity. The column was equilibrated 10 x the column volume with 1.0 M $(NH_4)_2SO_4$ in 0.02 M K phosphate pH 7.0. The applied sample was dialyzed in the same buffer before application. Elution was done stepwise with 10 column volumes of 1.0 M, 100 ml 0.5 M $(NH_4)_2SO_4$, 100 ml 0.25 M $(NH_4)_2SO_4$, and 100 ml 0.02 M K phosphate pH 7.0 containing 2% 1-Butanol.

2.2.4.2 Hydrophobic Interaction HPLC (HIC-HPLC)

The OSN-positive fraction from the Phenyl-Sepharose column was concentrated and dialyzed against 1.8 M $(NH_4)_2SO_4$, and 0.02 M K phosphate pH 7.0 (Buffer A) in a BRL microdialysis unit (BRL, Betnesda, Md) and then separated by HIC-HPLC. The HIC was Synchropac Propyl 250 x 4.1 mm with a guard column. The sample was eluted with a linear gradient (0-100%) between Buffer A and Buffer B (2% 1-Butanol in 0.02 M K Phosphate pH 7.0); then with 100% Buffer B for 15 minutes. Flow rate was 1 ml/minute. The collected fractions were concentrated in an Amicon Unit lined with a YM 10 membrane and dialyzed against deionized H_2O.

2.2.4.3 Blue Sepharose

Before application to the column, the OSN-positive fraction(s) from HIC-HPLC were dialyzed against 0.1 M Na phosphate buffer, concentrated in an Amicon cell with a YM10 membrane and applied to the Blue Sepharose column (Pharmacia) This column selectively absorbs albumin and proteases by affinity interactions. The unbound and bound fractions were collected with 0.1 M Na phosphate and 3 M KSCN, respectively.

Blue Sepharose did not remove OSN activity which eluted in the unbound fraction.

2.2.4.4 Anion Exchange (Ax300) HPLC

The OSN-containing fraction eluted from the Blue Sepharose column was dialyzed against Buffer A in a BRL microdialysis unit and applied onto a Synchropak Ax300 250 x 10 mm preparative HPLC column. The column (Waters Assocs.) equipped with a guard column was eluted with a gradient between Buffer A and Buffer B. Buffer A consisted of 0.003 M Tris acetate (pH 8.0) and Buffer B contained 0.1M sodium acetate in 0.003M Tris acetate (pH 8.0). Moving from 100% Buffer A to 100% Buffer B over 60 minutes, a linear salt gradient was formed. Protein peaks were collected, dialyzed and tested for antigen activity.

2.2.4.5 Affinity Column

Monoclonal Ab a-p25 was linked to affigel 10 (Bio-Rad). The gel (25 ml) was washed with 3x the bed volume with cold deionized H_2O in a sintered glass funnel. One hundred mg of protein in 50 ml of HEPES Buffer was added to the moist gel, agitated gently and rotated slowly for 4 hours at 4°C. Then the gel was drained and washed with 25 ml PBS. The gel was resuspended in 50 ml PBS containing 2.5 ml of 1M ethanolamine HCl pH 8.0, in order to block any remaining active ester sites. The mixture was incubated for 1 hour at 4°C. At the end of the incubation period the gel was decanted, resuspended in PBS and transferred to two glass columns. After the column was packed, it was washed successively with PBS, 0.05M diethylamine pH 11.5 and PBS. The washes were concentrated separately and the protein concentration of each was determined to find the amount of protein linked to the gel. After preparation of the column, a total of 4.635 mg of OSN active butanol material was applied and was left for 1 hr. At the end of this period the column was eluted with PBS containing 0.05% Tween 20 at 30 ml/hr speed to remove the unbound material to a total volume of 150 ml. The column was then washed with PBS till the optical density of the eluent reached 0.001. Then the bound fraction was eluted with 0.05 M diethylamine (Fisher Scientific) pH 11.5 at 60 ml/hour. Eluted diethylamine was neutralized with 1 M Tris pH 7.8. A total of 200 ml was collected followed by a washing with PBS with 0.05% Tween 20 which was added to the bound fraction. The bound material was immediately dialyzed against PBS and concentrated in an Amicon unit with YM10 membrane. The column was stored in PBS with 0.02% NaN_3 as a preservative.

2.2.4.6 Reverse Phase HPLC (RP-HPLC)

An analytical Ultrapore-C_3 75 x 4.6 mm column (Beckman) was used. The samples were dialyzed in 0.1% trifluoroacetic acid (TFA Pierce) in H_2O (Buffer A) and applied to the column. Buffer A was run for 5 minutes, then a linear gradient 0-80% of Buffer B (0.1% TFA in 100% CH_3CN) was run for 45 minutes. At the end of this period 80% Buffer B was run for 10 minutes and 80-0% of Buffer B for 5 minutes. Flow rate was 1 ml/minute. Fractions from successive runs were collected and pooled and concentrated in a SpeedVac (Model SVC 200H, Savant Instruments, Farmington, N.Y.).

3. Methods for P40 Peptide Generation

3.1 Purification of Antigens

P40 of colon or lung cancer was isolated from spent medium of tissue cultured colon or lung cancer cell lines HCT-15 and NCI-H69 respectively and purified by mAb affinity chromatography as described (6,279). Final purification was achieved by hydrophobic interaction HPLC to separate p40 OSN from any IgG which may have bled from the Ab affinity column (6,279).

3.2 Preparation of Reduced and Carboxymethylated P40

All cleavage reactions were performed on reduced and carboxymethylated p40 (CM p40). Derivatization of the cysteine residues in this manner simplifies further protein transformations by preventing cysteine dimerization. To a solution of colon or lung p40 (100 µg) in 100 mM Tris chloride, pH 8.5 containing 6 M guanidine hydrochloride was added 100 µl of a solution of DTT (50 mg/ml in H_2O). After standing 18 hours at room temperature, a solution of iodoacetic acid (20 mg in 20 µl of H_2O) was added, and the mixture was incubated at 37°C for 30 minutes. A second 20 mg aliquot of iodoacetic acid was added and incubation was continued for 2.5 hours. The solution was desalted by applying it directly to a reverse-phase HPLC Column (Method A, Section 3.4).

3.3 Protein Cleavage Methods:

The carboxymethylated colon and lung p40 antigen was cleaved by four different methods, as described below.

3.3.1 Cyanogen Bromide (CnBr) Cleavage

100 µg of CM p40 antigen was dissolved in 20 µl of a solution of cyanogen bromide (300 mg/ml in acetic acid) and allowed to stand 70 hours at 23°C. The mixture was applied directly to a reverse-phase HPLC column (Method B, Section 3.4).

3.3.2 Tryptic Digestion

Digestion of the p40 molecule by trypsin was performed either in the absence or in the presence of blocked lysines.

3.3.2.1 Tryptic Digestion without Blocking of Lysines

Immobilized TPCK Trypsin was washed with 1% ammonium bicarbonate and then 200 µl was added to 1 mg p40 OSN in 1% ammonium bicarbonate buffer, pH 8.1. After incubation and rotation for 18 hours at 37°C, the gel was spun down, the supernatant was removed and the gel was washed with 0.5 ml 1% ammonium bicarbonate. The supernatant and the wash were then pooled and rotated with 100 µl of washed immobilized Trypsin Inhibitor (Pierce) for 1 hour at room temperature. The gel was spun down, and the supernatant was removed and evaporated to dryness in a Speed-Vac and applied directly to a reverse-phase HPLC column (Method C, Section 3.4).

3.3.2.2 Tryptic Digestion after Blocking of Lysines

The blocking of lysines was done by the citraconic anhydride method as follows: To a solution of 1 mg of colon or lung p40 in 0.4 M Tris HCl buffer pH 8.1 was added 0.15 M 2-mercaptoethanol overnight and then alkylated with 0.25 M iodoacetamide (Sigma). The protein was precipitated with 20% tricloroacetic acid (TCA) for 4 hours at 4°C. The precipitate was washed twice with 5% TCA, once with 95% ethanol, once with anhydrous ether 1:1 and then twice with anhydrous ether and allowed to air dry.

The sample was then dissolved in 2 ml 7 M guanidine hydrochloride, 0.5 M Tris buffer, pH 8.0. Three additions of 6 μl citraconic anhydride (Fluka) were added with mixing with 10 minute incubation between additions. The sample was well dialyzed versus 3 liters of 1% ammonium bicarbonate in a BRL dialysis unit and digested with trypsin as described above. The acyl blocking groups were hydrolyzed in the TFA used for reverse-phase separation.

3.4 Peptide Purification

CM p40 and the peptidic fragments obtained after CnBr and tryptic fragmentation of lung and colon p40 were purified on RP-HPLC columns, according to the following methods. Peaks were detected with a variable wave length UV detector set at 214 or 220 nm.

3.4.1 Method A. Bio-Rad Hi-Pore RP318 Guard Column (C_{18}), 4.6 x 30 mm (Bio-Rad) was used with Buffer A: 0.05% aqueous trifluoroacetic acid (TFA), and Buffer B: 0.05% TFA in acetonitrile (Fisher Scientific). The gradient was held at 0% B for 15 minutes followed by a linear gradient to 60% B at 1% per minute. Flow rate was 0.5 ml/minute.

3.4.2 Method B. Bio-Rad Hi-Pore guard Column and Hi-Pore RP 318 (C_{18}) Column, 4.6 x 250 mm was used with Buffer A: 0.5% aqueous TFA and Buffer B: 0.05% TFA in acetonitrile. The gradient was held at 0% B for 15 minutes followed by a linear gradient to 60% at one percent per 1.5 minutes. Flow rate was 1 ml/minute.

3.4.3 **Method C**. Buffer A. The Ultrapore C_3 RP column was used with Buffer A: 0.1% TFA in H_2O and Buffer B: 0.1% TFA in CH_3CN. The gradient was held at 0% for 10 minutes followed by a linear gradient to 80% Buffer B in 45 minutes. The flow rate was 1 ml/minute. The OD was read at 220 nm. Individual peaks were collected and dried on the Speed Vac. Peaks were then reconstituted in 0.1% TFA in deionized H_2O (Buffer A) and injected on to the Hi-Pore RP 318 (C_{18}) column with a Hi-Pore guard column. The gradient was run from 0 to 80 % Buffer B in 120 minutes. Flow rate was 1 ml/minute and OD was read at 214 nm. Individual peaks were collected and evaporated to dryness in a Speed Vac (Savant Inc.). They were reconstituted in deionized H_2O and the protein was determined. Aliquots were then sequenced or tested in the LAI assay.

3.5 Peptide Microsequence Analysis:

Protein and peptides were subjected to automated Edmann degradation on a gas-phase microsequencer (AB1 470A), and phenylthiohydantoin (PhNCS) amino acid derivatives were separated and identified by reverse-phase HPLC using the protocol suggested by the manufacturer (PTH amino acid analyzer ABI). Amino acid compositions were determined using a Beckman 121MB amino acid analyzer.

3.6 Peptide Synthesis

Peptides utilized in this study were synthesized by the solid phase method of Merrifield (182) in Bio-Rad Laboratories. In this procedure the polypeptide chain is formed by attaching each amino acid by its C-terminus to the N-terminus of the previous one. The first amino acid of the chain is covalently anchored to a resin particle by its C-terminus.

4. Methods for P40 and P40-Peptide Distribution in Tumor and Normal Cultured Cell Lines and Fresh Tissues

4.1 Preparation of Cultured Cell Extracts

Cultured cells grown in monolayers were scraped with a rubber policeman spun down and rinsed four times with PBS. Each time the cells were spun down in a tabletop centrifuge (International equipment Co.) at 2000 rpm and resuspended in PBS. Alternatively, cells grown in suspension were decanted and spun down and then rinsed four times with PBS. At the final washing the cells were counted, spun down and placed on ice.

Initially three different extraction detergents were used on NCI-H69 lung and HCT-15 colon cancer cell lines: 1% Triton x-100 (153), 1% Nonidet-P40 (152), or 10% SDS (4). All three detergents gave identical results on western blots. So the subsequent extractions were performed with 1% Nonidet p40 as follows:

Five microliters extraction buffer consisting of 1% Nonidet P40 containing 2 mM PMSF were added per 1 x 10^6 cells to the pellet, mixed and incubated for 1 hour on ice. At the end of the incubation period, the cells were centrifuged for 20 minutes at 13,000 xg in a Sorvall superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall Inc) and the supernatant was removed and stored at -40°C for future study.

4.2 Preparation of Fresh and Frozen Tissue Extracts

Cancer and normal tissues were obtained form the Pathology Department of Montreal General Hospital. For each cancer specimen a comparable normal tissue of the same organ was obtained from the same patient. The tissues were either stored at -20°C overnight or immediately extracted. Extraction of solid tumors was performed in part according to the method of Endler (62). Before extraction each specimen was weighed then placed in a cylinder with extraction buffer (1% Nonidet P40 +2mM PMSF) at 4 µl/mg of tissue. The tissues were homogenized in a vessel surrounded by ice by a Virtis "45" homogenizer till liquification. Alternatively, frozen tissues were extracted with 1% SDS.

In this case the tissues were placed in the SDS extraction buffer at v/v ratio of 1:1 and the protein was determined. In both extraction protocols the homogenates were clarified by being centrifuged for 1 hour at 12,100 xg in a Sorvall Superspeed RC2-13 automatic refrigerated centrifuge. The lipid layer on the top of the supernatant was aspirated and discarded. The clear supernatant was collected, aliquoted and stored at -40°C.

5. Mycoplasma Contamination Testing Methods

5.1. Mycotect Assay

The presence of mycoplasma contamination in cell cultures was determined by the Mycotect kit assay (Gibco) according to the manufacturer's instructions. It is based on a mycoplasma property to be very rich in adenosine phosphorylase. This enzyme is only found in minute amounts in mammalian cells. Mixing of adenosine phosphorylase with 6-methyl-deoxyriboside (6-MPDR) a non-toxic analog of adenosine results in two products, 6-methylpurine and 6-methylpurine riboside which are toxic to mammalian cells. Thus when a mycoplasma non-infected fibroblast cell line which forms a dense monolayer is co-incubated with cells from mycoplasma-infected cultures and is subsequently given 6-MPDR, the fibroblasts will die. If the sample cells are not contaminated the addition of 6-MPDR will not have any effect on the fibroblast monolayer. The Mycotect assay, which is based on this reaction, is briefly outlined.

A mycoplasma free mouse fibroblast cell line 3T6 obtained from American Type Culture Collection (ATCC, CCL 96) was plated in 24 well cell culture plates (Starsdedt) at 30,000 cells/well in DMEM with 4.5 g/liter D-glucose with L-glutamine without sodium pyruvate (Gibco) or antibiotics, supplemented with 10% FBS, 2mM L-glutamine and 1 mM sodium pyruvate (Gibco). A set of four wells were allocated per test sample: two wells for testing the sample in duplicate and two for negative controls. There was also a set of four wells per plate to be used as positive controls. Two hours after plating, 0.2 ml of each samples to be tested including medium and cells (100-1000 cells/sample) were added to three wells of the set. The fourth well received 0.2 ml of plain seeding medium. At the same time three of the positive control wells in each plate received 0.2 ml of an adenosine phosphorylase stock solution provided with the kit. The fourth well received 0.2 ml of plain seeding medium. The cells were then incubated at 37°C in 5% CO_2 Twenty-four hours later duplicate wells containing test samples or adenosine phosphorylase were given 50 µl of a 10 mM stock solution of 6-MPDR. The other two wells (one of which had also received test sample) serving as negative controls did not receive 6-MPDR. The cells were then incubated undisturbed for 72-94 hours longer. At the end of the incubation period the supernatant medium was aspirated and the wells were filled with a solution of 10% crystal violet (Fisher) in formaldehyde (Baker analyzed). Twenty minutes later the dye was aspirated and the plates were washed with deionized H_2O until no more dye came off. Then they were air dried and observed. The duplicate test sample wells which had received mycoplasma contaminated samples and 6-MPDR showed a disrupted 3T6 fibroblast monolayer and sometimes the monolayer was completely non-existent, depending on the degree of contamination. The positive control duplicate wells which received adenosine phosphorylase and 6-MPDR exhibited complete lack of monolayer formation. On the other hand the wells which had received non-mycoplasma-infected test samples and 6-MPDR did not show any disturbance of the indicator fibroblast cell monolayer which had grown to confluency and stained evenly blue. The two control wells allocated per test sample served two purposes. The one which received the test sample but not the 6-MPDR indicated the effect of mycoplasma contamination alone on the indicator cells. Many times the indicator cell monolayer was torn even in these wells when a very heavily contaminated sample was tested. The second control well received neither sample nor 6-MPDR and played the role of a negative control in which the indicator cell line grew undisturbed to confluency, so these wells were stained with an even blue color at the end of the assay.

5.2. Culture Method

Culturing of mycoplasma was done in the Microbiology Department Laboratory at the Montreal General Hospital. Sterile cell samples were placed on specific mycoplasma broth and incubated for 72 hours at 37°C. They were then observed macroscopically and microscopically for mycoplasma colony formation.

5.3 ^{3}H-Thymidine Uptake Assay

This assay is based on ^{3}H-Thymidine uptake by cell free culture media containing infecting mycoplasmas according to the method by Kaplan et al.(132). Media from cell cultures were centrifuged at 400 xg for 6 minutes. The supernatant was removed to clean tubes and spun a second time at 700 xg for 8 minutes. The ^{3}H-Thymidine uptake assay was done in quadruplicate in 96 well plates. One hundred microliters of each centrifuge sample supernatant were placed in each well and 100 µl of fresh medium were added. The plates were incubated at 37°C in 5% CO_2 for 72 hours. One µCi of ^{3}H-Thymidine in 0.05 ml of fresh medium was then added in each well. The plates were incubated for 18 hours at 37°C. Then the contents of the well were harvested by an automatic cell harvester (SKATRON) on glass fiber filters which have the ability to retain DNA. The filters were placed in scintillation vials, 2 ml scintillation fluid were added, and the vials were counted in a β-counter (Beckman LS 60001C).

6. Methods for OSN Detection in Crude Mycoplasma Extract and Non-Contaminated Lung Cell Line NCI-H69.

6.1. Mycoplasma Culture

GDL strain of Mycoplasma hyorhimis obtained from ATCC was cultured and extracted at Dr. C. Stanners' laboratory. Briefly, it was grown in Brain-Heart Infusion Broth replenished with 20% horse serum and 2.5% (w/v) baker's fresh yeast extract. For extraction the micro-organisms were pelleted at 10,000 g for 20 minutes, washed extensively with PBS and lysed by sonication. The lysate then was used for testing in the LAI assay.

6.2. NCI-H69 Lung Cancer Cell Culturing (New Culture)

The new culture of NCI-H69 lung cancer cells was obtained from ATCC; the cells were grown in the RPMI medium identical to that described in Section 2.2.1 of this chapter and replenished with 10% FBS and the growth factors described previously but with no antibiotics. After the first burst of cell proliferation the cells were placed in serum and antibiotic free medium for two passages. The medium was changed twice a week. After the second passage, the cells were collected, pulverized and centrifuged at 8,000 g for 20 minutes. The supernatant protein was concentrated in an Amicon cell with PM-10 filter membrane and washed three times with 600 ml of PBS. Protein was determined and was tested for reactivity in the LAI assay.

CHAPTER III

RESULTS

1. Studies on Solid Tumor Extracts:

The validity of the LAI assay as a reliable in vitro assay that detects immune reactivity against animal and human tumor antigens has been well documented in the literature (93,107,122,124,213,284) and by coded studies (92,165,288). In view of the recent finding that P40 is a mycoplasma transport protein (56,240), the validity of the LAI assay as a means for tumor antigen detection was re-examined.

Parallel uncoded and coded studies were conducted. First the reactivity of lung, colon and breast extracts with leukocytes from patients bearing cancer of the same organ was verified.Second the organ specificity of the same extracts was verified in criss-cross testing. The coding was done to judge the impartiality of the tester. The samples were coded before testing by uninterested persons and the code was broken when the study was completed. The results of testing individual enriched colon, lung and breast tumor extracts are shown in Table 1. Leukocytes from the donor patients were always tested with our standard crude solid tumor extract that was proven to give appropriate positive responses. As well, leukocytes were tested against a negative control sample of FBS. The same enriched samples were tested in parallel, both uncoded and coded, to determine whether knowing the nature of the test sample resulted in any bias towards a particular result. Table 1 compares the results and shows that the crude extracts of solid tumor and the enriched extracts of the same tumors gave very similar positive responses in the LAI. FBS, an irrelevant protein source, did not exhibit reactivity with the leukocytes. Importantly, the results of the parallel testing of the coded and uncoded enriched isolates were equally positive. The results confirm previous studies (102,272,284) that solid tumors in the form of crude homogenates or enriched tumor extracts (as in the present study) contain substances which are recognized immunologically by leukocytes of patients with the same type of cancer and trigger a series of changes to inhibit the adherence of a proportion of the

TABLE 1

RESULTS OF UNCODED AND CODED STUDIES ON COLON, LUNG AND BREAST SOLID TUMOR HOMOGENATES BY LAI

Diagnosis of leukocyte Donor		NAI to crude solid tumor*	NAI to: uncoded samples of**				NAI to: coded samples of**			
			FBS***	Enriched isolates of			FBS***	Enriched isolates of		
				Colon 30 µg	Lung 20 µg	Breast 25 µg		Colon 30 µg	Lung 20 µg	Breast 25 µg
Cancer										
Colon	n = 3	87 ± 36	7 ± 7	80 ± 22[1]	NT	NT	1 ± 14	89 ± 34[4]	NT	NT
Lung	n = 2	40 ± 7	-5 ± 21	NT	96 ± 46[2]	NT	-5 ± 19	NT	52 ± 9[5]	NT
Breast	n = 2	61 ± 16	-1 ± 11	NT	NT	63 ± 12[3]	4 ± 14	NT	NT	94 ± 24[6]

*The crude solid tumor extract was identical to the diagnosis of leukocyte donor; tested at 100 µg.

**Comparing the responses of the tumor samples to those of the FBS, the differences are statistically significant, student's 't' dependent test: [1]p <0.005. [2]p <0.025, [3]p <0.01, [4]p <0.0025, [5]p <0.005, [6]p<0.01.

***Tested at 100 µg

n = Number tested.

NT = Not tested.

added leukocytes. Moreover, the loss of leukocyte adherence to glass can be quantitated. In the second study, the tumor extracts were tested simultaneously in a criss-cross fashion in order to verify their organ specificity (Table 2). As in the previous study, the same samples were tested in parallel coded and uncoded. There was no LAI response when enriched colon cancer material was tested with leukocytes from lung or with breast cancer patients. Similarly, the enriched material from lung tumor did not react with leukocytes from colon cancer patients. However, all three types of cancer patients' leukocytes (colon, lung, breast) do exhibit recognition of the tumor extracts of the same organ (Table 2). In summary, the results of those two studies show that colon, lung and breast solid tumors do express immunogenic substances which exhibit organ-specificity (OSN).

2. Studies on P40 Peptides

P40 was isolated from spent medium of lung NCI-H69 and colon HCT-15 cultured cancer cells. As will be discussed in more detail later, p40 is a mycoplasma transport protein (56) which is expressed on two mycoplasma infected cultured cancer cell lines that, when extracted, exhibited OSN activity in the LAI assay.

The combination of OSN expression by tumor extracts and the reliability of the LAI assay prompted the studies to purify the OSN and to find a constant cellular source for producing the large quantities of OSN needed (55,284). Spent medium from these two cell lines had been tested in the LAI assay. Both lines were found to exhibit OSN activity (6,55,279). A mouse mAb raised against OSN-enriched extract from the lung cancer cell line NCI-H69 was used to affinity purify a 40 kD molecule (p40) which exhibited OSN activity (279). The same mAb was used for affinity purification of a similar p40 molecule from OSN-enriched extract of the colon cancer cell line HCT-15. The colon p40 also contained OSN activity (6). However, in both instances of affinity purification of p40, the unbound fraction eluted from the affinity column and depleted of p40 also exhibited OSN activity (6,279). The purified p40 molecules from colon and lung cancer, when tested in the LAI assay, exhibited no cross-reactivity (6). The results had suggested that the mouse

TABLE 2

RESULTS OF CRISS-CROSS TESTING OF UNCODED AND CODED SAMPLES OF COLON, LUNG AND BREAST SOLID TUMOR HOMOGENATES BY LAI

Diagnosis of Leukocyte Donor		NAI to crude solid tumor***	NAI to enriched isolates of					
			Colon 30 µg		Lung 20 µg		Breast 25 µg	
			uncoded*	coded**	uncoded*	coded**	uncoded*	coded**
Cancer								
Colon	n = 2	111 ± 43	104 ± 41^{1}	114 ± 15^{4}	-27 ± 20^{1}	-1 ± 16^{4}	NT	NT
Lung	n = 3	77 ± 8	-12 ± 8^{2}	13 ± 15^{5}	74 ± 27^{2}	61 ± 27^{5}	NT	NT
Breast	n = 2	118 ± 40	17 ± 14^{3}	10 ± 17^{6}	NT	NT	111 ± 44^{3}	120 ± 32^{6}

*Comparing the samples of the uncoded studies the differences were statistically significant, student's 't' dependent test (^{1}p <0.005, ^{2}p <0.0005, ^{3}p <0.005).

