STUDIES ON TUMOUR ANTIGENS ASSOCIATED WITH

HUMAN BRONCHOGENIC CARCINOMATA

by

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B.Sc. University of British Columbia, 1970

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In the Department

of

Microbiology

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June, 1975

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ABSTRACT

Bronchogenic carcinomata of various pathologies were obtained either at post-mortem or post surgically for the purpose of this study. Normal lung tissue as control material was obtained from individuals who died from non-malignant causes. Antigenic materials were extracted from both normal and tumour tissue with 3.0 M KCl in order to determine whether bronchogenic tumours contained either tumour specific transplantation antigens (TSTA - cell surface antigens recognized as non-self by the host immune system) or tumour associated antigens (TAA - components not found in normal tissue which may or may not be antigenic in the host but which are antigenic in a heterologous species).

Attempts to identify tumour associated antigens by direct chemical comparisons between normal and tumour extracts were made using disc gel electrophoresis in conjunction with ion exchange and gel filtration chromatography of the extracts. A tumour associated antigen was detected in this way but it was not found in other tumour extracts and therefore its significance was limited.

Autoradiographic studies using sections of tumour or normal lung, patients' or normal sera, and ^{125}I-labelled anti-human Ig indicated that patients with bronchogenic carcinoma may produce antibodies reactive with their own and other tumours. Skin test reactions to fractions of homologous tumour extract also suggested that patients may react to tumour antigens, but neither of these methods were found to be suitable as an assay for tumour antigens.

Efforts to raise antisera against tumour antigens by rendering rabbits neonatally tolerant to normal lung antigens followed by immunization with tumour extract proved unsuccessful. Greater success was obtained by

hyperimmunizing rabbits to tumour extracts and passing resultant antisera through solid immunoadsorbents of normal lung extracts. The absorbed antisera, in many instances, reacted specifically with antigens in tumour extracts.

Broad studies using immunodiffusion with absorbed antisera and a panel of tumour extracts indicated that bronchogenic carcinomata may contain at least two tumour associated antigens which cross-react widely. One of these antigens appeared to share properties with an antigen in foetal lung extracts, but was neither carcinoembryonic antigen nor α_1-foetoprotein. When summarized, the results suggested that some TAA were associated with tumour pathology and possibly aetiology; squamous cell, oat cell and anaplastic carcinomas (carcinogen induced) appeared to contain related TAA which were distinguished from TAA in adenocarcinomas and alveolar cell carcinomas (not known to be associated with carcinogens).

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ACKNOWLEDGEMENTS

INTRODUCTION

I.   Historical Development

The attitude towards immunity and malignant disease has changed
radically during this century. As pointed out by Fairley (34), there have
been three phases in the approach to tumour immunology: the optimistic,
followed by the pessimistic, and finally the realistic. The optimistic phase
occurred around the turn of the century. Lymphocyte infiltration of tumours
was found to be important in the rejection of transplanted tumours. Later,
Woglom (140) in his literature review of six hundred papers published between
1913 and 1929 concluded: "Nothing may be hoped for at present in respect to
a successful therapy from this direction". The pessimistic phase was ushered
out with the breeding of syngeneic mice. Only with syngeneic animals could
the presence of tumour-specific transplantation antigens (TSTA) be proven,
as opposed to allo-transplantation antigens, thus suggesting the feasibility
of immunization against some types of carcinogenesis. TSTA can be defined as
those antigens against which the immune mechanisms of the host are directed
in the rejection of syngeneic tumours. Gross (39) and then Lewis and
Aptekman (69) demonstrated that tumour immunity could be induced in syngeneic
animals to transplanted isologous tumours. However, these results could also
be explained either in terms of heterogeneity of the inbred animals or a
mutation in the tumour lines as a result of transplantation. Foley (35)
subsequently showed that tumour immunity could be induced by tumour cells
which had not been repeatedly transplanted. Prehn and Main (102) proved that
tumour immunity was not a result of heterogeneity of the inbred animals by
showing that only tumour tissue and not normal tissue from the same inbred
animal, could immunize against isologous tumour challenge.