**Comparing the samples of the coded studies the differences were statistically significant student's 't' dependent test: (^{4}p <0.0005, ^{5}p <0.025, ^{6}p <0.0025).

***The crude solid tumor extract was identical to the diagnosis of leukocyte donor, tested at 100 µg.

n = Number tested.

NT = Not tested.

mAb was directed against a common epitope different from the immunogenic organ-specific epitope recognized by human leukocytes. In order to define the nature of the organ-specific epitope the lung and colon p40 were fragmented and sequenced.

Amino terminal sequencing of lung and colon p40 was performed by three major procedures for generation of individual peptides. Both the colon and lung p40 were subjected to cleavage by CnBr, which cleaves at methionine to generate few large fragments, given the fact that this amino acid (aa) was present at a very low frequency in the p40 molecule. P40 was digested by trypsin with and without prior lysine blocking, in order to generate long fragments. Many different samples were subjected to these cleavages on separate occasions.

Trypsin or CnBr did not destroy the antigenic sites of p40 (275). The peptide fragments generated from lung and colon p40 (shown in Tables 3 and 4 respectively) were HPLC-purified sequenced, and tested for OSN activity in LAI . A number of peptides from both lung p40 and colon p40 gave positive LAI responses (D.M.P. Thomson, unpublished observations). However, after separation by RP-HPLC, the quantity of individual peptides was limited. So, only a limited number of peaks from the column were tested in the LAI for OSN activity.

A trypsin-digested colon peptide labelled TC1D, did give repeatedly positive LAI responses as did a CnBr-cleaved peptide, CN5, from lung (D.M.P. Thomson, unpublished observations). Once the reactive peptide(s) were identified, the whole peptide or a portion of the peptide fragment was synthesized and the synthetic fragment was tested in LAI to determine whether it gave an identical response. A synthetic peptide corresponding to the sequence of colon TC1D (C1D) and two segments of lung CN5, designated peptide #1 and peptide #2, were synthesized, purified and resequenced to verify that the appropriate sequence had been synthesized. The amino acid sequences of these synthetic peptides are shown on Table 5.

TABLE 3

AMINO ACID SEQUENCE OF PEPTIDES FROM P40 OF LUNG CANCER CELLS

Cyanogen Bromide fragments

CN-2	NTFNETILHGKNA ( )Q
CN-3	GTFLN$\frac{I}{G}$ILNGK( )$\frac{N}{A}$
CN-5	EALIVTNPIPYDVGVFRKSVNQLEQNLIVQTFINLAKNKQDTYGPLGYNGYKKID
CN-9	LNKKKGGKEVNKEV
CN-11	LSKKKGGKEVNLEVTNTFLENFK( )EN

Tryptic peptides

L1-9	SATKPFETK
L4-6	VYDPTVQAD
L4-12	FTTLNEDR
L5-7	QSVYDPTVQAN
L6-6	EDVSVYQGQWDK
L6-7	YVDNTDSDPLVK
L8-12	GVSEAWLNK
L8-13	NFDDVDFK
L11-6	NNPGIITPALETTTN
L11-9	IFVETPY

Lysine blocked tryptic digests

L1-65	EVINTFLENFK
L2-62	NNPGIITPALQTTTNSFVLDKEK
L3-59	EINKIFVGTPVASWTDEN( )K
L4-60	EITFGVSEATLNK
L5-63	NNPGIITPALQTTTNSFVLDK

Amino acids are denoted by single letter code. All fragmentation procedures have been performed on intact affinity purified lung p40.

TABLE 4

AMINO ACID SEQUENCE OF PEPTIDES FROM P40 OF COLON CANCER CELLS

Cyanogen Bromide Fragments	
CCN-8	NTFLNVTNLQG
CCN-10	EALIVTNPIPYDVGVFRKSVNQLEQNLIVQTFINLAKNKQDTYGPL
CCN-13	LNKKKGGKEVNKEVSNT( )$\frac{G}{L}$EN( )KGG
CCN-15	LSKKKGGKEVNKEVVNTFLEN()K
Tryptic Peptides	
Tb1a	ASWTDENHKIDNEQK
Tb1c	VSQGQWDK
Tb1d	IFVETPY
Tb2a	FTTLNEDR
Tb2c	QSVYDPTVQAN
Tb2e	YVDGTDSDPLVK
Tb1b	IFVETPY
Tc1d	FYVDGTDSDPLVK
T9-17	QDEYGPE
T15-10	NNPGIITPALETTTN
Lysine Blocked Tryptic Peptides	
L1	NFGILHGKDNSSSKKFKLEE(T)ILKNHFQ
L2	SAEPNAYKQKSADTLGTLDDFHIAFSEF
L3	KSVNQLEQNLIVQTFINLAKNKQDTYGPLLGYNGYKKIDNFQKEIV
L4	GSVNQLEQNLIVQ( )F(I)N
L5	GVIWI( )GN( )ETLAKIK( )A(N)( )K
L6	SAHPNAYKQKSADTLGTLD
L7	GDNFQKEIVVYEKAIK

Amino acids are denoted by single letter code. All fragmentation procedures have been performed on intact affinity purified colon p40. The peptides start from the amino terminus to the carboxyterminus.

TABLE 5

SYNTHETIC PEPTIDE SEQUENCES FROM P40 OSN OF LUNG AND COLON CANCER CELLS

Peptide*	Amino acid sequence
1. Lung**	
Peptide #1	FINLAKNKQDTYGPL
Peptide #2	TNPIPYDVGVFRKSVNQL
2.Colon***	
Peptide C1D	FYVDGTDSDPLVK

*The peptides were synthesized according to Merrifield's solid phase method with an appendage of Cys-Gly-aAB for lung and Cys-Gly-Gly for colon peptides at the amino terminus to facilitate coupling to protein carriers for immunization purposes.

**The two lung peptides have been synthesized as copies of two segments of the LAI positive CnBr-cleaved 56aa lung peptide CN5 from lung intact affinity purified p40.

***Colon synthetic peptide C1D has been made as a copy of the respective 13aa long tryptic peptide of colon p40.

Two other cleavage procedures used on one occasion each for generating p40 peptides involved chymotrypsin (CH) and Staphylococcus aureus (SA) enzyme digestions. They were performed by Bio-Rad laboratories, producing chymotrypsin fragments from lung and colon p40 and SA fragments from lung only. There were insufficient fragments (shown in Tables 6 and 7) for testing in the LAI.

2.1 Testing of P40 Peptides as Inhibitors of the LAI Response

After purification, the synthetic peptides were tested in the LAI assay. Initially they were tested for their ability to negate a positive LAI response to the p40 molecule. It had been shown previously that when two antigens that gave an LAI positive response separately are combined together at their optimal LAI concentrations, they abrogate the positive response (274). Tables 8,9 and 10 show the results of peptides tested in blind studies for their ability to inhibit a positive LAI induced by crude cancer extracts. Table 8 shows criss-cross testing of two synthetic peptides. Lung #1 and colon C1D inhibited the response of lung and colon cancer patients leukocytes only to their respective crude cancer extracts of lung and colon (Table 8). In a similar criss-cross study, synthetic lung peptide #1 was tested with the natural CnBr cleaved CCN8 colon peptide which when tested alone was LAI positive (D.M.P. Thomson, unpublished data). Table 9 shows that each peptide inhibited the LAI positive response only to the cancer extract of the same organ. Table 10 shows the synthetic lung peptide #1 tested against a synthetic peptide with no sequence homology to either lung or colon peptides (labelled a nonsense peptide). The lung peptide inhibited the LAI response of lung cancer patients leukocytes to crude lung cancer extract whereas the nonsense peptide did not (Table 10).

TABLE 6

AMINO ACID SEQUENCE OF PEPTIDES OBTAINED BY CHYMOTRYPSIN DIGESTION OF LUNG AND COLON AFFINITY PURIFIED P40.

Peptide	Amino acid sequence
1. Lung	
CH-13	NNEANEFMEAL
CH-22	INENALDN
CH-29	IVTNPIPY
2. Colon	
CH-18	AAWK

TABLE 7

AMINO ACID SEQUENCE OF PEPTIDES OBTAINED BY STAPHYLOCOCCUS AUREUS DIGESTION OF LUNG AFFINITY PURIFIED P40.

Peptide	Amino acid sequence
SA-A	AWLNKKKGGKEVNKEVINTEL
SA-B	$\frac{\text{E}}{\text{S}}$DVSVSQGQWDKSO$\frac{\text{T}}{\text{H}}$FGVGE$\frac{\text{V}}{\text{T}}$ R( )
SA-C	NKVSFVFNYASD
SA-CM	EPNDPELGKEKQ
SA-D	( )WLNKKKGGKEVNKEV
SA-D1	EAVDPEAGNDK
SA-E1	$\frac{\text{T}}{\text{S}}$NKGVEFPYA$\frac{\text{E}}{\text{S}}$KTC
SA-E2	G( )LVQGTINL$\frac{\text{A}}{\text{S}}$KTK

TABLE 8

RESULTS OF TESTING CODED SAMPLES OF SYNTHETIC P40 PEPTIDES FOR THEIR ABILITY TO INHIBIT THE LAI RESPONSE TO THE SPECIFIC TUMOR

Diagnosis of Leukocyte Donor	**% Inhibition of LAI response***	
	Synthetic Peptides**	
	Lung Pept #1[1]	**Colon C1D[2]**
Cancer		
Colon n = 5	9	100
Lung n = 6	98	28

*%Inhibition of the LAI response to the test antigens when compared to the response of the same leukocytes reacting to the OSN of crude cancer extract.

**Both peptides were tested at 0.3 μg.

[1] Comparing the responses to test antigen synthetic lung peptide #1 the differences are statistically significant; student's "t" independent test ($p < 0.001$).

[2] Comparing the responses to test antigen synthetic colon peptide C1D the differences are statistically significant; student "t" independent test ($p < 0.005$).

n = Number tested

TABLE 9

RESULTS OF TESTING CODED P40 PEPTIDES FOR THEIR ABILITY TO INHIBIT THE LAI RESPONSE TO THE SPECIFIC TUMOR

Diagnosis of Leukocyte Donor	**% Inhibition of LAI response***	
	Synthetic lung peptide #1[1]	**CnBr cleaved natural colon peptide CCN8[2]**
Cancer		
Lung n = 4	100	0
Colon n = 4	39	96

*% Inhibition of the LAI response by the same leukocytes to the two test antigens compared by their response to the OSN of crude cancer extract. Both antigens were tested at 0.3 μg.

[1] Comparing the responses to synthetic lung peptide #1 the differences were statistically significant; student's "t" independent test ($p < 0.005$).

[2] Comparing the responses to colon CnBr-cleaved natural peptide CCN8 the differences were statistically significant; student's "t" independent test ($p < 0.0005$).

n = Number tested.

TABLE 10

RESULTS OF TESTING CODED SAMPLES OF SYNTHETIC PEPTIDES LUNG P40 PEPTIDE #1 AND A NONSENSE PEPTIDE FOR THEIR ABILITY TO INHIBIT THE LAI RESPONSE TO THE SPECIFIC TUMOR

Diagnosis of Leukocyte Donor	% Inhibition of LAI response*	
	Synthetic peptides[1]	
	Lung #1	Nonsense[2]
Cancer		
Lung n = 6	87	18

*% Inhibition of the LAI response given by the test antigens compared with the response given by the OSN of the crude cancer extract of the same organ

[1] Both peptides were tested at 0.3 μg Comparing the inhibition of the LAI response given by the same leukocytes to the two antigens, the differences were statistically significant; student's 't' dependent test ($p < 0.005$).

[2] Synthetic peptide of 17 amino acids unrelated to any sequences of p40.

n= Number tested.

2.2 LAI Testing of P40 Peptides as Antigens

The peptides were also tested for their ability to directly induce a positive LAI response. Synthetic lung peptide #1 was tested coded with the commercially obtained synthetic peptide T18 derived from myelin basic protein (MBP) (Table 11). MPB as described in the "Introduction", acts as an antigen in LAI for both cancer patients (71,72, 125) and animals with tumors (315,316). The results show that both peptides trigger LAI with leukocytes from lung cancer patients whereas only T18-MBP peptide triggers LAI with colon cancer patients leukocytes.

Table 12 shows the results of blind studies done with three synthetic peptides, lung #1, colon C1D and the nonsense peptide. Lung #1 peptide and colon C1D showed organ specificity (Table 12). Lung synthetic peptide #1 gave positive LAI response with lung cancer patient's leukocytes but not with colon cancer patient's leukocytes. Conversely, colon synthetic peptide C1D reacted positively with colon cancer patient's leukocytes but not with lung cancer patient's leukocytes. The nonsense peptide did not react with either patient's leukocytes. These results show that the synthetic peptide C1D of the LAI-positive colon peptide TC1D and the lung synthetic peptide #1, a synthetic peptide deriving from the LAI-positive larger fragment, exhibit OSN activity. Another synthetic peptide prepared from a different region of CnBr CN5 lung fragment (peptide #2) gave a negative LAI response (data not shown).

2.3 Anti-Peptide Antisera Binding to P40

The three synthetic peptides, colon C1D, lung #1 and lung #2 were used to raise mouse polyclonal as well as monoclonal antibodies (D.M.P. Thomson, unpublished data). The synthetic peptides and the intact lung p40 molecule were tested in ELISA with their respective monoclonal and polyclonal antisera (Table 13). With the exception of peptide #1, each peptide reacted only with its respective antiserum. Peptide #1cross-reacted slightly with the mAb raised against peptide #2. Only the polyclonal a-#2 Ab bound the whole p40 molecule in addition to the peptide #2 against which it was raised. The a-#1 and

TABLE 11

RESULTS OF BLIND TESTING BY LAI OF ANTIGENICITY OF LUNG P40 SYNTHETIC PEPTIDE #1 AND A SYNTHETIC MBP PEPTIDE

Diagnosis of Leukocyte Donor	NAI to: Synthetic peptide					
	Lung #1*			T_{18}-MBP**		
	0.15 µg[1]	0.175 µg[2]	0.2 µg[3]	0.15 µg	0.175 µg	0.2µg
Cancer						
Colon	-3 ± 5	15 ± 6	-4 ± 4	42 ± 12	-3 ± 8	-6 ± 8
	n = 5	n = 5	n = 2	n = 5	n = 2	n = 2
Lung	40 ± 9	51 ± 10	-5 ± 5	61 ± 14	-16 ± 5	-19± 5
	n = 5	n = 5	n = 2	n = 4	n = 1	n = 1

*For each concentration, comparing the responses to lung synthetic peptide #1 the differences were statistically significant, ([1]$p<0.001$, [2]$p<0.01$); [3]not significant, student's 't' independent test:

**For each concentration, comparing the response to synthetic Myelin Basic Protein peptide T_{18}, the responses were not significant at any level (student's 't' independent test).

n = Number tested.

TABLE 12

RESULTS OF BLIND TESTING BY LAI OF ANTIGENICITY OF COLON AND LUNG P40 SYNTHETIC PEPTIDES

Diagnosis of Leukocyte Donor	NAI to:		
	Synthetic peptides		
	Lung[1] #1	Colon CID[2]	Nonsense Peptide
	9-10 μl*	9-10 μl*	9-10 μl*
Cancer			
Colon	-5 ± 3 n = 11	42 ± 11 n = 6	-7 ±4 n = 6
Lung	38 ± 7 n = 10	7 ± 5 n = 6	8 ± 3 n = 6

[1] Comparing the responses to synthetic lung peptide #1, the responses were statistically different, student's "t" independent test ($p < 0.001$).

[2] Comparing the responses to synthetic colon peptide C10 the responses were statistically different, student "t" independent test ($p < 0.01$).

*Volumes of medium containing synthetic peptide added to the assay; for colon ClD 9 μl = 0.2 μg and 10 μl = 0.225 μg; for lung peptide #1 9 μl = 0.175 μg and 10 μl = 0.2 μg.

n = Number tested.

TABLE 13

BINDING OF THE INTACT LUNG P40 MOLECULE AND INDIVIDUAL P40 PEPTIDES BY THEIR RESPECTIVE MONOCLONAL AND POLYCLONAL ANTIBODIES AS MEASURED BY ELISA

Antigens[1]	**Antibody binding ($O.D_{410}$)**					
	a-P40[2] **mono**	**a-P40**[3] **poly**	**a-#1**[3] **poly**	**a-#2**[4] **poly**	**a-#2**[2] **mono**	**a-CID**[3] **poly**
Peptide #1	0.00	0.04	1.76	0.24	1.02	0.04
Peptide #2	0.01	0.00	0.02	1.67	1.76	0.00
Peptide C1D	0.04	0.00	0.06	NT	0.00	1.71
P40	0.89	1.76	0.03	1.60	0.00	0.05

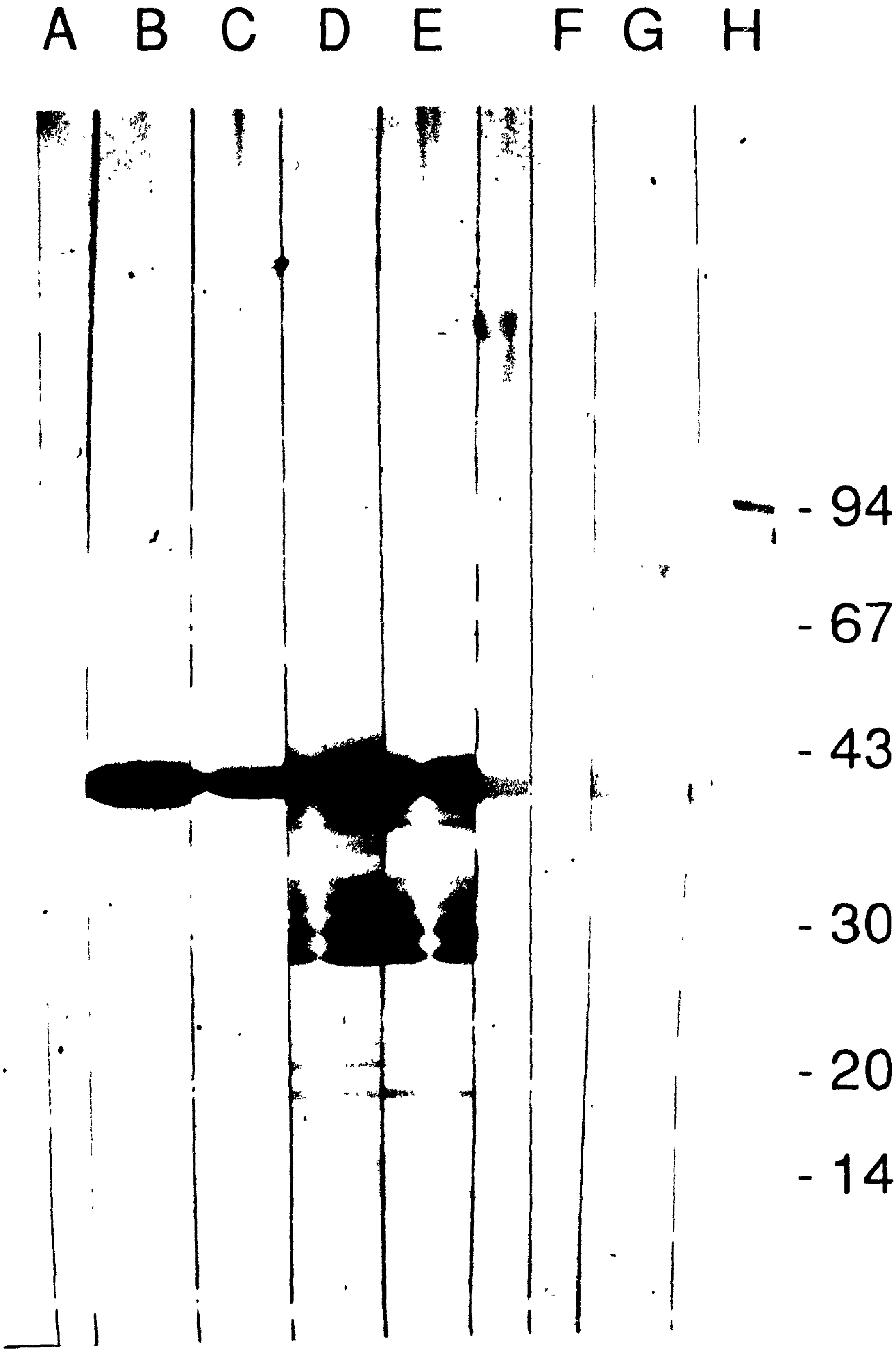
[1] Peptides #1,2 and C1D tested at 500 ng/well. Lung affinity purified p40 tested at 50 ng/well.
[2] Tested as neat culture supernatant.
[3] Tested at 1:1000 dilution.
[4] Tested as purified IgG at 20 µg/ml.
NT = Not tested.
mono = monoclonal.
poly = polyclonal.

a-CID pAbs and the mAb a-#2 bound peptides #1, C1D and #2 respectively, but they did not bind p40, suggesting that the epitopes were unavailable for binding in the whole molecule. One possibility for the unavailability is that the position of the epitopes in the whole molecule makes them unaccessible to the Abs. It has been shown that Abs to any peptide of a suitable size (>6 residues) may react with the native protein if the peptide represents a surface region in the three dimensional structure of the intact protein (19). However, it is also possible that the epitope recognized by the Ab was present in a different configuration on the synthetic peptide from that in the whole protein molecule attached to plastic. It is generally recognized that proteins become at least partly denatured when they are adsorbed to a layer of plastic during a solid-phase assay (300). The denaturation may have altered the conformation of the epitopes so that the Abs were unable to recognize them.

Affinity purified p40 was also analyzed on western blots with the a-p40 and a-peptide Abs. As shown in Figure 1, 2 µg of lung p40 was blotted with the following Abs: monoclonal negative control (lane A), monoclonal a-p40, neat supernatant and ascites (lanes B and C respectively), polyclonal a-p40 (lane D) polyclonal a-#2 (lane E), normal rabbit serum (lane F) and anti-mouse and anti-rabbit second Ab alone (lanes G and H respectively). It is of interest to note that all of the Abs used bound p40 with the specific ones binding strongly and the non-specific ones binding weakly, including the second Abs. This indicated that p40 has the ability to bind non-specifically to immunoglobulin, possibly in a manner similar to that of Staphylococcal protein A. Another point of interest is that the mAb a-p40 binds one only 40 kD band whereas the pAb a-p40 and polyclonal a-peptide #2 Abs bind to the same p40 band but also components of lower molecular weight which do not express the mAb a-p40 binding epitope. Whether these molecules represent p40 proteolytic fragments or contaminants is not known. However, these results have been reproduced many times with affinity purified p40 from different preparations, even after further purification on HIC-HPLC column.

Figure 1

Analysis of affinity purified lung p40 by western blotting. Antibodies of interest: Lane A, mouse mAb negative control; lane B, mAb a-p40 (neat supernatant); lane C, mAb a-p40 (ascites IgG, 4 µg/ml); lane D, pAb a-p40 at dilution 1:1000; lane E, pAb a-peptide #2 (20 µg/ml); lane F, normal rabbit serum at dilution 1:1000 and lanes G and H, sheep anti-mouse Ab and goat anti-rabbit Ab respectively, both used at dilution 1:5000. The numbers on the right side of the figure represent MW standards expressed in kD.


A
B
C
D
E
F
G
H
- 94
- 67
- 43
- 30
- 20
- 14

Affinity purified p40 was further analyzed by 2D western blotting. The pure p40 molecule on 2D-gels showed that it is composed of 6 or 7 different polypeptides with isoelectric points from 6.5 to 7.6 (279). Colon and lung p40 were separated on 2D gels and western blotted with monoclonal and polyclonal a-p40 Abs and with polyclonal a-peptide #2 Ab to compare the expression of the respective epitopes (Figure 2). The binding patterns of the pAb a-p40 and the a-peptide #2 Ab were similar for both colon and lung p40, although the a-peptide Ab bound fewer spots than the pAb p40. This is probably understood in view of the fact that the pAb a-p40 was raised against the whole p40 molecule and probably binds multiple epitopes on p40, whereas the pAb a-peptide #2 binds only a single peptide on the p40 molecule.

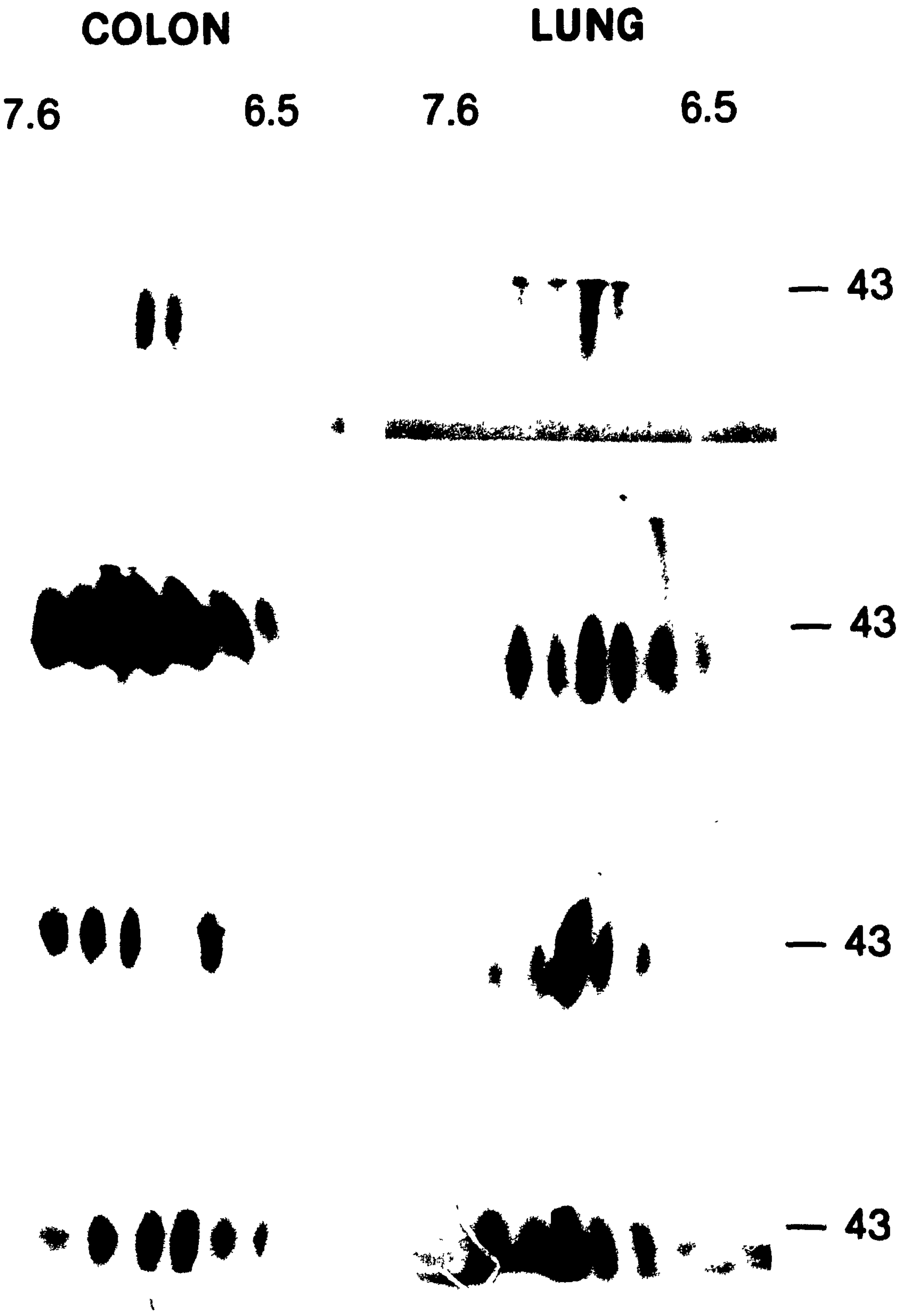
The mAb a-p40 binds to fewer spots than the pAb does and shows binding with different intensity on different spots of lung and colon p40. The mAb is directed to a single epitope of p40. Thus, the difference in binding with the different molecular forms of p40 may reflect the availability of the epitopes to be bound by the mAb a-p40 on the different molecules.

3. Studies on Butanol-extracted OSN

Animal TSTA can be selectively extracted with butanol from the surface of living cells without necessarily killing the cell (97,156,238,253). The mechanism by which butanol acts is not completely understood; it is believed that it competes effectively for the polar side chains of the protein with the alcohol displacing the lipid and thus causing the dissociation of the lipid protein complexes. Substances with OSN activity were extracted with 1-butanol from the surface of the two cultured human cancer cell lines: lung NCI-H69 and colon HCT-15 (146). Moreover, the OSN had physicochemical properties similar to the immunoprotective TSTA of animal tumors (146). When LAI-positive butanol extracts of NCI-H69 and HCT-15 cell lines were analyzed by SDS-PAGE, the extracts were found to contain many components and a p40 molecule was not conspicuously prominent. The question arose whether p40 was responsible for the OSN activity in the

Figure 2

2D-PAGE and western blotting of affinity purified lung and colon p40. From top to bottom: Coomassie Blue stained gel, and western blots with: pAb a-p40; mAb a-p40 and pAb peptide #2. There was no binding on the western blots of the mAb negative control and the normal rabbit serum acting as negative control for the pAbs (data not shown). The numbers on top of the figure represent isoelectric points. The numbers on the right side of the figure represent MW standards expressed in kD.



COLON
LUNG
7.6
6.5
7.6
6.5
— 43
— 43
— 43
— 43

butanol extracts or whether other substances also expressed OSN activity. To enrich and to isolate the butanol released OSN, the extracted material was separated by various physicochemical and immunochemical methods.

3.1 Hydrophobic Interaction HPLC (HIC-HPLC)

Before application to HPLC columns, the butanol extracted material was applied to a Phenyl Sepharose column. A batch method of Phenyl Sepharose chromatography recovered OSN in the 1.0M $(NH_4)_2SO_4$ fraction (data not shown). After concentration and dialysis, the active fraction was passed through Blue Sepharose affinity column. OSN did not bind to Blue Sepharose (data not shown). The eluted, unbound OSN was further resolved on HIC-HPLC with a gradient of 1.8-0M $(NH_4)_2SO_4$ The extent of LAI activity exhibited by the first four fractions of pooled lung butanol extracts eluted from HIC-HPLC column is shown (Table 14). Most of the OSN activity eluted in the high salt fractions of the gradient. No activity was observed in the later factions eluted with low or no $(NH_4)_2SO_4$ buffer. The combined LAI positive fractions, designated Fractions 1+2, were studied further. Colon butanol extracts showed similar physicochemical properties (data not shown).

3.2 Reverse Phase HPLC (RP-HPLC)

Further separation of butanol OSN was by C_3 RP-HPLC . Organic buffers used on RP-HPLC did not alter the OSN activity as shown when lung and colon butanol extracts were tested in blind studies in the LAI assay. The results of such blind criss-cross testing of two fractions eluted for RP-HPLC column are shown (Table 15). Both extracts retained their OSN specificity. The colon extract reacted with colon cancer patient's leukocytes but not with lung cancer patient's leukocytes. The results were reversed for the lung butanol extract. When the butanol extracts were separated on C_3 RP-HPLC, many peaks were observed. Figures 3 and 4 show the RP-HPLC profiles of two preparations of lung and colon butanol extracts, respectively. The LAI activity was distributed in material that eluted from 36-49 minutes and 34-41 minutes for lung and colon extracts, respectively.

TABLE 14

RESULTS OF LAI TESTING OF BUTANOL EXTRACTED MATERIAL FROM LUNG CANCER CELLS ANALYZED BY HIC-HPLC

Diagnosis of Leukocyte Donor	NAI to:					
	Crude Cancer Extract[1]	Control Blank[2]	Lung Butanol HIC-HPLC Fractions[3]			
			Fr#1 0.01-0.5 µg	Fr#2 0.5 µg	Fr#3	Fr#4
Lung Cancer n = 43	84 ± 7	4 ± 3	38 ± 9 to 91 ± 18	45 ± 15	Negative	Negative

[1] Crude cancer extract was the same as the diagnosis of leukocyte donor.
[2] Control sample containing medium 199.
[3] Fractions #3 and #4 were tested at a range of concentrations between 0.025 - 0.5µg.
Fr = Fraction.
n = Number tested.

TABLE 15

RESULTS OF LAI MEASURED ON CODED SAMPLES OF BUTANOL EXTRACTS FROM LUNG AND COLON CANCER CELLS SEPARATED BY RP-HPLC

Diagnosis of Leukocyte Donor		**NAI to:**	
		Butanol cancer extract*	
		Colon[1]**	**Lung**[2]**
Cancer			
Colon	n = 3	55 ± 6	1 ± 8
Lung	n = 3	-1 ± 3	56 ± 13

*Both extracts were tested at 0.75 µg.

**Comparing the two responses to: [1]Colon butanol extract eluted from RP-HPLC with retention time 37.08 minutes; the responses were statistically different, student's 't' independent test ($p < 0.001$); [2]lung butanol extract eluted from RP-HPLC with retention time 38.15 minutes; the responses were statistically different, student's 't' independent test ($p < 0.01$).

n = Number tested.

Figure 3

C_3 RP-HPLC isolation profile of the HIC-HPLC pooled Fractions 1 + 2 of one lung cancer butanol preparation extract. Individual peaks were collected and assayed for OSN activity (thin line) in the LAI assay and assayed for p40 expression by the mAb a-p40 in ELISA (thick line). Higher OSN activity is shown to be in peaks eluting with retention times between 36-38 mins and 41-48 mins outside the mAb a-p40 binding area.

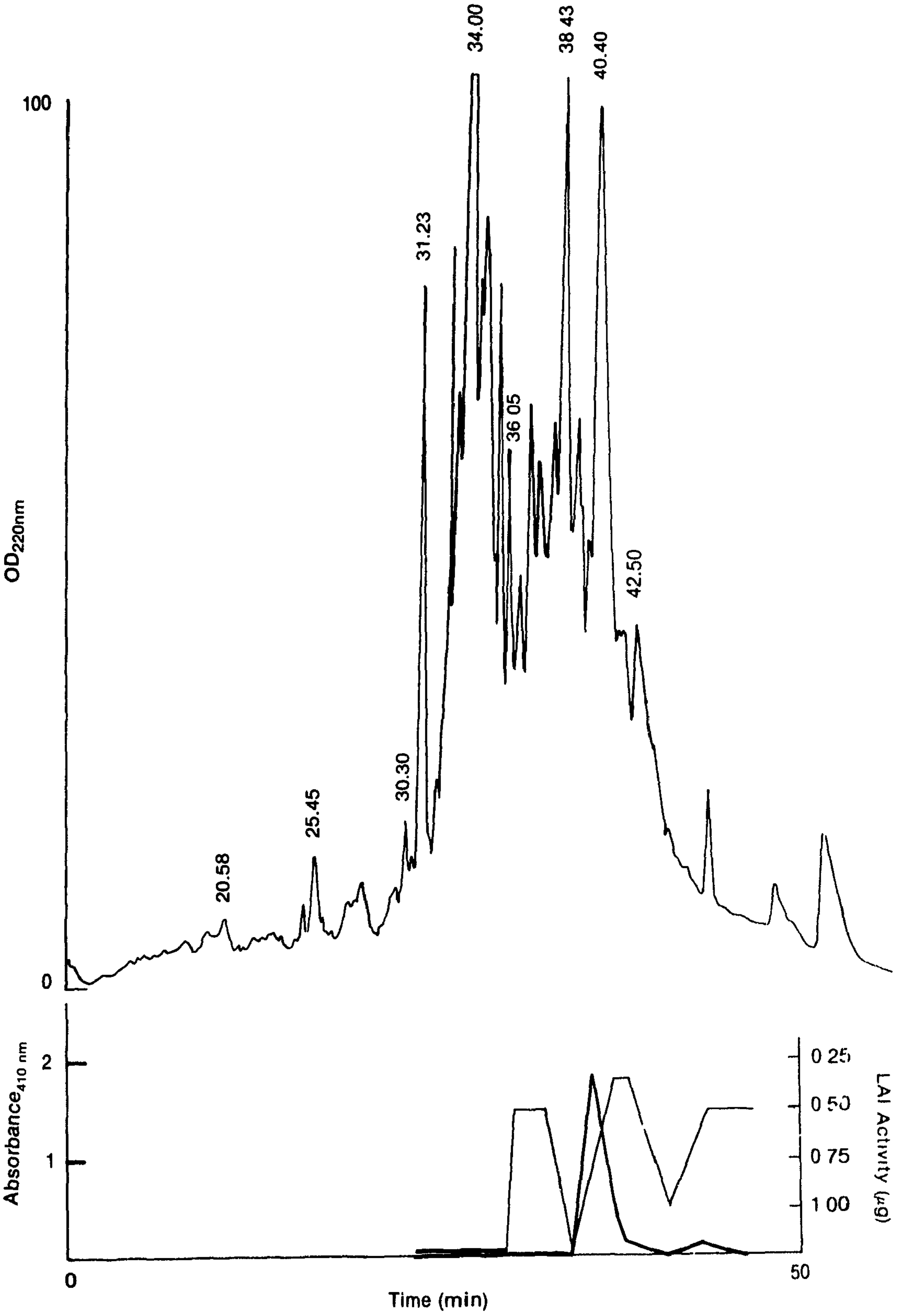
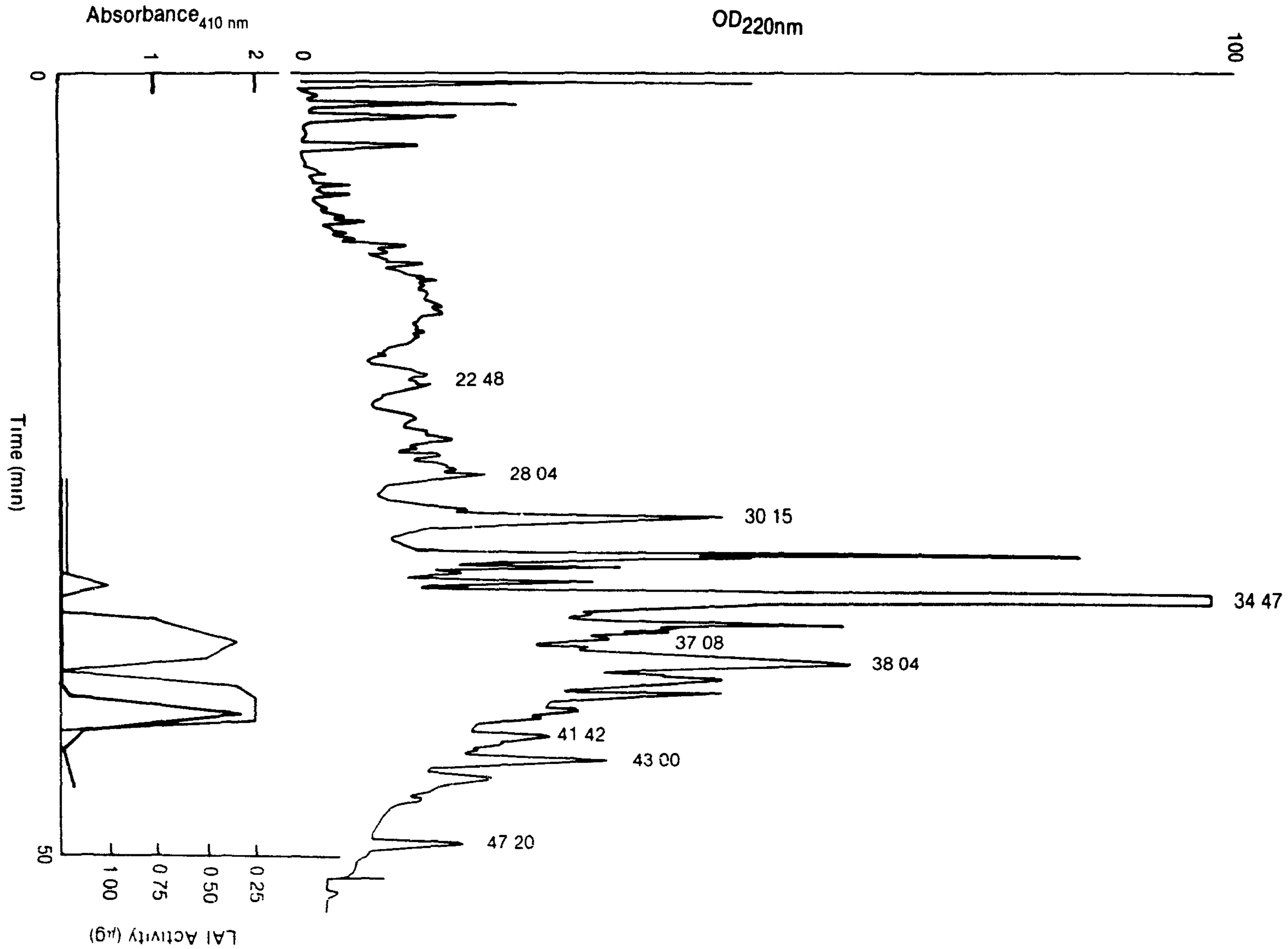

100
0
OD220nm
20.58
25.45
30.30
31.23
34.00
36 05
38 43
40.40
42.50
Absorbance410 nm
2
1
0 25
0 50
0 75
1 00
LAI Activity (µg)
0
50
Time (min)

Figure 4

C_3 RP-HPLC isolation profile of the HIC-HPLC pooled Fractions 1 + 2 of one colon cancer butanol preparation extract. Individual peaks were collected and assayed for OSN activity (thin line) in the LAI assay and assayed for p40 expression by the mAb a-p40 in ELISA (thick line). High OSN activity is shown to be in peaks eluting with retention times between 37-39 mins outside the mAb a-p40 binding area.


Absorbance$_{410 \text{ nm}}$
OD$_{220\text{nm}}$
0
100
1
2
0
50
Time (min)
1.00
0.75
0.50
0.25
LAI Activity (µg)
22 48
28 04
30 15
34 47
37 08
38 04
41 42
43 00
47 20

3.3 P40 in Butanol Extracts

SDS-PAGE analysis of individual peaks with different retention times from C_3 RP-HPLC showed that multiple components co-isolated (data not shown). ELISA tests were performed on the eluted fractions to detect p40 expression by mAb a-p40. The Ab detected p40 in both extracts which eluted with slightly different retention times, 40.40 minutes for lung (Figure 3) and 41.42 minutes for colon (Figure 4). Monoclonal Ab a-p40 did not bind to other areas of the chromatogram although they did possess LAI activity. This was the second instance of OSN activity in the absence of p40. The first time such activity was observed was during purification of p40 from spent medium from both NCI-H69 lung and HCT-15 colon cancer cells by mAb a-p40 affinity chromatography; there, the unbound material that eluted from the column exhibited LAI activity despite of the absence of p40 (279,6).

3.4 P25 in Butanol Extracts

Finding a distribution of LAI activity throughout the RP-HPLC chromatogram and the restricted elution profile of p40 suggested to us the presence of OSN entities independent of those carrying the mAb a-p40 binding epitope. Identification and isolation of one or more of those components was undertaken using immunochemical and physicochemical methods. Peaks from RP-HPLC with retention times 36-38 minutes and 37-38 minutes from lung and colon respectively exhibiting high LAI activity but no measurable p40 were used separately to immunize mice. A mAb was successfully produced from immunization with the colon enriched OSN. Designated a-p25, it bound a substance of approximately 25 kD (p25) in both lung and colon butanol extracts (data not shown).

The characteristics of p25 and a-p25 in relation to p40 and a-p40 mAb as determined by ELISA and western blots (data not shown) are summarized in Table 16. An interesting point is that p25 is expressed in the unbound fraction of spent medium from

TABLE 16

P25 AND ANTI-P25 FEATURES

Anti-p25

-binds to a 25 kD protein
-does not bind P40

P25

-does not cross-react with anti-P40
-is detected in the unbound fraction of spent medium from tissue cultures of NCI-H69 and HCT-15 cancer cell lines after passage through the a-p40 affinity column.
-is detected in butanol extracts of NCI-H69 and HCT-15 cancer cell lines.

HCT-15 and NCI-H69 cells eluted from the a-p40 affinity column which although devoid of p40 was LAI positive (6,279).

3.5 Analysis of P25-containing Lung Butanol Material by Affinity Chromatography Column.

The a-p25 mAb was tested by ELISA on lung butanol extracts eluted from anion exchange Ax-300 HPLC (Table 17). Monoclonal Ab a-p25 binds to peaks which do not contain measurable p40. Interestingly, LAI activity was high in fractions expressing little p25. Using an a-p25 affinity column, I attempted to purify p25. The fraction exhibiting the highest a-p25 activity was applied to the a-p25 affinity chromatography column. However, none of the p25 molecules was retained (Figure 5). A similar observation was made with colon butanol material applied onto a different, separately prepared a-p25 affinity column. When the two columns were prepared, the estimated amount of linked Ab was 95%. Despite that, neither column retained the p25 molecule, from either lung or colon butanol extracts. One possible explanation is that the Ab conformation was distorted or partially denatured upon linkage to matrix so that it could no longer bind the p25 molecule (300). A less likely possibility is that due to binding to the matrix the Ab adopted an orientation that obscured the antigen-binding sites. A third possibility is that the Ab was directed to a site accessible only on denatured molecules and it could not recognize the native molecule when in solution. The Ab reacts with denatured p25 in western blots and also binds p25 in ELISA where p25 may undergo changes in structure because of binding to plastic (300). Thus, it seems likely that the mAb did not retain p25 on the affinity column because the binding epitope was not exposed in the soluble molecule.

Consequently, alternative ways of purifying p25 were chosen which included separation on C_3 RP-HPLC. The eluted fractions were tested for p25 expression and LAI activity (data not shown). The one fraction exhibiting LAI activity was negative for mAb a-p25 binding in ELISA (Table 18). The two fractions containing the p25 molecule detected

TABLE 17

COMPARISON OF ANTI-P40 AND ANTI-P25 MAb BINDING TO LUNG BUTANOL EXTRACTED MATERIAL ELUTED FROM Ax-300 HPLC (MEASURED BY ELISA)

Fraction #	Retention Time (mins)	MAb binding ($O.D_{410}$)**		LAI activity (µg/assay)
		a-P40	a-P25	
Starting Material*		0.02	1.17	0.25
1	2-14	0.02	0.01	Negative
2	14-22	0.25	1.17	0.3
3	22-47.45	0.13	1.70	0.4
4	47.45-68	0.23	0.80	0.25
5	68-95	0.19	0.27	0.3
Affinity Purified p40		1.87		

*All samples were tested at 500 ng/well except affinity purified p40 tested at 50 ng

**The antibodies were applied as neat culture supernatant

Figure 5

LAI-active lung butanol material eluted from an Ax-300 anion exchange HPLC was applied on an a-p25 affinity chromatography column. The unbound fraction (lane 4) is almost identical to the material before application to the column (lane ST); lane 1 represents the bound fraction and lanes 2 and 3 represent PBS washings of the column. Lanes S show molecular weight standards. The gel was silver stained.

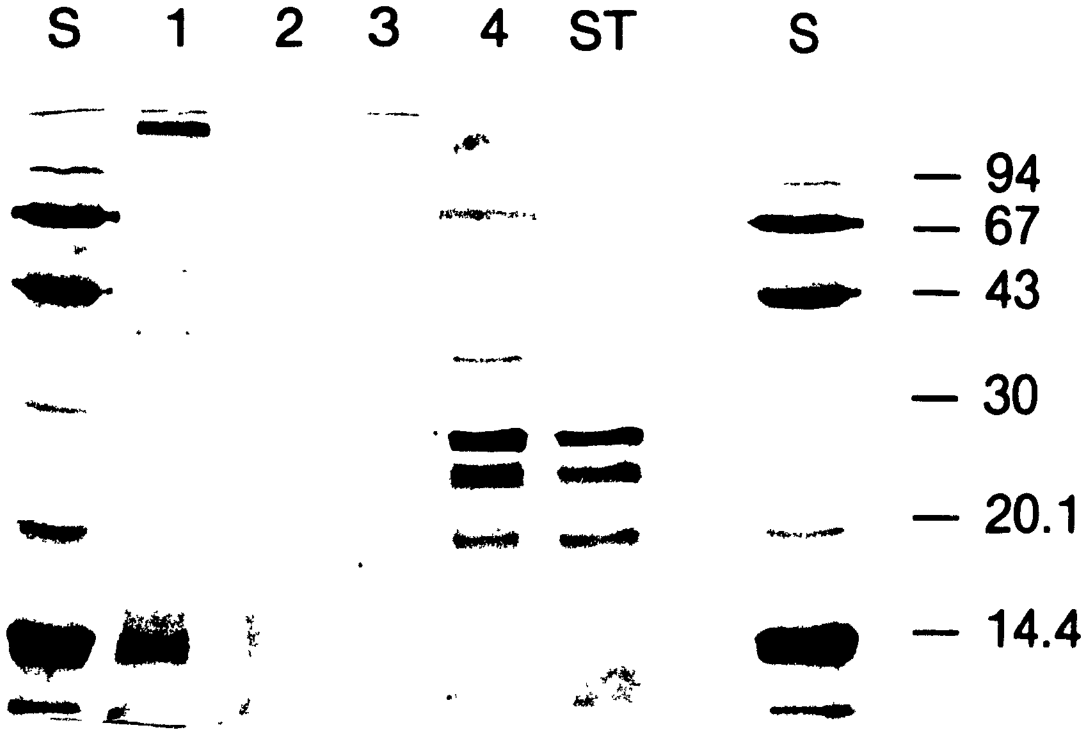

S
1
2
3
4
ST
S
94
67
43
30
20.1
14.4

TABLE 18

COMPARISON OF THE ANTI-P40 AND ANTI-P25 MONOCLONAL ANTIBODY BINDING TO RP-HPLC FRACTIONS OF ANTI-P25 UNBOUND LUNG BUTANOL EXTRACT IN THE LAI ASSAY

Fraction* #	RP-HPLC Retention Time (Min)	MAb binding ($O.D_{410}$)**		LAI Activity (µg/assay)
		a-P40	a-P25	
6	24.35-36.10	ND	1.84	Negative
7	26.10-27.35	ND	1.79	Negative
10	29.47-30.58	0.04	0.12	0.25

*Tested at 500 ng/well

**Used as neat culture supernatant

by mAb a-p25 tested LAI negative. Consequently, p25 was not an OSN-expressing molecule.