II. TSTA in Human Neoplasms

It has not been proven that TSTA in man exist, although there is
evidence supporting this possibility (20, 34, 65). This evidence may be
summarized as follows:

1.   Patients with abnormal immune systems have an increased
incidence of malignant diseases (37). For example, patients with hypogamma-
globulinaemia have an increased incidence of leukaemia and lymphoma (95),
autoimmune haemolytic anaemia is not infrequently associated with chronic
lymphocytic leukaemia and lymphosarcoma (27), and renal transplant patients
who have been treated with immunosuppressive drugs have a higher than normal
incidence of reticulosarcomas (63). The evidence is consistent with the
requirement for an intact immune system to effect the elimination of malig-
nant cells arising in the body, as postulated in Burnet's (20) immune
surveillance theory.

2.   Southam (122), by injecting autochthonous (from the same
individual) tumour cell suspensions, found that subcutaneous tumour nodules
would be produced if the cell dose were high enough. This dose is related
to the immunological status of the host.

3.   Histological evidence (14) demonstrates that for carcinoma of
the breast and stomach, lymphoid infiltration and sinus histiocytosis in the
regional lymph nodes are associated with a favourable prognosis.

4.   Specific lymphoid cell reactivity against autochthonous cultured
tumour cells can be detected in vitro by colony inhibition techniques (47).

Peripheral blood lymphoid cells from patients even with progressive malignant disease, can have a cytostatic effect upon either autochthonous or tumour cells of a similar cell type to the patient's neoplasm. These observations indicate not only the presence of TSTA on human neoplasms but also the possibility that common antigens may be present on tumours of similar morphology.

5.   Some cancer patients have antibodies in their serum, which appear to be specific for their tumour. Burkitt's lymphoma patients (64) can produce at least two specific populations of antibodies: one against the Epstein-Barr virus, thought to be responsible for the disease, and one against an antigen present on the tumour cell membrane. Many patients with other types of tumours have anti-tumour antibodies in their sera, either in the form of blocking factors or cytotoxic antibodies (48).

6.   Everson (33) has reported 130 cases of diagnosed cancer disappearing spontaneously or following trivial treatment. The tumours which undergo spontaneous remission will often induce inflammation in the vicinity of the tumour lesions upon immune stimulation. The simplest explanation for these phenomena is anti-tumour immunity.

III.   The Nature of TSTA and Tumour-Associated Antigens (TAA)

TAA can be defined as any material predominantly associated with neoplastic disease, which can be detected by an immunological method. This material is not necessarily antigenic in the tumour-bearer and includes TSTA. Information on the nature of TAA can possibly shed light on the little-known process of oncogenesis.

1.   Cell Membrane Chemistry

Emmelot (32) has reviewed cell membrane chemistry with emphasis on
the comparison of different tumour cell membranes with each other and normal
cell membranes for their content of various phospholipids and carbohydrates.
There was great variation between different tumours but no tumour-specific
changes in content were observed although reaction with plant agglutinins
was frequently increased (81).

According to the fluid mosaic model of the structure of membranes (119),
the surface membranes of cells are dynamic fluid structures in which membrane
components may migrate.

Malignant transformation is closely associated with a greatly
increased ability of the cells to be agglutinated by plant lectins. Lectins
(such as concanavalin A) are capable of agglutinating cells by binding to
specific sugar residues (such as α-D-glucopyranosyl or α-D-mannopyranosyl
residues). It has been suggested (119) that a difference between normal and
transformed cells could be the membrane fluidity. The lectin receptors
would be more mobile and produce clusters so that the lectin molecules can
become locally concentrated and thus able to agglutinate the cells.

Mild treatment of normal cells with proteolytic enzymes can induce
agglutinability similar to that of transformed cells. Perhaps the most
obvious explanation for this finding is that the enzyme cleaves protein and
exposes lectin binding receptors which were previously buried in cryptic
sites. However, it is also possible to explain this finding in terms of
fluidity differences between normal and transformed cells (119). The proteo-
lytic enzyme would preferentially cleave polar peptides from the membrane
proteins, thus making the proteins more hydrophobic and resulting in
aggregation of lectin receptors.