The observation that OSN activity was clearly exhibited in areas containing substances not expressing the mAb a-p40 binding epitope in Figures 3 and 4 suggests various possibilities:

(1) The OSN are derived from p40, but they do not express the epitope bound by the mAb a-p40.

(2) The OSN species are substances completely irrelevant to p40.

(3) The OSN are p40-like molecules, belonging to the same family of proteins.

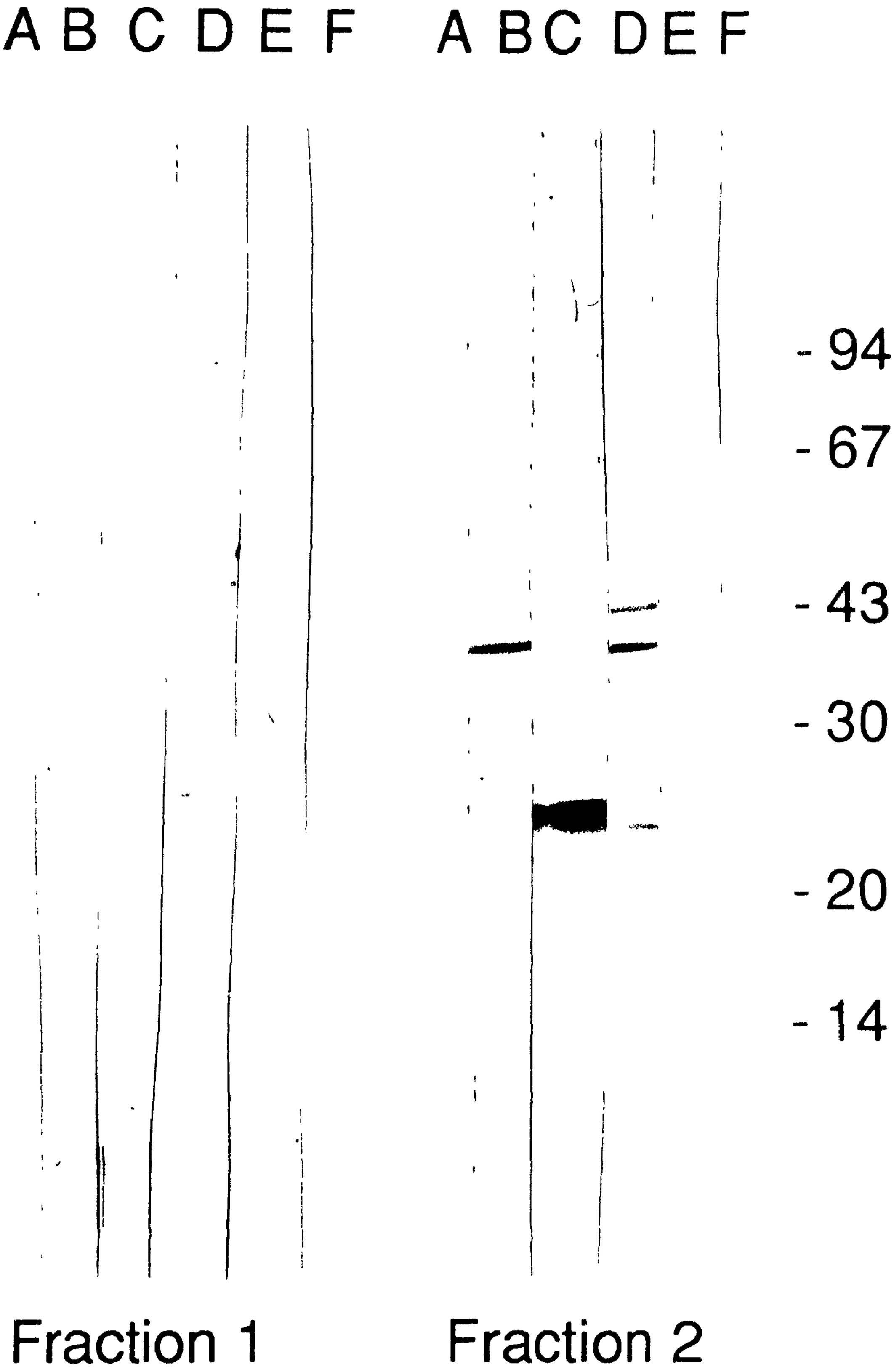
(4) Finally, the OSN might represent a combination of the preceding three options.

To examine the first possibility that the OSN were derived from p40, but not expressing the mAb a-p40 binding epitope, I performed western blots on LAI positive lung butanol material with the a-p40 peptide Abs. The results of a representative experiment are shown on two LAI positive lung butanol fractions eluted from Ax-300 HPLC (Figure 6). Fraction 1 does not bind mAb a-p40 (lane B), but Fraction 1 does bind polyclonal antibody (pAb) a-peptide #2 (lane D). The results suggest that there were fragments of p40 (or p40-like molecules) possessing the same peptide which is part of an LAI active CnBr cleaved peptide of lung p40. In contrast to this, Fraction 2 does contain p40 (lane B) as well as a-peptide #2 binding molecules (lane D).

In summary, butanol extracts of NCI-H69 lung and HCT-15 colon cultured cancer cells exhibit OSN activity. Although p25 is present in LAI positive butanol extracted material of the lung cell line, p25 is not an OSN. OSN activity in butanol extracts is partly accounted for by material which contains p40, later identified as a mycoplasma transport protein. However, OSN activity of the butanol extracts does not always correlate with p40 expression because LAI activity is exhibited by butanol extracted material from the same cell lines devoid of p40 as assayed by mAb a-p40 binding. This suggests that the lung NCI-H69 and colon HCT-15 cancer cell lines express OSN molecules independent of the

Figure 6

Western blots of two LAI positive fractions eluted from an Ax-300 anion exchange HPLC column. Fraction 1 was eluted with retention times 47-68 minutes and Fraction 2 immediately after with retention times 68-90 minutes. Lane A, mAb negative control binding; lane B, mAb a-p40 binding; lane C, a-p25 binding; lane D, pAb a-peptide #2 binding; lane E, normal rabbit serum binding. The numbers on the right side of the figure represent MW standards expressed in kD.


A B C D E F
A B C D E F
94
67
43
30
20
14
Fraction 1
Fraction 2

molecules bearing the a-p40 epitope. Possibly the OSN are molecules expressing epitopes cross-reacting with polyclonal antisera raised against p40 peptides.

4. Studies on P40 Tissue Distribution

Human tumor antigens have been divided in three classes (221). Class 1 includes antigens restricted to autologous tumor only and not expressed by normal or other malignant cells. Class 2 includes antigens expressed on the autologous tumor and also on a proportion of allogeneic tumors and some normal cells. Class 3 includes antigens expressed on normal and malignant cells with no restriction pattern. P40 is a molecule expressed by two tissue cultured cancer cell lines, lung NCI-H69 and colon HCT-15. P40 was later identified as a mycoplasma transport protein. Nevertheless, p40 exhibited organ-specific activity in the LAI assay (6,279). This meant that p40 was recognized only by leukocytes from cancer patients bearing tumor of the same organ as the cell line (lung or colon) from which p40 derived. Thus, p40 behaved in the LAI assay as a Class 2 tumor antigen with restriction to tumors of the same organ. However, it was possible that p40 was expressed on other tumor tissues which also exhibited organ-specific neoantigen activity. It was also possible that p40 was expressed on normal tissues in quantities below the immunogenic threshold. Finally, it was possible that only p40 peptides or p40-like molecules were expressed on other tumor and normal tissues, but the mAb a-p40 binding epitope was selectively expressed on lung and colon tissues.

Previously (Section 3.5) it was shown that the pAb a-peptide #2 detected several substances in LAI active cultured lung cell butanol material that did not contain the a-p40 mAb binding epitope. A possible conclusion was that the a-peptide #2 pAb (itself having been raised against a synthetic segment of an LAI positive CnBr-cleaved peptide of p40) detects fragments of p40 rather than p40-like molecules. To determine whether p40 or p40-like molecules were expressed in other tissues, the distribution of p40 and p40 peptides was examined. In a series of experiments, the qualitative tissue distribution of p40 and p40 peptides was examined by western blots of extracts from: 1) cultured cancer cell lines; 2) freshly obtained tissues from biopsies, cancer and normal; 3) freshly obtained aborted whole human fetuses; and 4) frozen human fetal organs. The Abs used were monoclonal and polyclonal a-p40, polyclonal a-peptide #2, monoclonal a-p25, monoclonal control Ab and normal rabbit serum.

4.1 P40 Expression on Cultured Cell Lines

First the expression of p40 was examined on cultured cell lines. The results of western blots performed on extracts of cultured cancer cell lines, as well as normal fibroblasts and fresh peripheral blood leukocytes (PBL), are shown in Table 19. Among eighteen cell lines, including one mouse cell line, six express the mAb a-p40 binding epitope. The cell lines also show a 40 and a 23 kD molecule with pAb a-peptide #2 . The pAb a-p40 binds a 40 kD band on all the cell lines in which the mAb binding epitope is present as well as a few others which are not bound by the mAb. Interestingly, a breast cancer cell line, Du 4475, which does not express the mAb binding epitope but does express the pAb a-p40 and a-peptide #2 epitopes possessed, as an extract, LAI activity. Figure 7 shows the western blots of extracts from Du 4475 breast cancer cell line (lanes A to I) in comparison with CaCo2 colon cell line (lanes J to R). The mAb a-p40 detects p40 on CaCo2 cells (lane K), but not on the Du 4475 cells (lane B). In contrast, the pAb a-p40 binds a 40 kD molecule on both extracts (lanes C and L). Binding of the a-p25 mAb to all extracts indicated that p25 is a common protein on human cultured as well as fresh cells (PBL) but is not expressed by the mouse myeloma cell line.

Another point of interest is that two different cultures of the same lung cell line NCI-H69 show two different p40 expressions (Table 19). These cells also exhibited two different types of behavior in culture. The one designated "new culture" NCI-H69 does not express the mAb a-p40 binding epitope (Lane B), as shown in Figure 8, but does express a 40 kD band detected by the pAb a-p40 (lane C). "New culture" NCI-H69 cells did not attach to the culture flasks nor did they grow in serum free medium, as did the

TABLE 19

RESULTS OF SDS-PAGE AND WESTERN BLOT ANALYSIS OF PROTEINS EXTRACTED FROM CULTURED CANCER AND NORMAL CELLS

Cell Line*	Antibody binding**			
	a-P40 mono	a-P40 poly	a-Pept #2 poly	a-P25 mono
NCI-H69 Lung[+, Φ]	40	40	40, 23	25
NCI-H69 (new culture)[+]	-	40	-	25
HCT-15 Colon[+, Φ]	40	40	40, 23	25
LS-180 Colon[+, Φ]	40	40	40, 23	25
Colo 320-HSR Colon	-	40	23	25
Colo 201 Colon	-	-	23	25
Colo 205 Colon	-	-	-	25
CaCo2 Colon[Φ]	40	40	40, 23	25
Du 4475 Breast[+]	-	40	23	25
MCF-7 Breast	-	40	23	25
MDA Breast	-	-	23	25
SK-Mel 5 Melanoma	40	40	40, 23	25
T-24 Bladder[+, Φ]	40	40	40	25
ECF-STT6 Astrocytoma[Φ]	-	-	-	25
HeLa Cervical	-	-	-	25
Fibroblasts	-	-	23	25
PBL (Fresh cells)	-	-	23	25
SP-2 Myeloma Mouse	-	-	-	-

*The cells were extracted with 1% Nonidet P40 in PBS containing PMSF as protease inhibitor.

**Approximate molecular weight of bands observed.
mono = monoclonal
poly = polyclonal

[+] Cell lines exhibiting OSN activity.

[Φ] Cell lines later shown to be contaminated with mycoplasma.

Figure 7

Western blot of breast Du 4475 (lanes A-I) and colon CaCo 2 (lanes J-R) cancer cell lines. Antibody binding of interest: Lanes A,J, mAb negative control; lanes B,K, mAb a-p40; lanes C,L, pAb a-p40; lanes D,M, pAb a-peptide #2; lanes E,N, mAb a-p25; lanes F,O, normal rabbit serum ; lane S, affinity purified p40 blotted with mAb a-p40 and lane T, p25 blotted with mAb a-p25. The numbers on the right side of the figure represent MW standards expressed in kD.

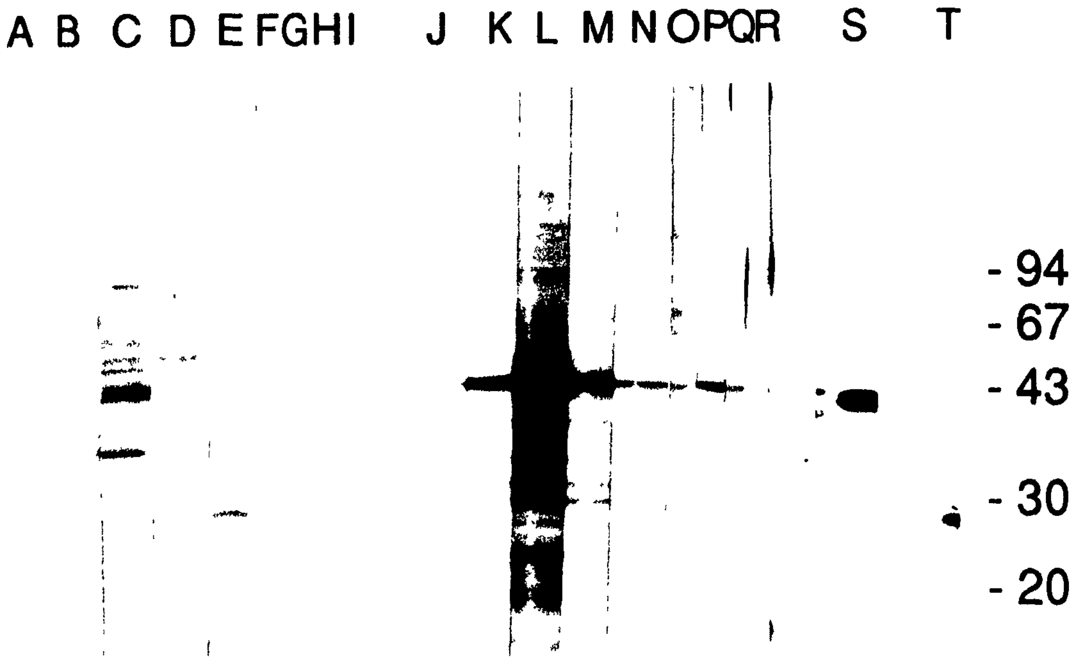
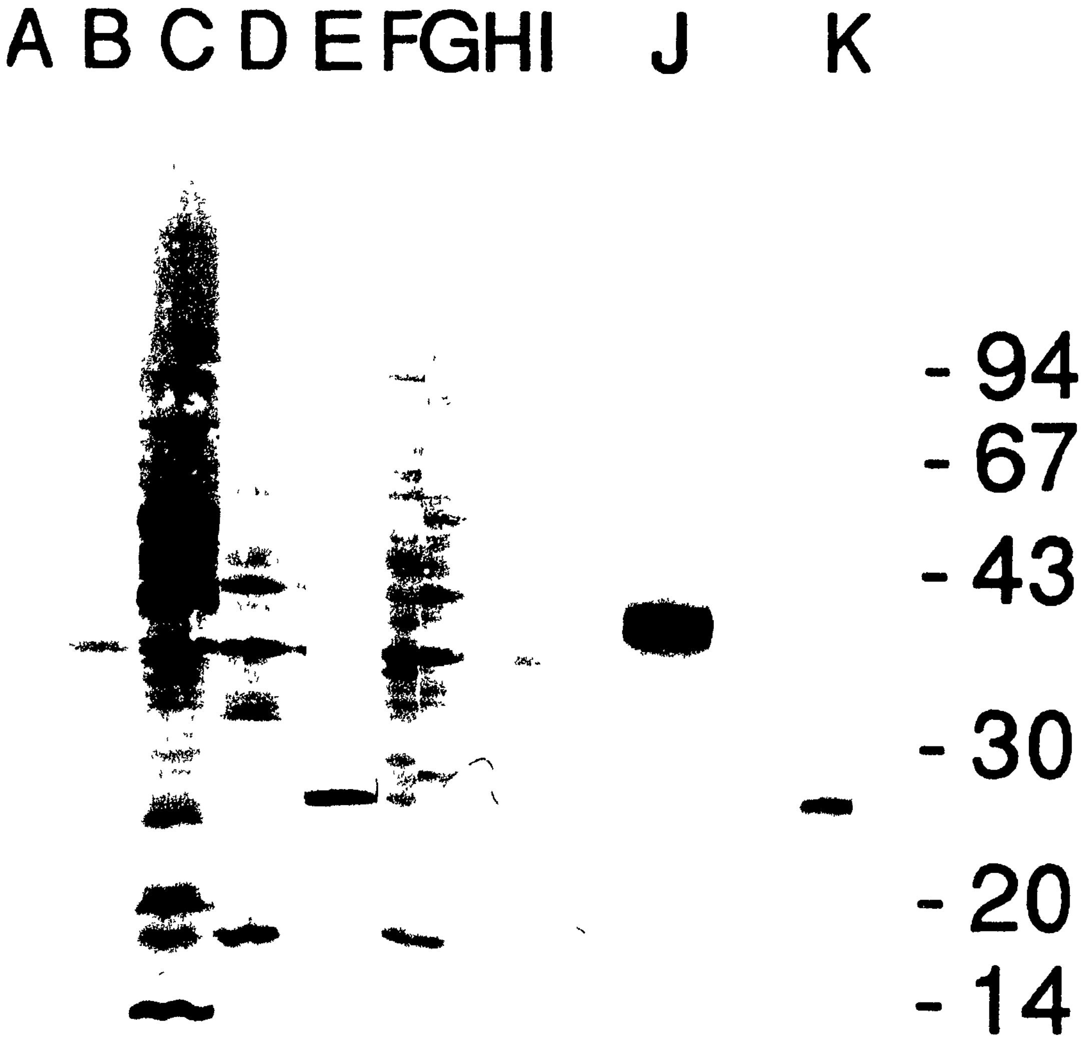

A B C D E F G H I J K L M N O P Q R S T
- 94
- 67
- 43
- 30
- 20

Figure 8

Western blot of lung cancer cell line NCI-H69 (new culture). Antibody binding of interest: Lane A, mAb negative control ; lane B, mAb a-p40; lane C, pAb a-p40; lane D, pAb a-peptide #2; lane E, mAb a-p25; lane F, normal rabbit serum; lanes J and K represent affinity purified p40 and p25 positive controls bound by mAbs a-p40 and a-p25 respectively. The numbers on the right side of the figure represent MW standards expressed in kD.


A B C D E F G H I J K
- 94
- 67
- 43
- 30
- 20
- 14

NCI-H69. NCI-H69 was later found to be contaminated with mycoplasma, whereas "new culture" NCI-H69 was not contaminated.

In summary, p40 detected by the mAb a-p40 is expressed only on certain human cultured cell lines. In contrast, the pAb a-p40 detects a 40 kD band on many cell lines and in particular cell lines which did not express the mAb binding epitope. Also, the a-peptide #2 Ab detected a 23 kD band on cancer cell lines as well as normal fibroblasts and PBL not expressing either the mAb or the pAb binding epitopes. The results suggest that a 40 kD molecule which cross-reacts with the pAb a-p40 is expressed on certain cultured cancer cell lines. Four LAI active cell lines expressed the molecule: both, mycoplasma-contaminated and non-contaminated lung NCI-H69 (see Section 7), mycoplasma-contaminated colon HCT-15 and non-contaminated breast Du 4475. Whether this 40 kD molecule bound by the pAb a-p40 is the same in all the cell lines, whether it is an OSN or whether it shares structural or functional similarities with mycoplasma p40 is not known. However, at the time the p40 distribution studies were done, p40 had not been identified as a mycoplasma protein, and the results had suggested that p40 detected by the mAb a-p40 behaves as a Class 2 human cancer antigen expressed only by certain cancer tissues. Also, since the pAb a-p40 bound specifically to a 40 kD molecule on more tissues than the mAb did, it seemed that the mAb a-p40 binding epitope was a unique determinant on a common molecule rather than a unique determinant on a unique molecule.

4.2 P40 Expression on Fresh Tissues

Among the tissues not containing p40 was a normal fibroblast cell line, as well as fresh normal PBL. Neither expressed the mAb or the pAb a-p40 binding epitopes.

Extracts of normal tissues do not react in the LAI assay (102,165,272,290) because they do not express the OSN epitope or express it at lower concentrations than cancers. Consequently, in the next study, I examined the distribution of p40 and related molecules on non-cultured cancer and normal tissues. Tables 20 and 21 summarize the results. In Table 21 the tissues have been extracted with SDS instead of Nonidet p40 which is used in

TABLE 20

RESULTS OF SDS-PAGE AND WESTERN BLOT ANALYSIS OF PROTEINS EXTRACTED FROM FRESH CANCER AND NORMAL TISSUES

Tissue*		Antibody binding**			
		a-P40 mono	a-P40 poly	a-Pept #2 poly	a-P25 mono
Colon					
cancer	n = 2	-	-/40***	23	25
normal	n = 2	-	-/40	23	25
Lung					
cancer	n = 3	-	40	23	25
normal	n = 3	-	40	23	25
Breast					
cancer	n =1	-	40	23	25
normal	n = 1	-	40	23	25

*The tissues were extracted with 1% Nonidet P40 in PBS containing PMSF as protease inhibitor.

**Approximate molecular weight of bands observed.

***-/40 indicates that some specimens did not express the 40 kD molecule and some did.

mono = monoclonal.

poly = polyclonal.

n = Number of specimen tested.

TABLE 21

RESULTS OF SDS-PAGE AND WESTERN BLOT OF PROTEINS EXTRACTED FROM SOLID TUMORS AND NORMAL TISSUES BY SDS

Tissue*		**Antibody binding****			
		a-P40 mono	**a-P40 poly**	**a-Pept #2 poly**	**a-P25 mono**
Colon					
cancer	n = 3	-	40	40	-
Lung					
cancer	n = 3	-	-/40***	40, 23	-/25***
Melanoma	n = 3	-	-/40	23	25
Breast					
cancer	n = 3	-	40	-/23***	-
normal	n = 1	-	40	-	-
Liver					
normal	n = 2	-	-/40	-/23	-/25

*The tissues were extracted with 1% SDS in PBS.

**Appropriate molecular weight of bands observed.

***-/40, -/23, -/25 indicates that some specimen did not express the 40, 23 or 25 kD molecule and some did.

mono = monoclonal.

poly = polyclonal.

n = Number of specimen tested.

all the other extractions. The mAb a-p40 binding epitope was not expressed by any cancer or normal tissues tested. However, as with the cultured cell lines some of the normal and cancer tissues express the polyclonal a-p40 and/or a-peptide #2 Ab binding epitopes. Figure 9 shows representative western blots of two of the extracted samples. Lanes A to G and H to N show the extract of lung cancer tissues and normal tissues, respectively, of the same specimen; they bound the Abs with very similar patterns. Monoclonal Ab a-p40 does not bind any molecule in either tissue. However, the pAb a-p40 binds a 40 kD band on both tissues and the pAb a-peptide #2 binds among others a 23 kD band.

Expression of p25 in the tissues was also examined. In some instances (Table 21) the lack of p25 expression may reflect differential expression (128,181) or loss of the p25 epitope as a result of tissue handling or extraction conditions.

4.3 P40 Expression of Fetal Tissues and Organs

Malignant transformation is associated with changes in gene expression, which can result in the activation of genes coding for fetal antigens normally expressed only in the fetus (25,42), such as CEA and α-fetoprotein (90,277). There is considerable evidence that animal fetal tissues express antigens which cross-react with virally or chemically-induced experimental tumors (29,42,89). In humans, in vitro studies of cellular and humoral immunity suggest that human cancers and fetal tissues share immunogenic molecules (90,104,119). By the LAI assay, an OSN molecule identical to that expressed by cancers of the same organ was expressed by fetal tissue (104). Consequently, I examined the possibility that p40 was expressed by human fetuses and/or fetal organs from human fetuses by western blotting (Table 22). Neither extracts of the whole fetuses nor the fetal organs expressed the mAb or the polyclonal a-p40 Ab binding epitopes. However, all expressed the 23 kD and the 25 kD bands detected by the pAb a-peptide #2 and mAb a-p25, respectively. The results indicate that the p23 and p25 are expressed in the human embryo.

Figure 9

Western blots of two lung extracts. Lanes A-G correspond to the cancer part, lanes H-N to the normal part of the same specimen. Antibody binding of interest: Lanes A,H, mAb negative control; lanes B,I, mAb a-p40; lanes C,J, pAb a-p40; lanes D,K, pAb a-peptide #2; lanes E,L, mAb a-p25; lanes F, M, normal rabbit serum; lanes O and P show the positive controls, affinity purified p40 and p25 blotted with mAb a-p40 and a-p25, respectively. The numbers on the right side of the figure represent MW standards expressed in kD.

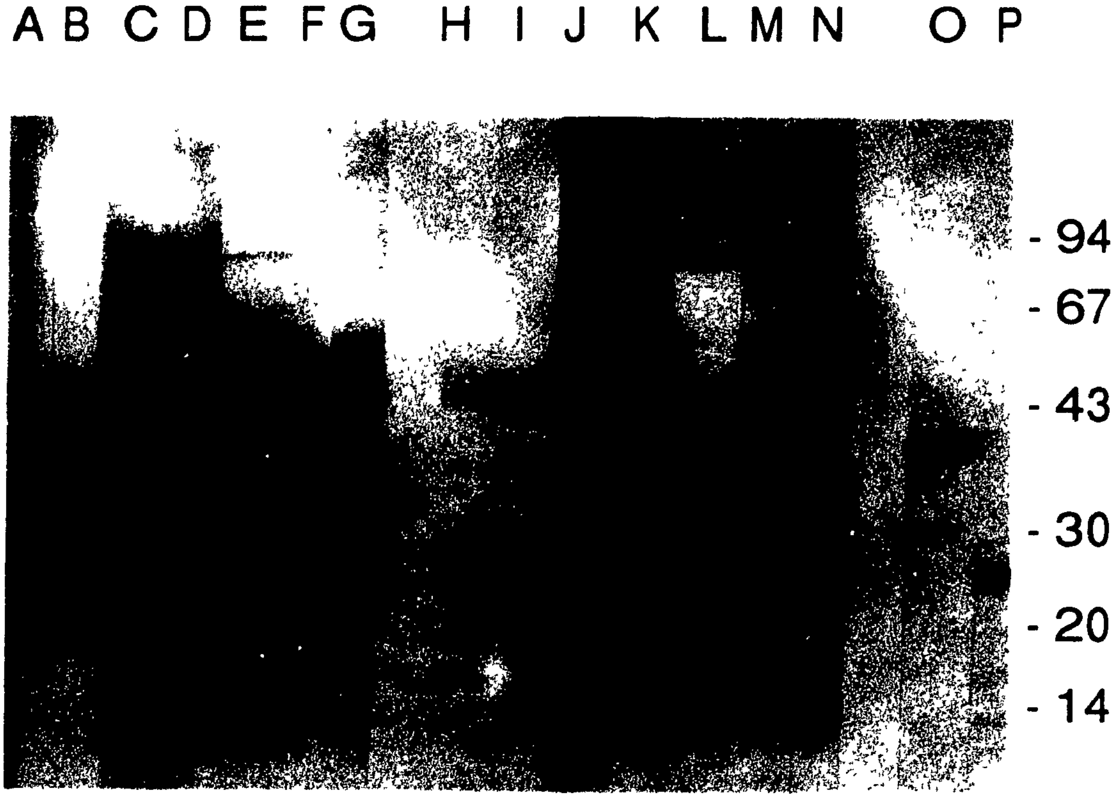

A B C D E F G H I J K L M N O P
- 94
- 67
- 43
- 30
- 20
- 14

TABLE 22

RESULTS OF SDS-PAGE AND WESTERN BLOT ANALYSIS OF PROTEINS EXTRACTED FROM FETAL TISSUES AND ORGANS

Protein Source*	**Antibody binding****			
	a-P40 mono	**a-P40 poly**	**a-Pept #2 poly**	**a-P25 mono**
Whole fetus/wks				
6	-	-	23	25
7	-	-	23	25
8	-	-	23	25
9	-	-	23	25
Fetal organs				
Stomach	-	-	23	25
Gut	-	-	23	25
Liver	-	-	23	25
Lung	-	-	23	25

*One sample of each tissue was tested except for the whole fetus of 6 weeks (wks) of gestation where two samples were tested.

**Approximate molecular weight of bands observed.

In conclusion, the studies of p40 tissue distribution showed that p40 detected by the mAb a-p40 is expressed only on certain cultured cell lines and not by fresh cancer, normal tissues, whole fetuses or fetal organs. Among the cultured cells expressing p40, the lung cell line NCI-H69 and the colon cell line HCT-15 were shown to exhibit OSN activity. The OSN was the affinity purified p40 molecule recently identified as a mycoplasma protein. However, these cell lines also express OSN activity distinct from p40, such as found in the unbound material from the a-p40 affinity column (279,6) and butanol extracted material from lung and colon cancer cells separated on RP-HPLC. Among the cell lines not expressing the mAb a-p40 epitope, the breast cell line Du 4475 tested positive in the LAI assay. Interestingly, some cell lines and fresh tissues, not expressing p40, as detected by the mAb a-p40, do express a 40 kD and a 23 kD molecules detected by both the polyclonal a-p40 and the a-peptide #2 Abs.

5. Studies on Mycoplasma Contamination and Association with P40

P40 behaved as an OSN in the LAI assay. However, it was disturbing to find that mAb a-p40 did not bind to either fresh or frozen human tissues, or to either fetal tissues or fetal organs and bound to only some cultured cancer cell lines. One of the cultured cell lines was the NCI-H69 lung cancer line, whose attachment properties and nutritional needs had changed during the first few months they were grown in culture. This observation, together with the observation that the polyclonal a-p40 Ab bound a 40 kD band on the majority of the fresh and cultured adult tissues tested, suggested the possibility that the mAb a-p40 epitope might represent an altered OSN moiety occurring as a result of culture conditions. Consequently, it might not necessarily be represented on human OSN in vivo. The possibility that the mAb a-p40 was raised against an infectious contaminant of the cell culture seemed improbable because the cells grew well and looked healthy. Admittedly, NCI-H69 cells had undergone a change in their attachment properties with the adaptation to serum-free conditions, but cytopathic effects were never observed. One agent which can infect cells with no conspicuous effects is mycoplasma. Because mycoplasma contamination of cell cultures and its effect on many different experimental results are well known (115,178,270,301), steps were taken to ensure that the cell lines being used were mycoplasma free. Hence, when p40 was originally discovered and isolated, tests for mycoplasma contamination were performed, and the results were negative. A summary of those tests is shown below.

1. Hoechst stain on cancer cells: NEGATIVE
2. Culture tests on mycoplasma agar: NEGATIVE
3. Effect of polyclonal a-mycoplasma Ab on butanol extracts of cancer cells measured by ELISA: NEGATIVE
4. Effect of a-p40 mAb on mycoplasma antigen: NEGATIVE

The first two tests seemed to eliminate the possibility of mycoplasma contamination and the next two seemed to rule out any relationship between p40 and mycoplasma proteins. However, when the cloning of the gene encoding p40 was subsequently undertaken, p40 mRNA isolation from human cancer cell lines through the poly A tail technique was unsuccessful. Alternatively, after the sequence of the affinity purified p40 tryptic digests and CnBr-cleaved peptides was determined, the two CnBr-cleaved colon peptides CCN10 and CCN15 were used as primers in the polymerase chain reaction assay in order to clone the p40 gene from the colon cell line LS-180 in the collaborating laboratory of Dr. C. Stanners. The isolated p40 cDNA fragment was used as a probe for p40 mRNA detection in northern blots in cultured cell lines. Total RNA extracts from a number of cancer cell lines were tested. Among them Colo 201 and Colo 205 were negative for p40 mRNA but LS-180, CaCo2, MCF-7 and NCI-H69 contained vast amounts of mRNA. I tested the same cell lines by western blots for p40 protein expression. P40 was expressed by LS-180, CaCo2 and NCI-H69 but p40 was not expressed by Colo 201, Colo 205 and MCF-7 (Table 19). With the exception of the breast cell line MCF-7, the cell lines which expressed p40 in my hands had shown to contain the message for p40 in the northern blots obtained in Dr. C. Stanners' laboratory. The discrepancy in the case of the

MCF-7 cell lines was not clear, but may relate to the fact that two separate sources of the MCF-7 cell line had been used for mRNA and p40 expression. With the employment of the polymerase chain reaction the p40 DNA was isolated, its sequence was determined, and consequently, the amino acid sequence of the p40 molecule became known.

Figure 10 shows the DNA and the corresponding amino acid sequence of the p40 molecule. Figures 11 and 12 show the same p40 sequence, as determined from the translated DNA with the tryptic and CnBr-cleaved peptides from lung and colon affinity purified p40 marked on it. When the p40 DNA sequence was determined and examined, it was found that it was rich in adenine and thymidine and it contained stop codons (TGA marked with asterisks in Figure 10) which, instead of acting as stop codons as they would in eukaryotes, coded for the amino acid tryptophan. Both properties are characteristic of mycoplasma DNA (56,115,220).

Consequently, further studies were undertaken to examine the relationship between p40 and mycoplasma contamination in our cell lines. Some of the tests performed previously were repeated. Table 23, shows the results of ELISA testing of lung affinity purified p40 and material extracted from a mixture of various mycoplasma species. Neither mAb or pAb a-p40, or pAb a-peptide #2 reacted with the mycoplasma extract. Furthermore, a commercially obtained polyclonal a-mycoplasma antiserum did not react with the affinity purified p40. The a-mycoplasma pAb gave a slightly elevated value of 0.33 on p40 at 1:100 dilution which was considered to be non-specific binding. Similar results were obtained by western blots on the lung cell line NCI-H69 (Figure 13). Lanes C and D show clearly the binding of the mAb and pAb a-p40, respectively, to p40. However, the a-mycoplasma Ab did not show any binding to the lung cell extract over the control rabbit serum; in particular no molecules at 40 kD were bound (Lanes E and G).

Additionally, culturing of live lung NCI-H69 and colon HCT-15 cells on mycoplasma broth gave no growth (Table 24) nor did the cells show increased thymidine uptake as indication of mycoplasma contamination (132). Although these results were

Figure 10

The DNA nucleotide sequence coding for p40 derived from LS-180 colon cancer cell line and the respective translated amino acid sequence of the molecule. Codons with asterisks represent stop codons which in this case of mycoplasma code for tryptophan. The nucleotide sequence spans a 986 base pair region and the translated protein bears no homology with any known protein sequence obtained through the National Biomedical Research Foundation data base.

```
  1  V   N   K   E   V   I   N   T   F   L   E   N   F   K   K   E     16
  1 GTG AAC AAA GAG GTT ATT AAT ACA TTT TTA GAA AAT TTC AAA AAA GAA     48

 17  F   N   K   L   K   N   A   N   D   K   T   K   N   F   D   D     32
 49 TTT AAT AAA CTC AAA AAT GCA AAT GAT AAA ACC AAA AAC TTC GAT GAC     96

 33  V   D   F   K   V   T   L   I   Q   D   F   T   V   L   L   N     48
 97 GTT GAT TTT AAA GTA ACT TTA ATT CAA GAC TTT ACT GTG TTG TTA AAC    144

 49  N   L   S   T   D   N   P   E   L   D   F   G   I   N   A   S     64
145 AAT TTA TCT ACT GAC AAT CCT GAA TTA GAT TTT GGA ATT AAT GCT TCA    192

 65  G   K   L   V   K   F   L   K   N   N   P   G   I   I   T   P     80
193 GGA AAA TTG GTA AAA TTT CTA AAA AAT AAT CCT GGT ATA ATA ACT CCA    240

 81  A   L   Q   T   T   T   N   S   F   V   L   D   K   E   K   D     96
241 GCA TTA CAA ACA ACA ACT AAT TCT TTT GTA CTT GAC AAA GAA AAA GAT    288

 97  K   F   Y   V   D   G   T   D   S   D   P   L   V   K   I   A    112
289 AAA TTT TAT GTT GAT GGT ACA GAT TCA GAT CCA CTT GTA AAA ATT GCT    336

113  K   E   I   N   K   I   F   V   E   T   P   Y   A   S   *   T    128
337 AAA GAA ATT AAT AAA ATT TTT GTT GAA ACT CCA TAT GCA AGT TGA ACT    384

129  D   E   N   H   K   *   N   G   N   V   Y   Q   S   V   Y   D    144
385 GAT GAA AAT CAT AAG TGA AAT GGT AAT GTT TAT CAA AGT GTT TAC GAT    432

145  P   T   V   Q   A   N   F   Y   R   G   M   I   *   I   K   G    160
433 CCA ACT GTT CAA GCT AAT TTT TAT AGA GGA ATG ATT TGA ATA AAA GGT    480

161  N   D   E   T   L   A   K   I   K   K   A   *   N   D   K   D    176
481 AAT GAT GAA ACT CTA GCT AAA ATT AAA AAA GCT TGA AAT GAT AAA GAT    528

177  *   N   T   F   R   N   F   G   I   L   H   G   K   D   N   S    192
529 TGA AAT ACA TTT AGA AAT TTT GGA ATT TTA CAC GGC AAA GAT AAT TCT    576

193  S   S   K   F   K   L   E   E   T   I   L   K   N   H   F   Q    208
577 TCT TCT AAA TTC AAG TTA GAA GAA ACT ATA TTA AAA AAC CAC TTT CAA    624

209  N   K   F   T   T   L   N   E   D   R   S   A   H   P   N   A    224
625 AAT AAA TTT ACA ACA CTA AAT GAA GAC AGA AGC GCA CAT CCA AAC GCA    672

225  Y   K   Q   K   S   A   D   T   L   G   T   L   D   D   F   H    240
673 TAT AAA CAA AAA TCT GCA GAT ACA TTG GGA ACT TTA GAT GAC TTT CAT    720

241  I   A   F   S   E   E   G   L   L   L   E   H   I   T   N   Q    256
721 ATT GCT TTT TCA GAA GAA GGT CTT TTG CTT GAA CAC ATA ACA AAT CAG    768

257  Q   A   K   P   F   E   T   K   A   N   E   K   M   E   A   L    272
769 CAA GCA AAA CCT TTT GAA ACT AAA GCA AAT GAA AAG ATG GAA GCA CTT    816

273  I   L   T   N   P   I   P   Y   D   V   G   V   F   R   K   S    288
817 ATA CTA ACT AAT CCA ATT CCG TAT GAT GTT GGA GTG TTT AGA AAA AGT    864

289  V   N   Q   L   E   Q   N   L   I   V   Q   T   F   I   N   L    304
865 GTT AAC CAA TTA GAA CAA AAT TTA ATT GTT CAA ACA TTC ATT AAT TTA    912

305  A   K   N   K   Q   D   T   Y   G   P   L   L   G   Y   N   G    320
913 GCT AAA AAT AAA CAA GAT ACA TAT GGT CCA CTT TTA GGG TAT AAT GGT    960

321  Y   K   K   I   D   N   F   H                                    328
961 TAT AAA AAA ATC GAT AAC TTC CAC AA                                986
```

Figure 11

The amino acid sequence of p40 deduced from the translated DNA sequence isolated from LS-180 colon cancer cell line. The individual peptides purified and sequenced from affinity purified p40 from the lung NCI-H69 cell line either by tryptic digestion, tryptic digestion in the presence of blocked lysine or cleaved by CnBr are marked. Red: cleaved by CnBr. Yellow: digested by trypsin. Green: digested by trypsin with blocked lysine.

LUNG

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
											E	I	T	F	G	V	S	E	A	T
1.	L	N	K	K	K	G	G	K	E	V	N	K	E	V	I	N	T	F	L	E
2.	N	F	K	K	E	F	N	K	L	K	N	A	N	D	K	T	K	N	F	D
3.	D	V	D	F	K	V	T	L	I	Q	D	F	T	V	L	L	N	N	L	S
4.	T	D	N	P	E	L	D	F	G	I	N	A	S	G	K	L	V	K	F	L
5.	K	N	N	P	G	I	I	T	P	A	L	Q	T	T	T	N	S	F	V	L
6.	D	K	E	K	D	K	F	Y	V	D	G	T	D	S	D	P	L	V	K	I
7.	A	K	E	I	N	K	I	F	V	E	T	P	Y	A	S	W	T	D	E	N
8.	H	K	W	N	G	N	V	Y	Q	S	V	Y	D	P	T	V	Q	A	N	F
9.	Y	R	G	M	I	W	I	K	G	N	D	E	T	L	A	K	I	K	K	A
10.	W	N	D	K	D	W	N	T	F	R	N	F	G	I	L	H	G	K	D	N
11.	S	S	S	K	F	K	L	E	E	T	I	L	K	N	H	F	Q	N	K	F
12.	T	T	L	N	E	D	R	S	A	H	P	N	A	Y	K	Q	K	S	A	D
13.	T	L	G	T	L	D	D	F	H	I	A	F	S	E	E	G	L	L	L	E
14.	H	I	T	N	Q	Q	A	K	P	F	E	T	K	A	N	E	K	M	E	A
15.	L	I	L	T	N	P	I	P	Y	D	V	G	V	F	R	K	S	V	N	Q
16.	L	E	Q	N	L	I	V	Q	T	F	I	N	L	A	K	N	K	Q	D	T
17.	Y	G	P	L	L	G	Y	N	G	Y	K	K	I	D	N	F	H	K	E	I
18.	V	E	V	T	E	K														

Figure 12

The amino acid sequence of p40 deduced from the translated DNA sequence isolated from LS-180 colon cancer cell line. The individual peptides purified and sequenced from affinity purified p40 from the colon HCT-15 cell line either by tryptic digestion, tryptic digestion in the presence of blocked lysine or cleaved by CnBr are marked. Red: cleaved by CnBr. Yellow: digested by trypsin. Green: digested by trypsin with blocked lysine.

P40 SEQUENCE

COLON

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	L	N	K	K	K	G	G	K	E	V	N	K	E	V	I	N	T	F	L	E
2.	N	F	K	K	E	F	N	K	L	K	N	A	N	D	K	T	K	N	F	D
3.	D	V	D	F	K	V	T	L	I	Q	D	F	T	V	L	L	N	N	L	S
4.	T	D	N	P	E	L	D	F	G	I	N	A	S	G	K	L	V	K	F	L
5.	K	N	N	P	G	I	I	T	P	A	L	Q	T	T	T	N	S	F	V	L
6.	D	K	E	K	D	K	F	Y	V	D	G	T	D	S	D	P	L	V	K	I
7.	A	K	E	I	N	K	I	F	V	E	T	P	Y	A	S	W	T	D	E	N
8.	H	K	W	N	G	N	V	Y	Q	S	V	Y	D	P	T	V	Q	A	N	F
9.	Y	P	G	M	I	W	I	K	G	N	D	E	T	L	A	K	I	K	K	A
10.	W	N	D	K	D	W	N	T	F	R	N	F	G	I	L	H	G	K	D	N
11.	S	S	S	K	F	K	L	E	E	T	I	L	K	N	H	F	Q	N	K	F
12.	T	T	L	N	E	D	R	S	A	H	P	N	A	Y	K	Q	K	S	A	D
13.	Y	L	G	T	L	D	D	F	H	I	A	F	S	E	E	G	L	L	L	E
14.	H	I	T	N	Q	Q	A	K	P	F	E	T	F	A	N	E	K	M	E	A
15.	L	I	L	T	N	P	I	P	Y	D	V	G	V	F	P	K	S	V	N	Q
16.	L	F	Q	N	L	I	V	Q	T	F	I	N	L	A	F	N	K	Q	D	T
17.	Y	G	P	L	L	G	Y	N	G	Y	K	K	I	D	N	F	H	K	E	I
18.	V	E	V	Y	E	K	A	I	K											

TABLE 23

REACTIVITY OF ANTI-P40 MONOCLONAL AND POLYCLONAL ANTIBODIES WITH MYCOPLASMA EXTRACT AND ANTI-MYCOPLASMA POLYCLONAL ANTIBODY WITH AFFINITY-PURIFIED P40 MEASURED BY ELISA

Antigen	**Antibody binding ($O.D_{410}$)**				
	a-P40 mono neat	**a-P40 poly 1:1000**	**a-Pept #2 poly 20 µg/ml**	**a-Mycoplasma poly 1:100**	**1:1000**
P40[1]	1.60	1.59	1.62	0.33	0.08
Mycoplasma[2]	0.00	0.04	0.00	1.29	1.30

[1] Affinity purified lung p40 tested at 50 µg/well.

[2] Tested at 50 µl/well of 1:1000 dilution of crude mycoplasma antigen extracted with 0.5% (w/v) SDS in PBS. Preliminary antigen titrating tests with polyclonal a-mycoplasma antibody showed no change of binding capacity at antigen dilutions in a range of neat - 1:10,000 and antibody dilutions 1:100 - 1:1000 (data not shown).

mono = monoclonal.
poly = polyclonal.

Figure 13

Western blot analysis of binding of polyclonal a-mycoplasma and a-p40 antisera to lung cell line NCI-H69. Lane A, binding of mAb a-p40 to affinity purified p40; lane B, no sample; lanes C-G, binding of Abs to NCI-H69 lung cell extract; lane C, mAb a-p40 binding; lane D, pAb a-p40 binding; lane E, pAb a-mycoplasma binding; lane F, mAb negative control binding; lane G, normal rabbit serum (pAb negative control) binding. The numbers on the right side of the figure represent MW standards expressed in kD.