It is possible to make firmly established tumours regress in syngeneic mice if the host is challenged with tumour cells which have been reacted with Vibrio cholerae neuraminidase in vitro (118). The effect is augmented if non-specific immunostimulants such as Mycobacterium bovis (strain BCG) is injected also. Tumour regression could not be induced with cells treated with inactivated neuraminidase or with tumour cells of a different type, suggesting that the neuraminidase is making the cells more immunogenic. Neuraminidase cleaves a portion of the sialic acid from cell membrane gangliosides. This results in a decrease in negative charge and could lead to increased immunogenicity by decreasing the steric hindrance to antigen, or increasing cell deformability or susceptibility to phagocytosis (118).

Concanavalin A treated tumour cells can induce tumour regression similar to neuraminidase treated cells. There is considerable parallelism between the effects of these agents even though concanavalin A does not remove groups from the membrane (like neuraminidase) but is bound to specific sugar residues on the membrane. Therefore, it is possible the increased immunogenicity induced by these agents is via the same mechanism. Both agents have a mitogenic effect on lymphocytes. If mitogens were created on tumour cell membranes, reactions with immunoreactive cells could be supplemented. Both agents could disrupt cell surfaces non-specifically, thereby exposing neo-antigens (118). It is also possible that both agents could reduce steric hindrance to antigens by different mechanisms: neuraminidase by removing sialic acid residues, and concanavalin A by aggregating glycoproteins and leaving the antigens exposed.

A wide variety of enzymes has been tested for the ability to modify tumour cells and induce tumour rejection (101). Besides neuraminidase, only trypsin had any effect, and this was augmented by reacting the trypsinized cells with neuraminidase (neuraminidase followed by trypsin gave no protection).

It is possible to gain a further understanding of membrane chemistry by studying the effects of modifying membranes. Besides modification with enzymes, chemicals capable of reacting with specific chemical groups have also been used. Prager and Baechtel (101) have reviewed several catagories of specific chemical group modifications and their ability to augment tumour rejection. In general, only the addition of small, non-haptenic reagents are effective. Alteration of sulfhydryl groups with reagents such as iodo-acetate have been shown to make murine tumour cells more immunogenic. Sodium periodate, which cleaves certain monosaccharide units, results in a decrease or loss of tumour cell immunogenicity. (It is of interest to note that like concanavalin A and neuraminidase, lymphocytes reacted with periodate undergo blastogenesis as if they were reacted with a mitogen. However, only tumour cells reacted with concanavalin A or neuraminidase can induce tumour reject- ion (118), suggesting that the increased immunogenicity of these agents is not by the creation of mitogens on tumour cell surfaces).

Reagents capable of reacting with amino groups have given a range of results. Reaction with fluorodinitrobenzene has generally not induced tumour rejection while reaction with formaldehyde can frequently give some protection (101). Tumour extract reacted with diketene has been shown to induce tumour rejection (70).

Because of the interdependence of membrane components for their location, configuration and exposure, changes in tumour antigenicity induced by specific reagents does not necessarily mean that these groups are part of the antigen itself. Most of these studies (101) have been done by modi- fying tumour cells and looking for increased immunogenicity, whereas more definitive studies on the nature of tumour antigens could be gained by trying to diminish the antigenicity of soluble tumour antigen.

2.   TSTA and Normal Histocompatibility Antigens

TSTA are almost certainly associated with the cell membrane. It is difficult to conceive how intracellular antigens could function in immunological rejection, since the tumour cell membrane is largely impermeable to antibody, and cell-cell contact interactions are thought to be required for tumour cell killing mediated by sensitized lymphocytes (5). Normal histocompatibility antigens are known to reside on the cell membrane (58) and are capable of stimulating an immunological response analogous to (although more effective than) that stimulated by TSTA. Methods used for the extraction and purification of normal alloantigens have been successfully applied to studies of TSTA. We might therefore expect to find similarities between normal alloantigens and TSTA. Considerable work has already been done on the chemical characterization of normal alloantigens. Mouse H-2 alloantigens have been extracted from membrane fractions by papain, purified, and found to be glycoproteins with molecular weights of 65,000 to 75,000, 90% of which is protein and 10% is a variety of different carbohydrates (117). Although the antigenic determinants have not yet been characterized, their susceptibility to protein denaturants and prolonged proteolytic enzyme digestion implies that the protein configurations are important. Treatment with different glycosidases produces no loss in antigenicity. The loss of antibody binding ability by chemical modifications of the glycoprotein molecules with reagents specific for tyrosine residues or amino groups provides further evidence that the protein structure determines H-2 reactivity (96). Guinea pig transplantation antigens (MW 15,000) extracted by sonication contain no more than one carbohydrate residue per molecule and HL-A antigens (MW 34,600) extracted by 3M KCl contain no more than two carbohydrate residues (107).