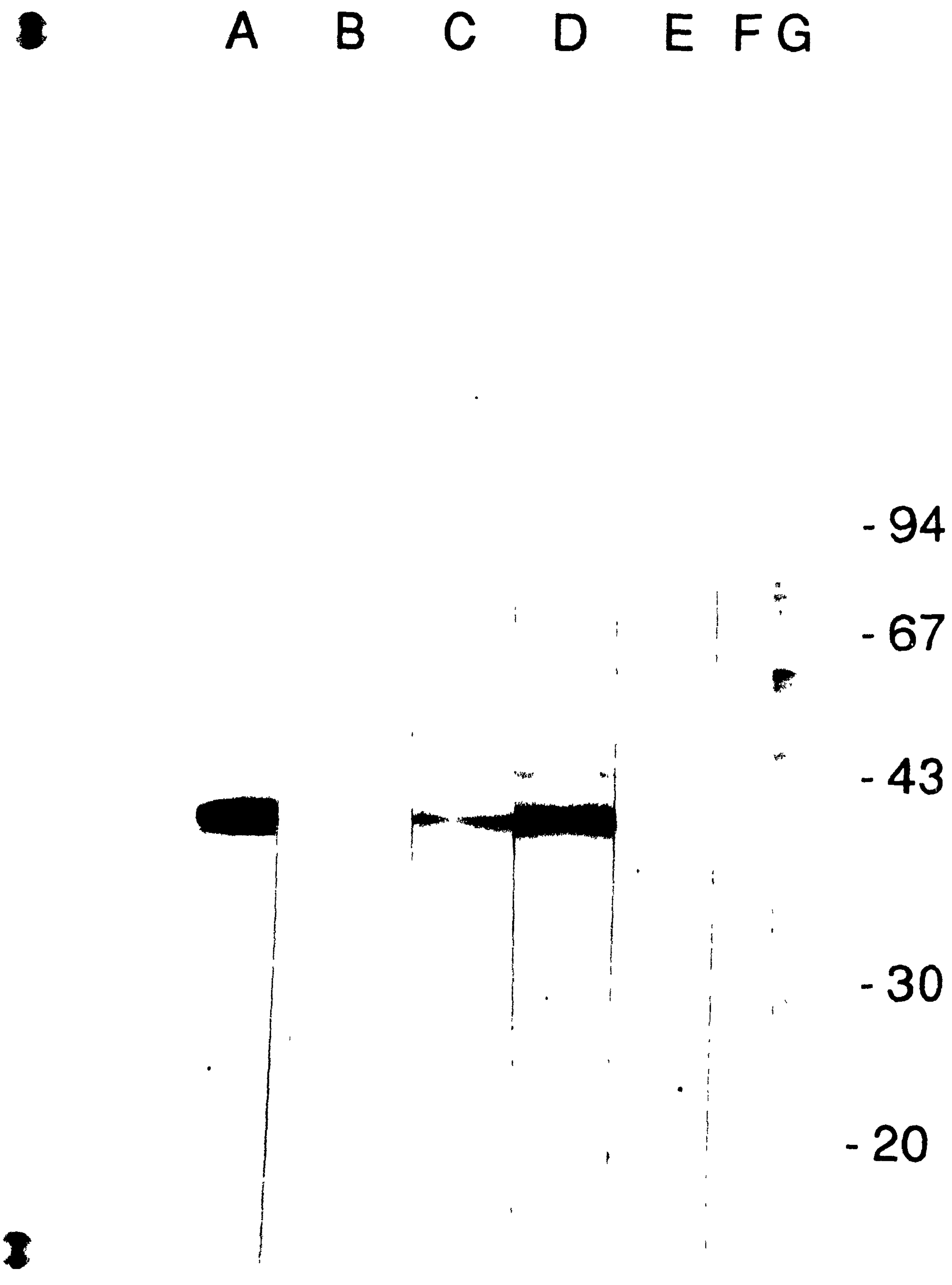

A
B
C
D
E
F
G
- 94
- 67
- 43
- 30
- 20

TABLE 24

ADDITIONAL TESTING FOR CONTAMINATION OF LUNG NCI-H69 AND COLON HCT-15 CELL LINES

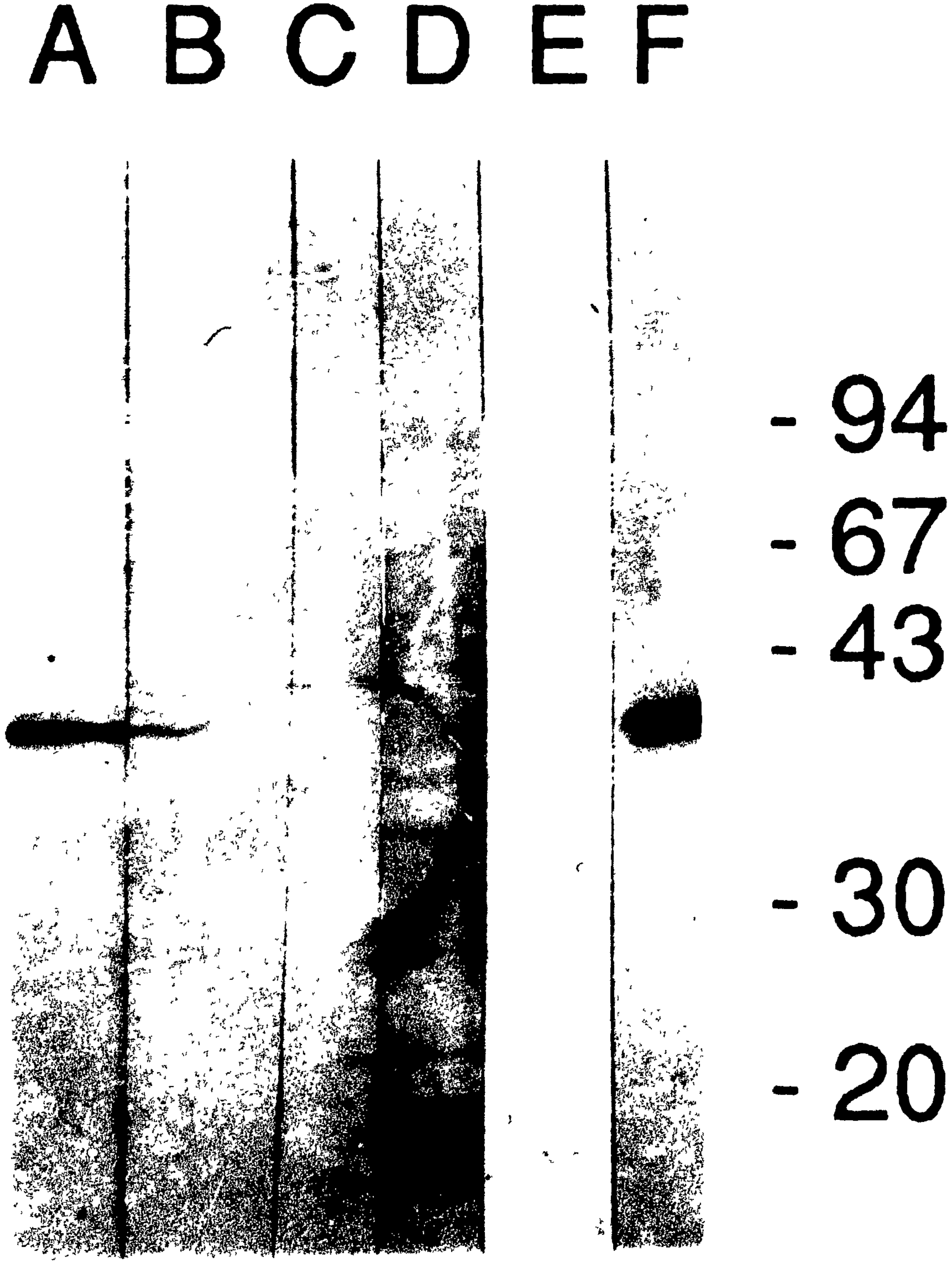
Test	Test result
-Culturing of samples from lung NCI-H69 and colon HCT-15 cells on mycoplasma agar.*	NEGATIVE
-^{3}H-Thymidine uptake of spent medium from lung and colon cancer cells:	NEGATIVE

*Samples of cells were cultured under sterile conditions on specific mycoplasma broth agar by experienced staff of the Microbiology Department of the Montreal General Hospital.

negative, the p40-coding DNA exhibited mycoplasma DNA properties and the amino acid sequence of the p40 peptides agreed with the amino acid sequence of the translated p40 DNA with the exception of a few random amino acid residues. Consequently, I determined the effect of an anti-mycoplasma compound (BM-cycline) on the expression of p40 by both the lung NCI-H69 and colon HCT-15 cell lines. The reagent was used according to the manufacturer's instructions. Figure 14 shows the western blot performed on the lung cell line extract before and after the BM-cycline treatment. Lanes A and C show mAb a-p40 binding before and after treatment. In lane A the Ab detects p40 well, whereas in lane D p40 is absent. Similar results were obtained with the colon cell extract (results not shown). The results indicated that the BM-cycline inhibited the expression of p40. While this might have been attributed to some metabolic effect of the reagent on the cells, the most plausible explanation was that BM-cycline was inhibiting mycoplasma growth and their expression of p40. Consequently, a second experiment to confirm the origin of p40 was undertaken. The Mycotect kit was purchased to detect mycoplasma contamination of our cell lines. The function of the Mycotect kit is based on a biochemical difference between mycoplasmas and mammalian cells. Mycoplasmas are rich in the enzyme adenosine phosphorylase but mammalian cells contain only small quantities of this enzyme. Adenosine phosphorylase converts 6-methylpurine riboside (6-MPDR), a non-toxic analogue of adenosine, into two compounds: 6-methylpurine and 6-methylpurine riboside, both toxic to mammalian cells. A mycoplasma-infected culture can be detected by inoculation of a mycoplasma-free indicator mammalian cell monolayer with a sample of the contaminated cells, and subsequent incubation with 6-MPDR. The adenosine phosphorylase present in mycoplasma converts 6-MPDR to the two analogs that destroy the mammalian cell monolayer. Staining of the monolayer with crystal violet shows the degree of contamination. A confluent, strongly stained monolayer indicates healthy cells and, hence, no mycoplasma infection. A sparse, weakly stained monolayer indicates destruction of the cells, hence, mycoplasma infection. The results are summarized in Tables 25, 26, 27 and

Figure 14

Western blot analysis of p40 expression in extracts of lung cell line NCI-H69 before and after treatment with BM-cycline. Lanes A-B, cell extract before treatment. Lane A, mAb a-p40 binding. Lane B, mAb negative control binding. Lanes C-D, cell extract after treatment. Lane C, mAb negative control binding. Lane D, mAb a-p40 binding. Lane E, no sample. Lane F, positive control affinity purified lung p40 blotted with mAb a-p40. The numbers on the right side of the figure represent MW standards expressed in kD.


A
B
C
D
E
F
- 94
- 67
- 43
- 30
- 20

28. Table 25 shows the results of Mycotect assays performed on a number of our cultured cell lines. The lung NCI-H69 and colon HCT-15 cell lines were both contaminated. In parallel, the infected and BM-cycline treated cells were also tested by Mycotect assay. The results were inconsistent as shown in Table 26. Some flasks were cured, some were not. Mycoplasma-free NCI-H69 lung cells were reinfected with cell-free supernatant from their infected counterparts and then tested for expression of p40 by mAb a-p40. Initially the BM-cycline treated cells were negative for mycoplasma contamination and p40 expression, but one week after mycoplasma contamination they were Mycotect positive and negative for p40 expression and after three weeks later tested positive for both (Table 27).

It has been observed that routinely used antibiotics may suppress mycoplasma contamination and its effects (115). Thus, one of the conditions required for successful results in the Mycotect assay is culturing the cells for at least two passages in medium free of antibiotics. The Mycotect testing of mycoplasma contamination and expression of p40 was done with cells grown for three passages in antibiotic free medium. In order to examine whether routinely used antibiotics affected p40 expression I tested our cultured cell lines by western blots: (1) when the cells were grown in medium containing antibiotics and (2) when the cells were grown for at least two passages in medium free of antibiotics.

Culturing of the cells in medium containing antibiotics or free of antibiotics was done concomitantly in sister flasks and monitored in parallel by both western blots and Mycotect tests. The results are shown in Table 28.

Of six original cell cultures which tested positive for mycoplasma contamination by Mycotect test, four expressed p40 in medium containing antibiotics. Moreover, when non-infected NCI-H69 was reinfected with mycoplasma, the cell line became positive both in the Mycotect test and for p40 expression. Hence most mycoplasma-contaminated cell cultures, as determined by Mycotect test, expressed p40 despite the presence of routinely used antibiotics. There were some exceptions to Mycotect positivity and expression of p40. ECF-STT61 was Mycotect positive and negative for p40 without and with antibiotics,

TABLE 25

RESULTS OF MYCOPLASMA CONTAMINATION TESTING OF CULTURED CANCER CELL LINES AS ASSESSED BY MYCOTECT ASSAY

Cell line	Mycotect assay result
HCT-15 colon	+
NCI-H69 lung	+
NCI-H69 lung (new culture)	-
MDA breast	-
Du 4475 breast	-
MCF-7 breast	-
Colo 205 colon	+
Colo 201 colon	-
Colo 320 HSR colon	-
LS-180 colon	+
ECF-STT61 astrocytoma	+
3T6 mouse fibroblasts	-
SP-2 mouse myeloma	-

TABLE 26

RESULTS OF MYCOPLASMA TESTING ON COLON HCT-15 AND LUNG NCI-H69 CANCER CELL LINES AFTER BM-CYCLINE TREATMENT AS ASSESSED BY MYCOTECT ASSAY

Cell Line	Mycotect assay results
HCT-15 Colon	
Flask #7	-
Flask #2	+
NCI-H69 Lung	
Flask #3	+
Flask #lC	-

TABLE 27

RESULTS OF DETECTION OF P40 BY WESTERN BLOTS AND MYCOPLASMA CONTAMINATION BY MYCOTECT ASSAY ON LUNG CANCER CELL LINE NCI-H69 BEFORE AND AFTER INFECTION WITH SUPERNATANT FROM MYCOPLASMA-INFECTED TISSUE CULTURED CELLS*

Cell Line	Mycotect assay result	Anti-P40 mAb binding
NCI-H69 Lung		
Before	-	-
After	+	+

*The mycoplasma used to infect the cells was derived from frozen stock cells of the same cell line. The frozen cells were thawed, resuspended in a small amount of fresh medium to wash away the DMSO of the freezing medium and were then centrifuged. After resuspending in a small volume of fresh medium, the suspension were forced through 0.2 μ filter. Due to their small size and lack of cell wall mycoplasmas can pass through a 0.2 μ filters while eukaryotic cells cannot. The filtering procedure was repeated twice.

TABLE 28

CORRELATION BETWEEN PRESENCE OF MYCOPLASMA AND P40 EXPRESSION ON CULTURED CELL LINES AS ASSESSED BY MYCOTECT ASSAYS AND WESTERN BLOTS

Cell Line	Mycotect assay	P40 on cells cultured in medium	
		without antibiotics[1]	with antibiotics[2]
HCT-15 Colon			
Original Culture*	+	+	+
BM-cycline treated			
Flask #7	-	-	-
Flask #2	+	-	-
NCI-H69 Lung			
Original Culture*	+	+	+
BM-cycline treated			
Flask #3	+	+	-
Flask #1a**	-	-	-
Flask #1 infected with mycoplasma +ve supernatant	+	+	+
NCI-H69 Lung (new culture)	-	-	-
MDA Breast	-	-	-
Du 4475 Breast	-	-	-
MCF-7 Breast	-	-	-
Colo 205 Colon	+	+	-
Colo 201 Colon	-	NT	-
Colo 320 HSR Colon	-	-	-
LS-180 Colon	+	+	+
CaCo2 Colon	+	NT	+
ECF-STT61 Astrocytoma	+	-	-
3T6 Fibroblasts (Mouse)	-	-	NT
SP-2 Myeloma (Mouse)	-	-	NT

*Original culture refers to cells not treated with BM-cycline.

**Flask #1a is the cured negative control to Flask #1. The two flasks were cultured in parallel and mycotect tested and extracted at the same time.

[1] The cells were passaged twice in medium free of antibiotics; [2] in parallel, sister flasks with cultured cells in medium containing antibiotics were kept.

NT = Not tested.

suggesting that a mycoplasma species not expressing p40 contaminated this cell line. Colo 205 cell line was the only original culture that was Mycotect positive and did not express p40 in the presence of antibiotics. This result suggested that mycoplasma contamination of this particular cell line was low, and the antibiotics had some suppressive effect on mycoplasma growth. Both colon HCT-15 and lung NCI-H69 were treated with cycles of BM-cycline, resulting in complete eradication of mycoplasma contamination as shown by a negative Mycotect test and negative p40 expression. However, flask #2 of colon HCT-15 continued to be positive by Mycotect test despite BM-cycline treatment. The mycoplasma contaminated cells did not express p40 either in the presence or absence of antibiotics. The results suggested that mycoplasma contamination persisted and that either the mycoplasma contamination was too low for sufficient p40 expression to be detected or a mycoplasma species not producing p40 was present. In the instance of flask #3 of NCI-H69, mycoplasma contamination persisted after BM-cycline treatment and p40 was expressed in the absence of antibiotics, indicating that there was probably a low level persistent mycoplasma contamination that flourished after removal of antibiotics.

In summary, it seems that most of the contaminated original cell lines were contaminated with Mycoplasma hyorhinis which expressed p40 and inclusion of antibiotics in the medium had no effect on the growth of mycoplasma or its expression of p40.

In conclusion, p40 was found to be a protein coded for by a DNA segment which exhibits mycoplasma DNA properties. Testing of the lung and colon cells (from which p40 was purified) by a mycoplasma-specific assay indicated that these cells were contaminated with mycoplasma. Moreover, p40 expression was eliminated from these cells by treatment with an anti-mycoplasma agent (BM-cycline) and reappeared upon reinfection of non-contaminated cells with cell-free supernatant from infected cells of the same line. Finally, the extent of p40 expression was at times influenced by antibiotics routinely added to the culture medium.

6. Studies on Pure Mycoplasma Extract

The question arose as to whether the OSN activity of the purified p40 was the result of contamination by a true OSN or whether the p40, a mycoplasma protein, expressed OSN epitopes. From a literature search, I found that p40 is a transport protein in the Mycoplasma hyorhinis strain (56). Most Mycoplasma hyorhinis species are not cultivatable in vitro, but one species (GDL) can be cultivatable in vitro. GDL was obtained from ATCC. Affinity purified p40 and GDL mycoplasma extract were analyzed by western blots. Anti-p40 Ab bound to a molecule at 40 kD in the mycoplasma extract in the same position as p40 was bound. P40 in the crude extract was quantitated on western blots by comparison with the affinity purified p40. These tests were done in Dr. C. Stanners' laboratory (data not shown). Using the quantitation of p40 in the crude mycoplasma protein extract by western blot, we tested the extract by LAI (Table 29). The mycoplasma extract was tested coded. The concentration chosen was to give p40 concentration, deduced from the quantitative western blot analysis, equivalent to that previously shown to stimulate positive LAI. First the mycoplasma antigen and a control FBS were tested with leukocytes from patients with different types of cancer. Leukocytes of cancer patients gave positive LAI responses with the mycoplasma antigen but none with FBS. On the other hand, when leukocytes from patients with non-malignant diseases (controls) were used, they reacted with neither the mycoplasma antigen nor FBS. The results suggest that molecules present in the Mycoplasma hyorhinis extract were capable of eliciting LAI positive reactions, although they had no organ specificity.

Although the preceding results clearly proved that Mycoplasma hyorhinis stimulated positive LAI with leukocytes from cancer patients, it was possible that the leukocytes from control subjects may not have shown any reaction due to a bias of the tester, since he was aware that the leukocytes were from controls. To obviate this problem, both the extracts as well as the leukocytes were coded, so that the tester was unaware of the leukocyte source or the antigen. The leukocytes were from lung cancer patients and patients with

TABLE 29

RESULTS OF CODED TESTING BY LAI OF ANTIGENICITY OF MYCOPLASMA EXTRACT

Diagnosis of Leukocyte Donor		NAI to: crude cancer extract*	NAI to:	
			Mycoplasma extract**	FBS
Cancer				
Lung	n = 4	58 ± 6	57 ± 11[1]	8 ± 13
Colon	n = 4	97 ± 28	72 ± 21[2]	-19 ± 5
Breast	n = 3	82 ± 11	47 ± 7[3]	-1 ± 8
Control***	n = 2	-11 ± 11	6 ± 8[4]	-2 ± 2

*Crude cancer extract was identical to the diagnosis of leukocyte donor and it was tested at 100 µg.

**Mycoplasma extract was tested at 0.5 µg (p40 equivalent)

[1,2,3] Comparing the responses of each type of leukocytes to mycoplasma proteins against FBS (≈100 µg of protein) the differences were statistically significant, student's 't' dependent test ($^1p < 0.0005$, $^2p < 0.0025$, $^3p < 0.0025$).

***Control donors are patients with benign disease. The control donors were tested against cancer extract of the same organ as the diagnosis of their disease.

[4] Comparing the responses of controls to mycoplasma extract against FBS, the differences were not statistically significant.

n = Number tested.

non-malignant diseases (Table 30). The lung cancer patients' leukocytes responded according to the pattern established in the previous study; that is, they gave positive LAI responses with the mycoplasma antigen but not with FBS. In contrast, the leukocytes from non-cancer patients gave negative LAI responses with both mycoplasma antigen and FBS. In summary, these results indicate that crude extracts of Mycoplasma hyorhinis, which had been cultured in a eukaryotic cell-free environment, contain a molecule that is recognized by lung, colon and breast cancer patients leukocytes. This recognition results in release of mediators which inhibit the adherence of bystander cells, producing positive LAI reactivity, but there is no organ specificity. On the other hand, p40 from the tumor cell lines infected with mycoplasma exhibits OSN activity.

7. Studies on Mycoplasma-Free Lung Cancer Cell Line NCI-H69 Extract

In the next experiments, the antigenicity of the uncontaminated cultured lung cancer cell line NCI-H69 was examined in LAI. A new culture was obtained from ATCC and grown in our laboratory. The cell morphology of the new cells as well as their growth requirements were different from that of the mycoplasma infected NCI-H69 cell line (Figure 15). The new mycoplasma-free cells grew in clusters of round cells (A), whereas the old mycoplasma-infected cells grew in a monolayer with many cells acquiring a spindle-like shape (B). The new uninfected cell line required 10% FBS to grow normally and its deprivation rendered the cells incapable of sustaining rapid growth, despite the supplemented growth factors described in the "Materials and Methods" chapter. The infected cells, on the contrary, did not require FBS for growth. Even after complete cessation of feeding for approximately four months these cells were capable of resustaining new growth.

The new uninfected cells were maintained according to the ATCC instructions. Before extraction, the cells were passaged twice in serum-free medium and, monitored for mycoplasma infection by Mycotect assay and for p40 expression by western blots. Neither infection nor p40 expression were detected. The cells were then extracted, and tested in the

TABLE 30

RESULTS OF DOUBLE BLIND STUDIES TESTING THE ANTIGENICITY OF MYCOPLASMA EXTRACT*

Diagnosis of Leukocyte Donor		NAI to: crude cancer extract*	NAI to:	
			Mycoplasma Extract[1]	FBS[2]
Cancer**[3]	n = 2	89 ± 54	65 ± 16	-15 ± 13
Control[4]***[4]	n = 2	-15 ± 11	-11 ±13	-7±12

*In this study both, the leukocytes and the antigens were coded so the tester was completely impartial.

**Each cancer patient gave positive LAI responses with cancer extract of the same diagnosis.

[1,2]Comparing the responses of the two types of leukocytes to mycoplasma antigen, tested at 0.5 μg and FBS (≈100 μg of protein) the differences were [1]statistically significant, independent student's 't' test, ($p<0.005$); [2]not significant.

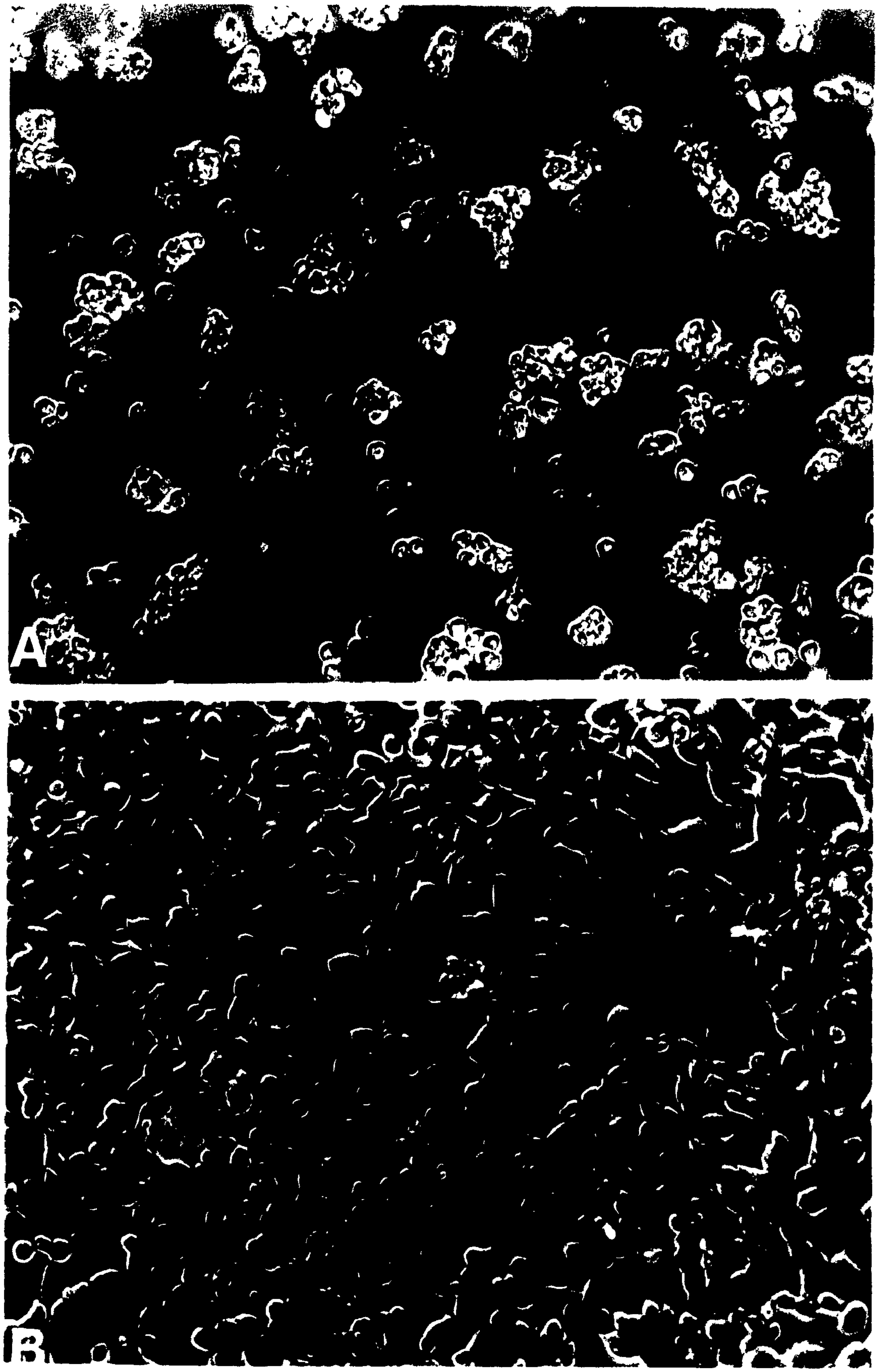
[3,4]Comparing the responses of each type of leukocytes to mycoplasma antigen against FBS the differences were [3]statistically significant for cancer patients, dependent student's 't' test ($p<0.0125$); [4]not significant for control donors.