Singer and Nicholson (119) have categorized membrane proteins as integral or peripheral. Briefly, peripheral proteins are bound by weak, non-covalent forces and are easily solubilized free of lipid. Integral proteins are much more closely associated with membrane lipids, more difficult to dissociate from membranes, and then usually insoluble or aggregated. These proteins possibly have appreciable α-helical content and a significant portion of their amino acids buried in lipid by hydrophobic interactions. It has been suggested (133) that H-2 alloantigens are integral proteins. However, this is only on the basis of the close association of H-2 antigens with membrane lipid as determined by the lack of shedding of these antigens by the cells. This was compared to the rapid shedding of peripheral molecules such as immunoglobulin and Θ antigen. It is also possible that HL-A antigens are integral proteins in spite of the relatively large quantities of HL-A antigens detected in the spent medium of cultured lymphocytes (99). This could be due to membrane fragments of dead cells rather than shedding from viable cells.

HL-A antigens solubilized by papain have a molecular weight of 48,000 and can be dissociated into 33,000 and 11,000 MW fragments. The smaller fragment is β_2-microglobulin which has also been found in urine and is the same for all HL-A specificities studied (126). It has 28% sequence homology with parts of the γ chain of IgG-1 and is not required for the reaction of HL-A antigen with antibody (99). (This protein is also suspected as a possible link between histocompatibility and immune response gene loci (99,103)). β_2-microglobulin is not essential for the binding of HL-A antigens to membranes because HL-A antigens (33,000 MW) can be solubilized with papain, from the Daudi cell line. This cell line does not produce or contain β_2-microglobulin (103). Thus, it appears that the 33,000 MW. fragment (which contains the HL-A antigenicity) could be the antigenic part

of the protein bound to the cell membrane and cleaved by papain.

HL-A antigens solublized by detergent (NP-40) have a molecular weight of about 150,000 which upon further purification and removal of the detergent is reduced to about 70,000. The β_2-microglobulin is associated with these fractions. If this smaller fragment is reduced and alkylated, the predominant component is indistinguishable in size (by gel chromatography) from IgG heavy chains (24,103). This evidence suggests that HL-A antigens may form a complex similar to immunoglobulin molecules. Similar findings are reported for H-2 antigens (103).

The 33,000 MW fragment which retains the HL-A antigenicity has a very similar amino acid analysis, regardless of the HL-A type. However, some HL-A specificities can be separated by isoelectric focusing (126). Therefore, the wide range of HL-A antigen specificities is determined by minor changes in the protein.

Characterization of TSTA is still at a primitive stage of development and therefore it is difficult to draw sound analogies between them and normal alloantigens. There is evidence that in rat hepatomas, protein, carbohydrate, and lipid moieties are all involved in the arrangement of the TSTA (MW 55,000) in the cell membrane. However, the purified antigenically active component is protein with only a trace of carbohydrate (5).

3.   Mutational Basis for the Origins of TAA

In order to explain the difference in antigenicity between spontaneously arising tumours and methylcholanthrene induced tumours, Prehn and Main (102) suggested that a potent mutagen would be more likely to produce simultaneous antigenic and neoplastic changes than the agent(s) responsible for the induction of occasional spontaneous tumours. The expression of TSTA was

not concommitant with the malignant process _per se_ since one of the tumour lines lost its antigenicity after numerous transplants. The possibility that TSTA represent a replacement of or modification of normal alloantigens is unlikely since the H-2 alloantigens from mouse spleen and tumour cells are indistinguishable (79). However, it is possible that TSTA might result from manipulations on weak histocompatibility gene loci (58).