***Control donors are patients with non-malignant diseases. Each control was tested with cancer extract of the same organ as the diagnosis of their disease.

n = Number tested

Figure 15

Inverted microscopy of lung cancer cell line NCI-H69: (A) without mycoplasma infection where it grows, as clusters in suspension and (B) with mycoplasma infection where cells grow as a monolayer with many cells displaying a spindle-like shape (original magnification 200x).


A
B

LAI assay. The titrated cell extract gave positive responses at 20 µg (data not shown). The cell extract was then tested, coded in parallel with FBS (Table 31). The extracted uninfected NCI-H69 lung cell line gave positive LAI responses with leukocytes from lung cancer patients, but did not give positive LAI responses with leukocytes from patients with non-malignant diseases. A third LAI testing of the extract was performed with cancer patients bearing tumors of organs other than lung. The extract was first titrated coded; and the LAI results of the titration were negative (not shown). Subsequently the extract was tested coded at the same concentration which induced LAI positive reactivity to lung cancer patients. None of the non-lung cancer patients tested exhibited positive LAI.

The results suggest that the uncontaminated lung cancer cell line NCI-H69 expresses molecules which exhibit OSN activity. The cells do not express substances that carry the mAb a-p40 epitope which defines p40, indicating that the OSN activity of the uncontaminated cell line is distinct from that of p40.

TABLE 31

RESULTS OF BLIND TESTING BY LAI OF THE ANTIGENICITY OF MYCOPLASMA-FREE NCI-H69 LUNG CANCER CELL EXTRACT

Diagnosis of Leukocyte Donor		NAI to: crude cancer extract*	NAI to:	
			Lung cancer cell line extract	FBS
Cancer				
Lung	n = 4	48 ± 4	75 ± 6	13 ± 5
Unrelated**	n = 5	87 ± 18	4 ± 6[1]	7 ± 10
Control***	n = 5	-7 ± 6	-8 ± 5[2]	-16 ± 9

*Crude cancer extract was identical to diagnosis of leukocyte donor.

**Unrelated cancer patients included breast, melanoma and colon.

***Controls were patients with benign diseases. They were tested with crude cancer extract of the same organ as their disease.

[1,2] NCI-H69 lung cancer cell line extract was tested at 20 μg in parallel with FBS (≈100 μg of protein); [1]the differences in the responses between unrelated cancer patients leukocytes and lung cancer patients leukocytes were statistically significant, independent student's 't' test ($p < 0.001$); [2]the differences in the responses between control subjects leukocytes and lung cancer patients leukocytes were statistically significant, independent student's 't' test ($p < 0.001$).

n = Number tested.

CHAPTER IV

DISCUSSION

The original aim of my research was the isolation and characterization of organ-specific neoantigens (OSN) present in butanol extracted material from the surface of cultured cancer cells. Following this, the distribution of the isolated and characterized molecule in human cancer would be examined. The unexpected results, however, carried my research in an entirely unforeseen direction. In this discussion, I will review my findings and will provide several possible explanations for mycoplasma protein seemingly exhibiting tumor antigen activity.

Tumors express molecules recognized as antigens by the immune system which mounts an immune response against them (106,140,284). The antigenic properties of tumors of both animals and humans have been the subject of research for more than 50 years. As reviewed in the "Introduction" a variety of in vivo and in vitro assays are used to measure the immune response against tumor antigens. Tumor antigens have been isolated and characterized from animals (258) but the human counterparts still remain elusive. A vast variety of cell surface structures have been detected on human tumors by means of mouse (16,77,131,170,181,234,265,306) and human mAbs (196,266). Histologically, most of these structures are quantitatively specific for the tumor and are not expressed by normal tissues. The structures detected by the xenogeneic mAbs are not responsible for T cell-mediated in vivo cytotoxicity, which is similar to the tumor transplantation rejection response of animals. On certain occasions Abs present in human sera specifically detect, in the autochthonous host, antigens in tumor tissues which are not present in the normal part of the tissue (221). A class of human neoantigens expressed by tumor cells, unlike the unique TSTA of chemically and virally induced animal tumors, are organ-specific . The immune response against OSN is detected by in vitro assays such as LAI (284). OSN are not expressed on normal tissues as shown by the absence of a measurable immune response in individuals free of cancer (102,165,272,284). An OSN, p40, was isolated by mAb affinity-chromatography from two human cancer cell lines: lung NCI-H69 (279) and colon HCT-15 (6). My studies described here indicate that p40 is not a human cell OSN as was originally thought, but actually a molecule from mycoplasma contaminating the two cell lines. Nevertheless, p40 was shown to possess in vitro stimulating activity in the LAI assay.

1. P25

LAI active butanol extracted material from the two cell lines was shown to contain a p40 and a p25 molecule. P25 was always present in material with OSN activity whether from spent medium that had passed through the a-p40 affinity column or from partially purified butanol extracts that did not contain p40. However, when p25 was enriched by RP-HPLC, the p25 fraction did not exhibit LAI activity, indicating that p25 itself was not an OSN. Subsequently, p25 was shown to have wide tissue distribution, being expressed by both cancer, normal adult and fetal tissues. The nature and function of p25 is unknown. Although it is possible that p25 complexes with the OSN molecule, I have no data to support this hypothesis. Since p25 is extracted with butanol from living cells, it must be a peripheral cell membrane protein and is expressed from early fetal life.

2. LAI Assay for Detecting Anti-Tumor Reactivity.

In the present study the principal findings with the LAI assay can be summarized as follows: 1) Crude extracts of fresh cancer exhibit OSN activity; 2) p40 synthetic peptides exhibit OSN activity; 3) mycoplasma extract containing p40 mediates LAI activity but not OSN activity; and 4) crude extract from lung cancer cell line NCI-H69, not contaminated with mycoplasma, exhibits OSN activity. Since p40, a molecule that exhibited OSN activity in the LAI assay (p40-OSN), was found to be a protein coded for by a mycoplasma DNA (Ilantzis, submitted for publication) and in my hands to be associated with mycoplasma infected cell cultures, the validity of the LAI assay as a competent tumor immunodiagnostic assay was put in doubt. This situation generated the need to reconfirm that the LAI assay, which has been in use since 1972 (111), is a valid detector of the human immune response to cancer. In the first of two series of new tests performed with both uncoded and randomly coded samples of colon, lung and breast solid tumors, we showed that leukocytes from colon, lung and breast cancer patients reacted positively with the crude homogenate of their respective tumor and with homogenates of the same type of tumor partly enriched for OSN activity. Moreover, in the second series of experiments, cancer patients leukocytes were shown to react specifically with the sensitizing cancer, indicating that the immune response was directed against substances which were specific for the organ in which the cancer arose. The findings are in agreement with previous reports from our laboratory (100,102,131,272) in that: 1) the leukocytes of cancer patients recognize in vitro sensitizing substances in the human tumor extracts of the same organ and become non-adherent, and 2) the antigenic substances of the tumors of different histologic origin do not cross-react, thus showing specificity for the organ of tumor origin (272,280,287). Furthermore, reports from other laboratories have confirmed specific anti-tumor immunity is detected by the LAI assay in animal (284) and human cancers (70,92,108,160). Since the two groups of tests were needed only to confirm previous results, they were done with few but significant number of patients. The values obtained when testing the uncoded and coded samples for the same tumor extract were nearly identical, suggesting that the uncoded LAI tests were both unbiased and reproducible. In conclusion, the LAI assay is able to detect a tumor host response to an OSN expressed by human cancers.

3. OSN Activity of p40 Synthetic Peptides

OSN activity is exhibited by cultured cell lines (6,55,150,279). OSN activity expressed by lung NCI-H69 and colon HCT-15 cancer cell lines was purified by mAb affinity chromatography (279,6). The mAb seemed to be directed against a common framework determinant of p40 since it bound p40 from both lung and colon cancer. In the present study, p40 is always the molecule identified by the expression of the epitope bound by the mAb that was used for its affinity purification. For p40 to trigger LAI, participation by both CD4$^+$ T cells and Class II MHC molecules on APC is needed (275). T cells (CD4$^+$ and CD8$^+$) are known to recognize short sequences of amino acids (about nine) in the context of MHC products (Class II and Class I, respectively) (9,21,28,251). Generation of peptides by cleavage of p40 with CnBr or trypsin and testing of the p40 peptides had previously shown that p40 cleavage did not destroy the OSN activity (275). Since the OSN epitope had survived cleavage, it was present on one of the generated peptides. Therefore by cleaving the two p40 molecules from both lung and colon into peptides, separating and then testing the individual peptides in the LAI, the OSN epitope(s) should be identified. After purifying, sequencing and generating synthetic copies of the OSN bearing peptide, mAbs could be raised to recognize the individual OSN epitopes for lung and colon cancer. Such a specific mAb could be a useful tool for cancer immunodiagnosis (275). Interest in the antigenic properties of synthetic peptides derives from the fact that peptides are able to mimic the antigenic sites of whole proteins (19, 300). Moreover, use of synthetic peptides eliminates the possibilities of contamination by co-isolating components and shortage of natural peptide.

The cleavage of the two p40 molecules yielded many peptides which, after separation and purification by RP-HPLC, were not always obtained in sufficient amounts to allow thorough testing in the LAI. Although the cleavage procedures were identical for lung and colon p40, the peptides generated from one did not always correspond with the peptides generated by the other. For this reason, corresponding peptidic fragments from the two p40's could not be tested in LAI for OSN epitope comparison. However, one CnBr peptide sequenced to 56 amino acids from lung p40 (CN5) and one (TCID) digested with trypsin from colon p40 proved to be LAI positive and were obtained in sufficient quantities for extensive LAI testing.

Recent results indicate that the length of antigenic peptides presented in the groove of Class I or Class II MHC molecules is about nine amino acid long (82,203). Furthermore, studies with short synthetic peptides have indicated that the T cell immunodominant epitopes on a protein tend to be amphipathic structures on an α-helix and that residues on the hydrophilic side are necessary for T cell recognition. On the other hand, exposure of hydrophobic sites of an immunogenic epitope is necessary for interaction with the MHC products (17). Based on these two pieces of information two stretches of the 56 amino acid long peptide were selected for their amphipathicity as potential T cell epitopes and consequently potential OSN epitopes (peptides #1 and #2) and were synthesized and purified by RP-HPLC. A synthetic peptide (CID) for colon was prepared in an identical fashion. Synthetic peptide #2 elicited a good Ab response but was LAI negative; synthetic peptide #1 elicited a poor Ab response but was LAI positive. A possible explanation for the differences in the Ab response to the two synthetic peptides may relate to their nature; peptide #2 may represent a B cell epitope and peptide #1 may contain a T cell epitope (19). The LAI results indicated that the synthetic peptides, lung #1 and colon CID, possessed OSN epitopes. However, as shown by ELISA, neither peptide was bound by the a-p40 mAb. The overall conclusion drawn from the studies on the p40 synthetic peptides was that at least two synthetic fragments of p40 can mimic the OSN activity of the corresponding natural peptides.

4. P40 Cross-Reacting or Mimicking the Real OSN

The LAI activity of the synthetic p40 peptides has been a puzzling finding in view of the later developments showing that the p40 DNA is of mycoplasma origin and the p40 amino acid sequence is identical to that of a mycoplasma transport protein (56). I propose here several lines of reasoning that could possibly explain the results that were obtained. P40-OSN exhibiting OSN activity and identified by the mAb a-p40, was only expressed by cultured cell lines infected with mycoplasma. P40 was not expressed by non-mycoplasma-infected cell lines, fresh cancer, or normal tissues, or fetuses. On the other hand, OSN activity was exhibited by the non-infected lung cell line NCI-H69. Furthermore NCI-H69 did not express p40, indicating that OSN molecules are present on this cultured cell line regardless of the presence of p40. Given the two findings that OSN is expressed by the uncontaminated lung cells and that p40, either intact (6,279), fragmented (275) or its synthetic peptide, expresses OSN activity, one could speculate that molecular mimicry may be involved in the phenomenon of OSN activity of p40.

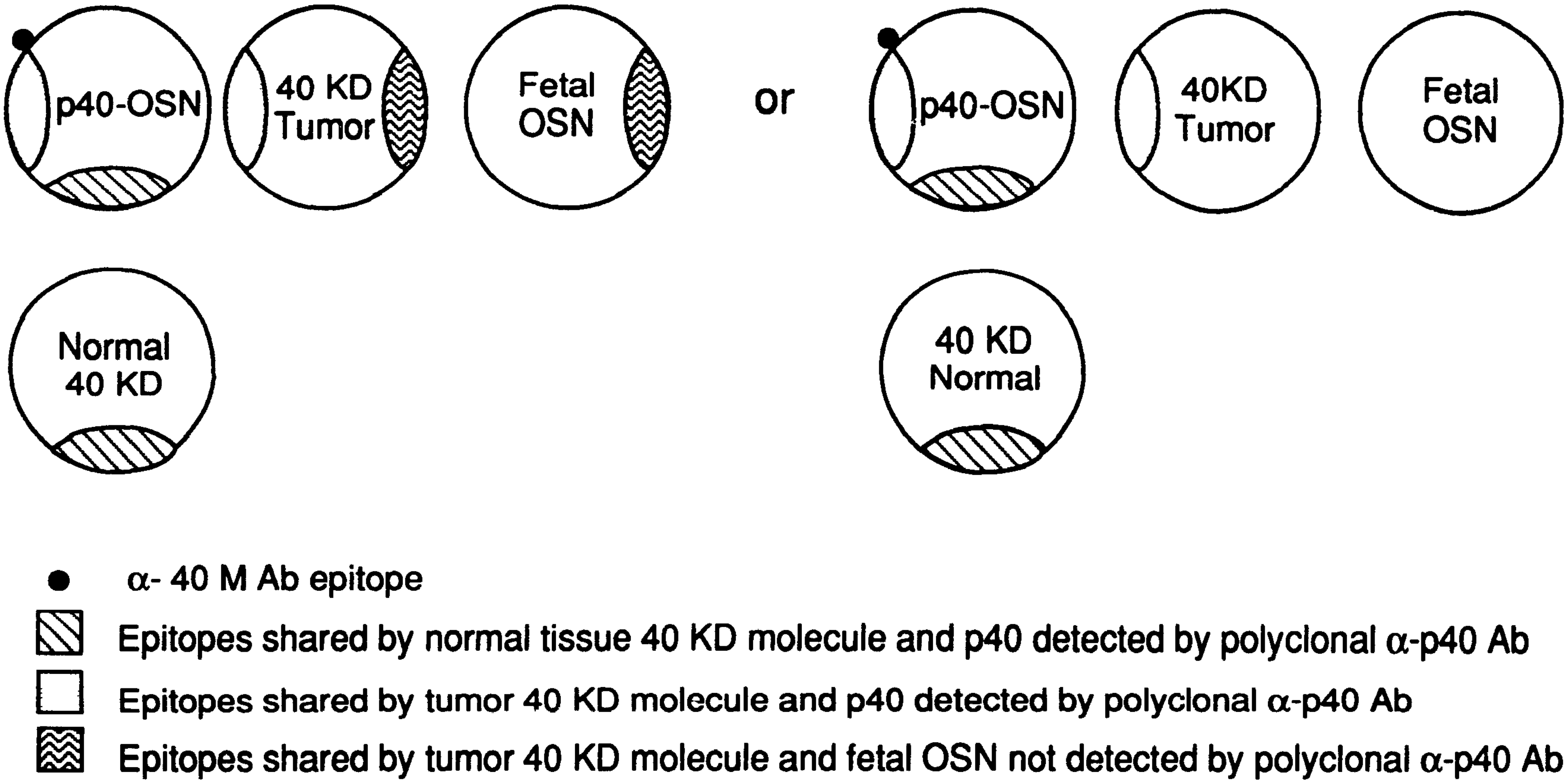
Cross-reactivity and mimicry of antigenic determinants in animals and plants by various microbes is widespread and is important in the compatibility of hosts and parasites (49). One theoretical idea concerning the origin of common antigens is that the interaction between host and parasite results in the antigenic alterations of either the host cells (262,264,269,296), or the parasite (271,312), or both (310,312). These changes may be genetic or epigenetic, and as a consequence, there is a convergent change in antigenicity toward that of the host by cells of the parasite (41,52,53). Early studies focused on the concept that tolerance to parasites by the host increases with increasing antigenic similarity, whereas resistance of the host is characterized by increasing disparity of antigenic determinants (49). Serological cross-reactions between organisms of diverse phylogenetic origin have been observed repeatedly, such as Mycoplasma arthriditis and rat cardiac and voluntary muscles and Mycoplasma hyopneumoniae and porcine lung (49). Vaccinia virus (80) and Mycoplasma hyorhinis share determinants with human intermediate filaments (IF) (309). Cross-reactivity between parasite antigens and human proteins has also been observed in Mycobacterium leprae and HLA-A2, Epstein-Barr virus and Ig gene superfamily domains and HIV envelope and monomorphic Class II MHC structures (105). Moreover, it was recently reported that a membrane protein of Schistosoma mansoni resembles a human melanoma tumor-associated antigen (13).

In the present study the tissue distribution results of p40 showed that fresh cancer tissues (which exhibit OSN activity) do not express the mAb a-p40 epitope that defines p40-OSN. Nonetheless, there are substances on non-infected culture cell lines and on fresh human tissues which specifically cross-react with the polyclonal Abs a-p40 and a-peptide #2 raised against p40-OSN and a synthetic peptide of p40-OSN, respectively. The two epitopes are expressed on a 40 and a 23 kD molecules. The 23 kD molecule is consistently expressed on most human tissues, including fetal tissues, suggesting that p23 is a protein that appears early in human development and does not lose expression in the adult. Animal fetal tissues express molecules that can act as TSTA as shown by their ability to confer immune protection against experimentally transplanted tumors (29,42). Human fetal tissues are known to express OSN as detected by the LAI assay, but the fetal OSN has not been isolated (104). The fact that p23 is identified by an Ab (a-peptide #2) raised against an OSN peptide (the CnBr-cleaved peptide from lung p40-OSN) might imply a relationship between p23 and fetal OSN. However, whether the p23 molecule that carries the peptide #2 epitope also carries the fetal OSN epitope is unknown. P23 is also present in both normal and cancer tissues. Since leukocytes from cancer patients do not react with normal tissue extracts (8,169,272), the p23 of normal tissues lacks the OSN epitope. If p23 can express an OSN epitope, the detection of p23 on both normal and cancer tissues implies that p23 shares common epitopes in both cancer and normal tissues, whereas the OSN-specific epitopes may be expressed only by cancer tissues. It is quite feasible that p23 is a common molecule expressed by all tissues which acquires OSN epitopes in fetal life and upon malignant transformation. Nonetheless, it is not known whether the p23 detected in cancer tissues and fetal tissues actually has an OSN epitope.

The other instance of possible cross-reactivity involves the 40 kD molecule detected by the a-p40 pAb on cultured cell lines and human tissues but not on fetal tissues. The consistent presence of a 40 kD molecule in tumor tissues was originally observed when papain-solubilized LAI active tumor material from cell membranes of breast, bowel, melanoma and hepatoma was analyzed on SDS gels (291). However, it is not known whether this 40 kD molecule and the 40 kD molecule detected by the a-p40 pAb on the tumor extracts in the western blots are related. A 40 kD molecule is also detected by the a-p40 pAb on non-contaminated breast Du 4475 and lung NCI-H69 cell lines and both exhibit OSN activity as determined by LAI. LAI reactivity of cultured tumor cell lines has been observed by other investigators as well (229).

Extracts of the uninfected NCI-H69 lung cancer cells, when tested with leukocytes from lung cancer patients, other cancer patients and controls, showed that as with fresh cancer tissues, OSN activity is expressed by these cultured cells in the absence of p40 expression. It is not known whether the OSN of fresh cancer extracts is expressed on the 40 kD molecule of fresh cancer tissues that is detected with the pAb a-p40 (itself raised against p40-OSN). Neither is it known whether the 40 kD molecule becomes an OSN by expressing different epitopes on cancer tissues than those expressed on normal tissues. Nor is it known whether the 40 kD molecule which cross-reacts with the a-p40 pAb is structurally or functionally related to mycoplasma p40-OSN. Possibly, the cross-reactivity between p40-OSN and the 40 kD molecule is accidental. However, if the 40 kD molecule is an OSN, it lacks the mAb a-p40 binding epitope that characterizes p40-OSN. Fetal tissues express OSN activity but they do not express the 40 kD molecule as defined by the pAb a-p40. Consequently, if the 40 kD molecule is an OSN, it may share epitopes with the fetal OSN which are not detected by the pAb a-p40. Alternatively, the fetal OSN may exist on a distinct molecule that is unrelated to either the 40 kD or the p40-OSN molecules. To determine whether the 40 kD and 23 kD molecules are OSN would require their purification and testing for OSN activity. The antigenic cross-reactivity and the putative epitopes are depicted in Figures 1 and 1a.

The changes at the protein or even gene level that could account for molecular mimicry are not known. It has been observed that phenotypic and antigenic variation can occur in several Mycoplasma species including Mycoplasma hyorhinis (69,228,310,312). These phenomena are important for the study of pathogenic mycoplasmas since genomic organization and variation dictate phenotypic characteristics which may determine the outcome of mycoplasma-host interactions (69,271,311,312). It was recently found that Mycoplasma hyorhinis incorporates pieces of genetic material which is suggested to be of extrachromosomal origin, possibly a component of a phage (69). The ability for changes at the gene level that could be responsible for mimicry of a mammalian cell component has not



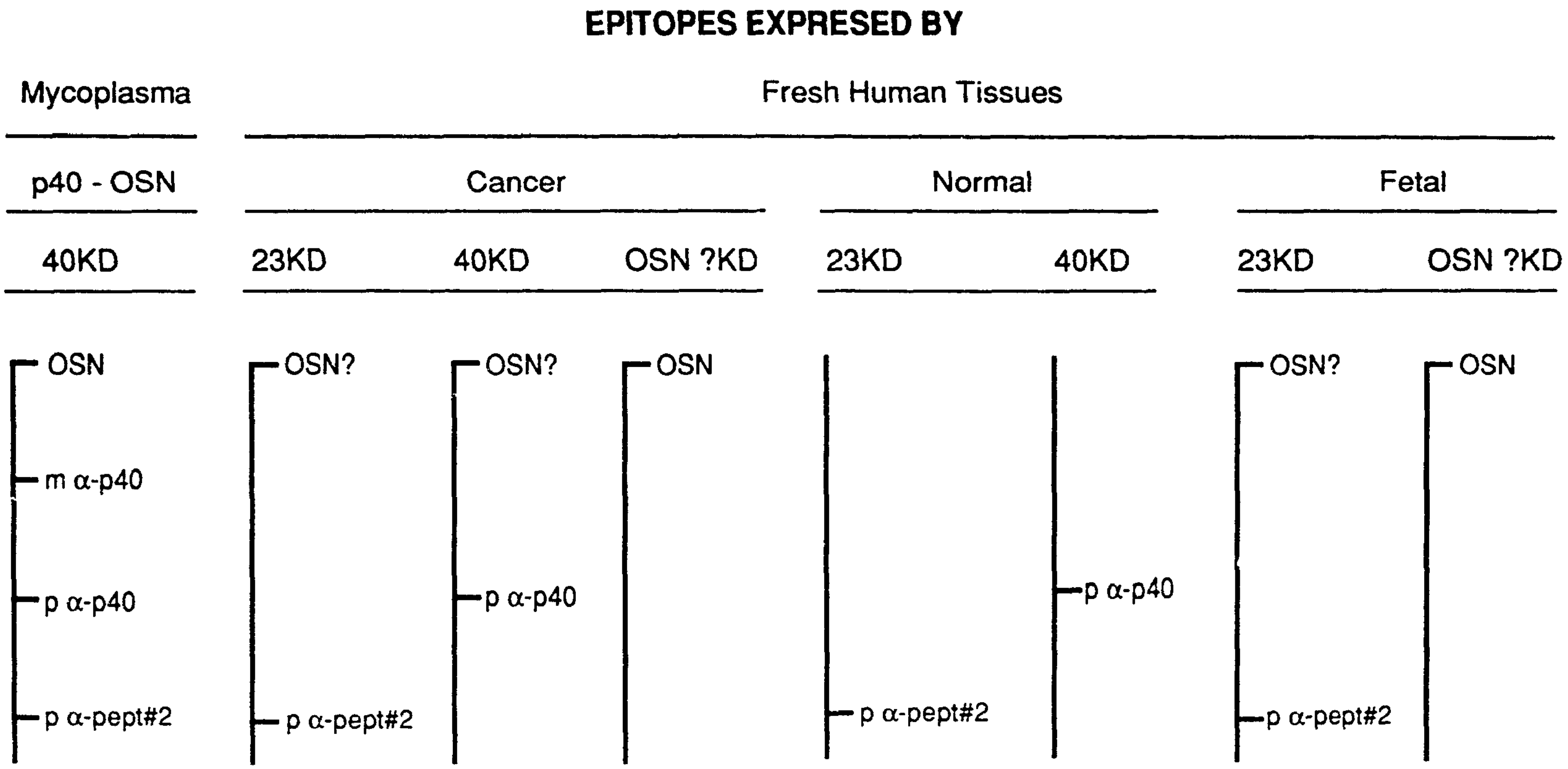
● α- 40 M Ab epitope

▨ Epitopes shared by normal tissue 40 KD molecule and p40 detected by polyclonal α-p40 Ab

☐ Epitopes shared by tumor 40 KD molecule and p40 detected by polyclonal α-p40 Ab

▩ Epitopes shared by tumor 40 KD molecule and fetal OSN not detected by polyclonal α-p40 Ab

Figure 1



m=monoclonal
p=polyclonal

Figure 1a

been observed so far. A search of the literature showed that another molecule like p40 had been isolated a year earlier than p40 from a mouse sarcoma cell line (56,240,267,268). In the mouse sarcoma system, this molecule (p37) behaved as a metastasis promoting (adhesion?) molecule. P37 seemed to share epitopes with an unidentified cellular molecule with metastasis promoting properties. It is interesting to note that in our system mycoplasma-contaminated lung cells NCI-H69 adhere to plastic, whereas their non-contaminated counterparts do not. Moreover, SP2/0 mouse myeloma cells grown in suspension deliberately infected with mammalian cell-free mycoplasma-contaminated culture medium expressed p40 and adhered to plastic about three weeks after infection (data not shown). In both cases, the attachment could be due to p40 or to modulation of the cell membrane by mycoplasmas or to both. One incident favoring the modulation of the cell membrane is that the cells of the infected NCI-H69 lung cell culture did not lose their attachment properties after being cured of the mycoplasma infection.