Since a large portion of a cell's genetic material is probably involved in regulatory functions, a genetic change capable of interfering with the control of cell division could also interfere with protein synthesis control. This genetic change could occur at a location in the cell's DNA specific for the particular agent and result in TSTA specific for the onco- genic agent. Oncogenic virus induced tumours possess common group-specific TSTA (62). Alternatively, the genetic change could occur at random, in which case only rarely would tumours possess common TSTA. Tumours induced by chemical carcinogens or physical agents only rarely cross-react immuno- logically (62).

The genetic changes associated with oncogenesis could lead to production of molecules not normally produced, an increase in production of normally produced molecules or even a change in existing molecules. Neoantigens associated with oncogenesis could be either immunogenic or non- immunogenic in the tumour bearer. Even molecules normally produced by a cell could be immunogenic if more was produced or if exposed to the immune system in a different manner (40). Examples of TAA which can also be detected in non-malignant conditions are the various polypeptide factors associated with cancers, particularly lung cancer (92), and the T-globulin which can be detected in the sera of cancer patients and pregnant women (125). Another TAA in normal tissue is the brain-associated tumour antigen. Lymphocytes from cancer patients are capable of responding specifically

to heterologous basic protein of myelin (30). A similar antigen can be
detected on rat tumours by immunofluorescence with xenoantiserum against
rat brain. This antigen is not the theta antigen (129). Other TAA could be
produced by normal tissue in response to neoplastic disease. An example of
such a substance, although not tumour specific, is alpha 1-antitrypsin.
High levels can be detected in the serum of patients with obstructive lung
disease and lung cancer (44).

The carcinogens themselves do not constitute TAA (at least in experi-
mental animal tumours) since the tumours can be transplanted over many
generations without a loss of antigenicity.

4.   Tumour-associated Foetal (embryonic) Antigens

Some TAA can also be found in normal foetal tissue. A suggested
name for them is 'onco-foetal antigens' (OFA) (1). OFA include carcino-
embryonic antigen (CEA), originally thought to be specifically associated
with human colon carcinoma, alpha-foetoprotein (AFP), associated with rat
and human hepatomas, and foetal alkaline phosphotase (Regan iso-enzyme).
These TAA could be produced by the tumour cells as a result of derepression
associated with oncogenesis or by normal tissues in response to neoplastic
growth or tissue damage. Although originally thought to be highly specific
for tumours of the digestive tract and hepatomas (1), CEA and AFP respect-
ively, were found widely associated with non-malignant diseases when highly
sensitive radioimmunoassays were developed. While no longer thought to be
useful for the mass-screening of cancer, ascertaining the level of these
foetal antigens can be useful clinically by aiding prognostication (82,143).

Other OFA have been found in experimental animals with tumours.
These tumours also possess TSTA which are distinct from the OFA in both

rats (8) and guinea pigs (17). OFA can be associated with lymphocyte
mediated cytotoxicity (104) and can stimulate cell-mediated immunity in
cancer patients as detected by delayed cutaneous hypersensitivity (52).
Thus it appears that OFA may function as TSTA as well as serving as aiding
the clinician as an index of neoplastic disease.

5.   Viral Antigens

Although it is not yet proven whether the viruses associated with
human cancers are oncogenic or opportunistic, there is considerable evidence
to suggest that viruses can play a causative role in cancer. Much of this
evidence is based on immunological studies on the viral-associated antigens
and antibodies found associated with certain tumours. Whether viruses are
a primary or secondary oncogenic agent, or merely a neoplastic tissue-specific
passenger, the virus-associated antigens and antibodies are important to
study like any other TAA. For this reason, I will summarize the types of
antigens associated with the viruses which are most likely oncogenic in
humans.