The amino acid sequences of p40 and p37 exhibited almost complete homology except for some isolated amino acids and one continuous stretch of 11 amino acids (Figure 10, Row 16, LLLEHITNQQA to SFAWTHNKSAT). Whether this 11 amino acid difference between our p40 and the mouse sarcoma p37 is due to an artifact of the cDNA isolation procedure or to differential interaction between mycoplasma and two different mammalian cell systems is not known. However, mycoplasmas have the ability to diversify phenotypically under various environmental conditions by genetic mechanisms not yet understood (228,271).

5. P40 as an OSN Transport Protein

In mycoplasmas, p40 appears to be part of a high affinity transport system protein complex (56), which is suggested to act as a receptor of unknown ligands. The complex consists of two other proteins and all three are coded for by one operon. The complex is similar to the periplasmic transport systems (permeases) of gram-negative bacteria. The mammalian p-glycoprotein coded for by the multidrug resistance gene is structurally similar to the bacterial permeases. P-glycoprotein acts as an energy dependent export pump in tumor cells that exhibit resistance to certain drugs, but its normal role is not known (240). One could hypothesize that p40 could act as a peptide carrier in the cultured cell lines from which it was isolated. OSN fragments transported by Class I and II MHC molecules to the cell surface during normal protein turnover could in turn bind to p40 and be co-isolated with p40. However, this hypothesis does not explain the OSN activity of p40 peptides coded for by mycoplasma DNA.

6. P40 as a Non-Specific Activator by Cross-Linking Cytophilic Ab.

The induction of organ-non-specific LAI with leukocytes from cancer patients as opposed to leukocytes from non-cancer patients by crude mycoplasma extract indicates a differential response. The molecule responsible for triggering the response is unknown but is presumed to be p40. One explanation for the organ-non-specific reactivity of the crude mycoplasma extract may relate to the mechanism of the LAI phenomenon. LAI is stimulated when leukocytes from cancer patients encounter in vitro the cancer antigen to which they have been sensitized in vivo. Reactive monocytes "armed" with antigen-specific cytophilic Ab and $CD4^+$ T cells in association with APC expressing Class II molecules are known to recognize tumor antigen. One (or more) components present in the extract may bind non-immunologically with cytophilic Ab bound to cancer patients' monocyte Fc receptors and cross-link the Fc portion. The cross-linking by the mycoplasma component would imitate the cross-linking induced by the binding of tumor antigen which, in turn, triggers the monocytes to release the mediators that induce LAI. Protein A from Staphylococcus aureus (60) and streptococcal protein G (20,60) are two bacterial surface proteins described to exhibit such non-immunologic reactions with immunoglobulins. The LAI triggered in this fashion would depend on quantitative differences in cytophilic Abs present on the monocytes of cancer patients compared to cancer-free individuals. In fact, affinity purified p40 was shown to bind non-specifically to both sheep anti-mouse and goat anti-rabbit immunoglobulin conjugate, indicating that p40 can react non-specifically with IgG. Supporting my observation is a recent report that Mycoplasma hominis and arginini possess a 95 to 105 kD protein which reacts non-immunologically with animal IgG (2).

7. P40 as a Non-Specific Activator in a Manner Similar to that of Superantigens.

The organ-non-specific LAI could also be stimulated by cross-linking of the T cell receptor (TCR). A class of molecules, termed "superantigens", have been demonstrated to be capable of stimulating lymphocytes at a much higher rate than conventional antigens (73). Staphylococcal enterotoxins, other toxic substances derived from bacteria and the minor lymphocyte stimulatory antigens (Mls) are substances that react with murine TCR which carry certain specific variable regions (48). It has been shown that in the Mls system, the CD4$^+$ T cell clones respond independent of their antigen specificity or the T cell receptor and MHC restriction (177). Superantigens stimulate T cells by binding outside the antigen groove of the Class II MHC molecules and, in turn, they bind a portion of the T cell receptor seemingly independent of the antigen receptor (48,73).

Mycoplasmas secrete factors which have various non-specific immunological properties. One factor secreted by mycoplasma arthriditis (MAS) has been found to act in a way very similar to that of bacterial enterotoxin superantigen (43,177), requiring Class II molecules on human accessory cells and a structure on T cells that appears to be the TCR. MAS seems to activate T cells by non-immunologically cross-linking the TCR and the Class II molecules of the APC. The cross-linking takes place independent of the MHC restricted, antigen-specific receptor (14, 43). Applying this model to p40 in the crude mycoplasma extract, I would suggest that p40 acts as a cross-linker between Class II molecules and T cell receptors in the LAI assay. The differences in the responses between cancer patients and normal subjects would have to be attributed to quantitative differences in activated immune cells between the two groups. Few immune cells might be expected to be upregulated in normal humans, but in tumor bearing individuals, an ongoing immune response to other antigens such as an OSN might upregulate many immune cells (63,277). Thus the cancer patients might have far more activated APC and T cells and, as a consequence, express higher levels of Class II molecules than the non-activated immune cells of normal persons (1). In this environment, p40 would then have sufficient numbers of Class II and TCR molecules to cross-link, thereby triggering the release of mediators responsible for LAI.

Various mycoplasmas also secrete factors that induce expression of Class II molecules on a macrophage cell line (269), on rat B cells (269), and Class I and Class II on primary bone marrow macrophage cultures (269). If p40 acts as both a Class II inducer on APC and cross-linker between the induced Class II and the TCR, I would expect that both cancer patients and controls should exhibit LAI. However, the control patients may not express appropriate levels of activated T cells for cross-linking with the excess of induced Class II molecules. Moreover, the two hour time incubation period of the LAI assay is probably insufficient for induction of Class II products on the APC of either cancer patients or controls. In this regard, mycoplasma induction of Class II molecules in a mouse macrophage cell line takes 24 hours (269). Consequently, the LAI activity of the cancer patients' leukocytes is likely due to cross-linking of TCR with pre-existing Class II molecules rather than Class II molecules that are upregulated by mycoplasma. Whether p40 acts as an inducer of MHC products cannot be determined by means of the LAI assay.

8. Synergy between P40 and OSN for LAI Activity.

So far three conflicting results of LAI activity are a major issue in the present study: (1) Crude mycoplasma extract exhibits organ-non-specific LAI activity. 2) Crude extract from non-contaminated lung NCI-H69 cells exhibits OSN activity. 3) Purified p40 exhibits OSN activity (6,279). The organ-non-specific LAI activity of the crude mycoplasma extract which contains p40 is in conflict with the OSN activity of the purified p40 molecule. For this reason, I have used crude extracts expressing p40 and/or OSN as a common basis for comparing the organ-specific and non-specific LAI activity exhibited by material containing p40. Figure 2 shows the three extracts tested: (A) crude mycoplasma extract exhibiting organ-non-specific LAI activity and containing p40; (B) crude extract of spent culture medium derived from mycoplasma-contaminated NCI-H69 lung cancer cells exhibiting OSN activity and containing p40; and (C) crude extract of non-contaminated NCI-H69 lung cancer cells exhibiting OSN activity. The mechanism of the LAI response is mediated in all three instances both by interaction of $CD4^+$ T cells with APC and monocytes armed with cytophilic Ab. The mycoplasma-infected lung extract shows that this extract (B), which expresses OSN activity shares the p40 and OSN molecules of extracts (A) and (C), respectively. The organ-non-specific LAI activity due to components from pure mycoplasma is overshadowed by the specific OSN activity. The question that arises is why the organ-non-specific effect given by the mycoplasma components is overshadowed by the specific effect of the OSN in the extract (B) when the extract contains both antigens. One explanation could be that the mycoplasma components in extract (B) might be negligible compared to OSN so that the response is mediated by almost pure OSN. However, p40 was isolated from extract (B) implying that mycoplasma substances are at a high enough concentration to allow their purification. Another explanation for the absence of the organ-non-specific effect in extract (B) may lie in the mechanism of the LAI response. When two antigens, which separately give optimal LAI responses, are added together, the LAI response is negated (274). A gradual addition of a second antigen first induces a decrease in the intensity of the LAI response (274) and then completely negates the LAI response when the two antigens reach their optimum concentrations for LAI. LAI induced by a combination of the p40 and OSN may occur in two ways. First, when the two antigens are present together, a combination of the two may give a specific OSN response. The bell-shaped dose-response curve would be shifted to a lower range of OSN concentration required to stimulate the same magnitude of LAI because the organ-non-specific material potentiates the response (Figure 3A). Second, as the antigen concentration

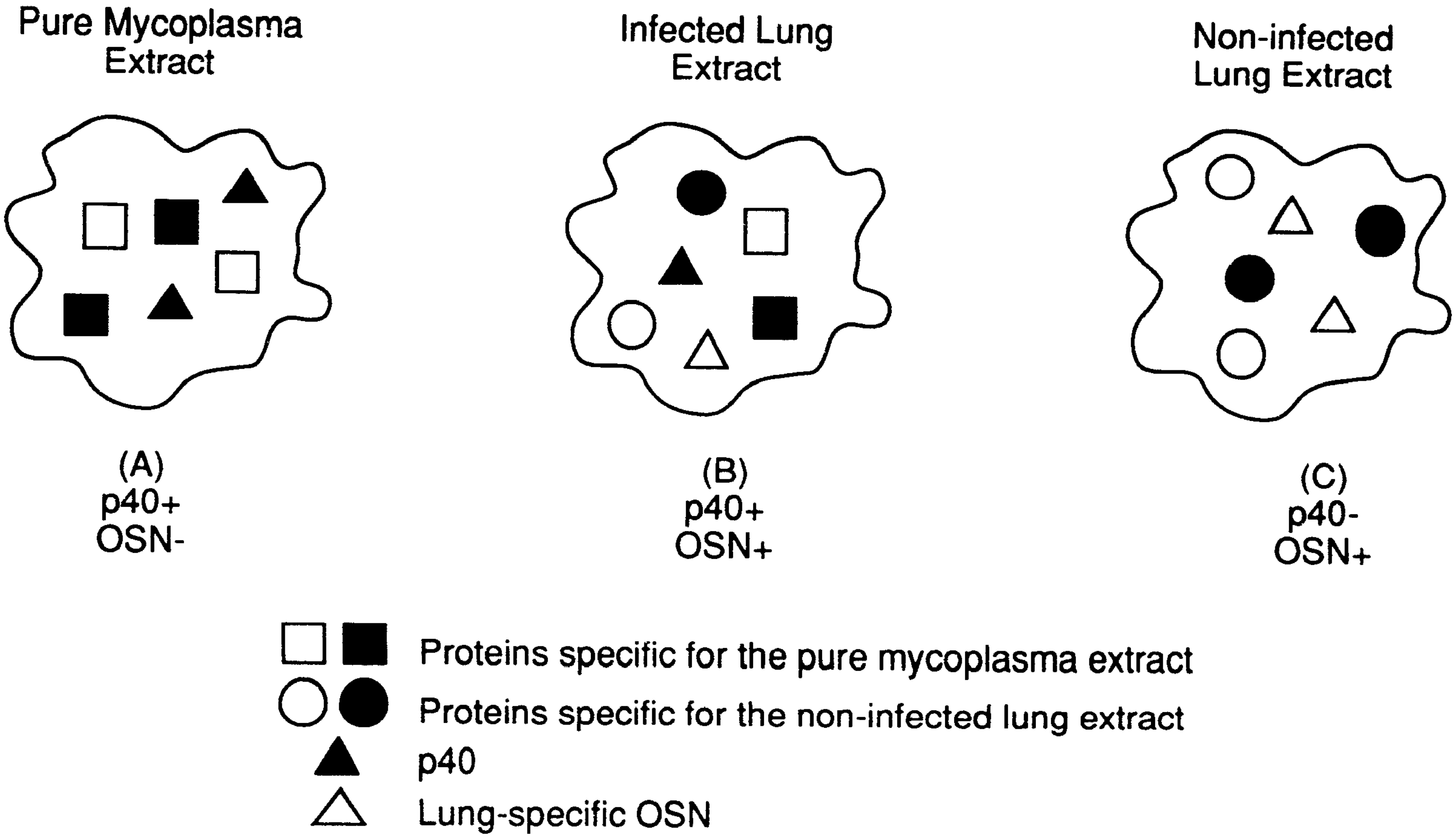

Pure Mycoplasma Extract
Infected Lung Extract
Non-infected Lung Extract
(A)
p40+
OSN-
(B)
p40+
OSN+
(C)
p40-
OSN+
□ ■ Proteins specific for the pure mycoplasma extract
○ ● Proteins specific for the non-infected lung extract
▲ p40
△ Lung-specific OSN

Figure 2

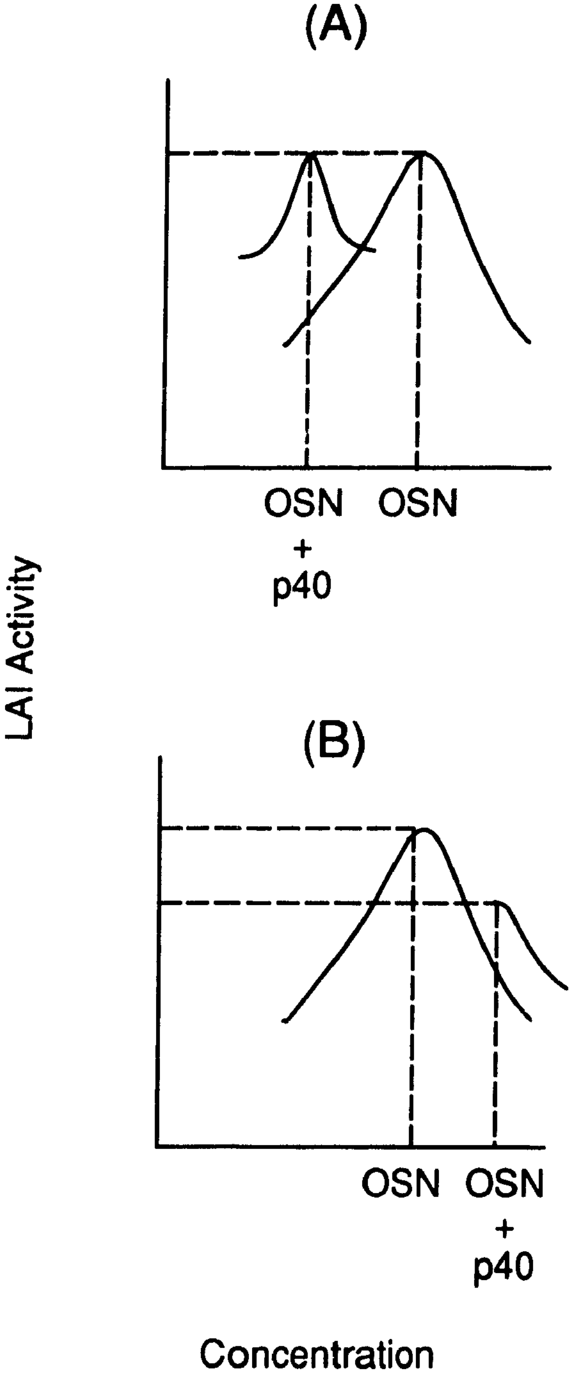


Figure 3

of the combined p40 and OSN increases, the combination will, in fact, diminish the LAI response (Figure 3B).

The above theory provides a basis for explaining the organ-non-specific LAI activity of the pure mycoplasma extract and the OSN activity of infected lung extract when both contain p40. While it explains the mechanism that may underlie the lack of organ-non-specific LAI activity of the infected lung extract, it does not explain the OSN activity of purified p40 and p40 synthetic peptides.

9. Cross-Reactivity between Human Mycoplasma Proteins and p40

Since mycoplasmas are common parasites in animals and humans, there may be in vivo recognition of the mycoplasma antigenic substances by the immune system of the parasite host under conditions of immune activation during malignancy. In the past, as reviewed in the "Introduction", mycoplasmas have been repeatedly associated with tumors. However, this concept has not been generally accepted since there has been no consistent evidence in the literature that patients with tumors also host pathogenic mycoplasma species. Well known are the common interactions between mycoplasmas and humans with Mycoplasma pneumoniae, which causes upper respiratory infections and atypical pneumonia (237), Mycoplasma hominis, which causes genital infections (239), and Mycoplasma genitalium, which causes genitourinary infection (188). Recently mycoplasma infections have been found in AIDS patients, but their role in the illness is still undefined (233). Inconclusive evidence has implicated mycoplasma species in ankylosing spondylitis (269) and chronic septic arthritis (269). However, mycoplasma species such as Mycoplasma fermetans inhabit the body, but do not act as pathogens (233). Either pathogenic and/or non-pathogenic strains of mycoplasmas inhabiting the body could be targets of non-specific activation of immune reactivity that is elicited by the effect of antigenic cancers (277). An immune response to tumor antigens may initiate a non-specific immune response to other antigens in the tumor host (94,274,277). Non-specific activation of cancer patients' immune cells (63,277) could result in the upregulation of existing immunity to the mycoplasmas. For the non-specifically activated immune response to recognize p40 in vitro, the protein epitopes which serve as targets in vivo must cross-react with p40 of Mycoplasma hyorhinis which is a swine pathogen. Cross-reactivity between proteins of various mycoplasma species including Mycoplasma hyorhinis (309), Mycoplasma pneumoniae (188,237), Mycoplasma genitalium (188, 237), and Mycoplasma fermetans (309) is known. A 74 kD protein of Mycoplasma hyorhinis has been reported to cross-react with proteins of mycoplasma strains infecting humans (309) but, to my knowledge, p40 has not been assessed for cross-species reactivity. Human sera against Mycoplasma hominis recognized a 40 kD band in western blots of this strain (188) and mAbs recognize a 38 kD protein on extracts of Mycoplasma genitalium (237). These are two examples of human mycoplasma proteins of similar size to p40 which might have homology and share cross-reacting epitopes.

The pure Mycoplasma hyorhinis extract tested in LAI assay reacted non-specifically with leukocytes from cancer patients with tumors of various organs. This extract contained a mixture of proteins, including p40, some of which may be identical with mycoplasma proteins from other species and be recognized in vivo by activated T cells or Ab-dependent monocytes. In normal subjects, leukocytes did not react to mycoplasma extract as assessed by their LAI activity.

10. OSN as a Co-isolate with P40

Another possible explanation for the OSN activity of mycoplasma contaminated cell lines is that purified p40 is contaminated with OSN. Evidence for this possibility is that the unbound material that eluted from the a-p40 affinity column, although devoid of p40, exhibited OSN activity (6,279). This suggests that the material applied onto the affinity column contained cellular OSN (OSN derived from human cells) which was not retained by the mAb. Moreover, when p40 was isolated by mAb affinity chromatography, analysis of the affinity purified p40 indicated that other minor components of 32, 25 and 13 kD also co-isolated. It was believed that they represented proteolytic products of p40 because they immunoprecipitated with mAb a-p40. In the present study p40 was expressed as a single band on western blots bound by the mAb a-p40. However, the pAbs a-p40 and a-peptide #2 bound other components of lower molecular weights of approximately 28-30 kD, 20-25 kD and 13-15 kD. The molecular weights of the bands bound by the pAbs are similar to those originally reported to be precipitated by mAb a-p40. Since these molecules are not bound by the mAb a-p40 in western blots, it suggests that they may not be the same molecules as those immunoprecipitated with the mAb. However, the possibility cannot be excluded that these molecules are not bound by the mAb in western blots because of denaturation, which did not occur during immunoprecipitation. Whether the molecules bound by the a-p40 and a-peptide #2 pAbs represent cellular OSN or fragments of OSN is not known. If they are cellular OSN, then they do not cross-react with the mAb a-p40, but they do cross-react with pAb a-p40 and pAb a-peptide #2. This is another instance where the a-p40 and a-peptide #2 pAbs cross-react with molecules in OSN active preparations which do not express the mAb epitope, in spite of the fact that the two pAbs were raised against p40 or a synthesized fragment of p40. It is more likely that the bands in the western blots represent proteolytic fragments of p40 which do not express the mAb epitope rather than being cross-reacting OSN. Nonetheless, the mere presence of molecules other than those carrying the mAb a-p40 epitope (which identifies p40-OSN) in affinity purified p40 preparations suggests that a contaminant might have co-isolated. This theory suggests a possibility for cellular OSN involvement in the p40 preparations, but it does not explain the OSN activity of the p40 synthetic peptides.

CHAPTER V

SUMMARY

The results of the present study indicate that OSN are expressed on cancer tissues and are also expressed on at least one cultured lung cancer cell line. P40-OSN was identified to be a mycoplasma protein by three criteria: 1) DNA sequence analysis. 2) Loss of expression from cells cured of mycoplasma infection and reappearance after reinfection of the same cells. 3) Comparison of p40 cDNA and amino acid sequence with published observations from other laboratories (56,240). Moreover, the overall findings, in the present study and in the literature, ascribe two properties to mycoplasma p40: the property of an OSN and that of a metastasis mediating molecule (56, 240). Both properties are associated with malignant transformation of mammalian cells. Herein, certain speculations were made about the OSN character of p40. However, the results of the present study were unable to resolve the problem of how a single protein derived from a procaryotic organism can acquire the ability to act as two different OSN molecules expressed by human cancer cells. Resolution of the p40-OSN question requires certain additional information.

1. Establishing whether there are essential differences in the DNA sequence of p40 isolated from: 1) pure mycoplasma extract, 2) lung cancer cells and 3) colon cancer cells and, if so, how these differences could relate to the OSN character of the molecule.
2. Assessing whether p40 or a different molecule in the crude mycoplasma extract is responsible for the organ-non-specific LAI activity. Affinity purification of p40 from pure mycoplasma culture and subsequent LAI testing might resolve this point.
3. Resolving whether the 40 kD molecule expressed by fresh tissues and detected by polyclonal a-p40 Ab is an OSN. Similarly, resolving whether the 40 kD molecule expressed by non-infected tumor cell lines and detected by the polyclonal a-p40 Ab, is an OSN. If this is the case, these molecules should be analyzed for homology with each other and with mycoplasma p40.

4. Establishing whether infection of cultured lung cancer cells with mycoplasma from colon (and vice-versa, i.e. infection of cultured colon cancer cells with mycoplasma from lung) alters the organ-specificity of p40. This would indicate a potential influence of mammalian cell components on the mycoplasma protein expression.

CHAPTER VI

STATEMENT OF ORIGINALITY

1. P40 had previously been identified to be an OSN. In this study I established that p40 was in reality a known mycoplasma transport protein.
2. I used a detergent extraction procedure and western blotting for detection of p40 in (i) cultured cell lines, (ii) fresh cancer and normal tissues, and (iii) fresh fetal tissues. P40 was expressed by some cultured cancer cell lines, but p40 was not expressed by any of the fresh tissues.
3. I established that p40 expression was associated with mycoplasma infection of cultured cancer cell lines. I accomplished this by monitoring the cells for mycoplasma contamination and concomitant testing of cell extracts for p40 expression in western blots. P40 was only expressed by mycoplasma-contaminated cell lines.
4. P40 expression by the contaminated cell lines was not affected by routinely used antibiotics.
5. I established that the mycoplasma-free lung cancer cell line NCI-H69 exhibited OSN activity which, due to absence of mycoplasma contamination, was distinct from p40. Thus it was established that at least one cultured cancer cell line expresses OSN.
6. Pure mycoplasma extract containing p40 was shown to express non-organ-specific LAI activity with cancer patients' leukocytes but did not exhibit LAI activity with normal persons leukocytes.
7. A 25 kD molecule of unknown function was identified as a common protein of human cultured cell lines and normal, cancer and fetal tissues.

CHAPTER VII

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