Of the DNA viruses, Herpesviruses are most likely to be oncogenic in
man. The Epstein-Barr virus (EBV) is closely associated with Burkitt's
lymphoma and nasopharyngeal carcinoma (80). The virus is very common in
humans and many people develop antibodies against the viral capsid antigen.
However, patients with Burkitt's lymphoma have 5 to 10 times as much antibody
as do matched normal controls. Early antigens are another EBV-related
antigen complex and are so-called because they appear early in the viral
replication cycle when cultured cells are infected with EBV. Antibodies
against early antigen are rarely detected in healthy people but reach high
levels in patients with Burkitt's lymphoma. If, after chemotherapy the

antibodies disappear or decline, the prognosis is generally good; if the antibody concentration does not decline, the prognosis is generally poor. These antibody populations may be present even though no EBV particles can be detected. The antigens against which these antigens are directed must either be or have been present in the patient, and are probably coded by the viral DNA which can also be detected (80). Burkitt's lymphoma cells also produce an EBV associated nuclear antigen that may be analogous to the T-antigens produced in cells infected with oncogenic animal viruses such as SV40. T-antigen, an early viral gene product, is present in the nucleus of a trans-formed cell but not in the virus itself (2). Burkitt's lymphoma patients can also produce antibodies against this nuclear antigen. A cell membrane antigen can be detected in biopsies from these patients as well as specific antibody against this non-virion antigen (49).

Other herpesviruses also are possibly oncogenic. Herpes simplex (hominis) viruses types 1 and 2, cause most oral and cutaneous herpetic infections, and genital infections, respectively. Patients with carcinoma of the uterine cervix frequently have in their serum, antibodies which react with early antigen from type 2 infected cultured cells.

There have also been reports that patients with lip and cervical carcinomas have antibodies capable of reacting with herpesvirus non-virion antigens (112) and that the tumours contained these antigens (54). These findings could provide strong evidence for oncogenesis by herpes simplex viruses, but the results were later not confirmed (111).

IV.   TSTA and Tumour Rejection

If many tumours possess TSTA and the host organisms have immunity against these antigens (45), why are the tumours not rejected? Burnet's

immune surveillance theory (20) suggests that most malignant cells are rejected. Tumours that do arise have escaped the immune surveillance by a defect in the immune response of the host to the malignant cells. On the basis of the finding that tumours which arise in immunosuppressed mice are no more antigenic than tumours arising in normal mice, the immune surveillance theory has been challenged, although the implications are not fully understood (88). There could be a number of reasons for a defective immune response which allows malignant cells to escape immune surveillance and tumours to progress and metastasize.

1.   The TSTA produced could be weak, or at a low concentration on the tumour cell membrane (109). This could impair immunization and/or killing by sensitized lymphocytes or cytotoxic antibody. It is also possible that there is only a small number of lymphocytes capable of responding to tumour antigens.

2.   The carcinogen involved may be immunosuppressive. Some oncogenic viruses have been shown to depress cell-mediated immunity (90) and many chemical carcinogens are also immunosuppressive (5).

3.   The neoplastic cells could produce an immunosuppressive factor (19).

4.   The immune system could be temporarily incapacitated by a cause unrelated to the malignancy. Thus, in renal transplant recipients, because of the required immunosuppression, cancer could be an iatrogenic (related to treatment) disease (97).

5.   The individual may have a genetic incapacity to respond immunologically to certain groups or types of TSTA.

6.   Neonatal tolerance to TSTA can be developed. Tolerance has

been demonstrated in the case of several tumours induced by vertically

transmitted viruses, such as lymphomas induced by the Gross virus, and

leukaemias induced by the Moloney virus (62). By transplanting the tumours

into adult animals of the same inbred strain, the tumours were shown to

possess TSTA and were rejected, while animals infected neonatally with the

viruses were incapable of reacting against them.

7.   A simple explanation for the escape from immune defences is

that the immune system cannot cope with the rapid growth of the tumour.

Closely associated with rapid growth is the tumour load. As the tumours

get larger, anti-tumour immunity and even general immunity decreases (139,

45). This could be related to any of the factors already mentioned.

V.   TSTA and the Roles of Antibody in Tumour Rejection.

1.   Immunologic Enhancement

Immunological enhancement of tumour development has been a subject

of much study and controversy. This phenomenon was originally observed as

enhanced tumour growth in animals which had been either actively immunized

with the same tumour or passively immunized with hetero or isoantiserum.

There were two main mechanisms suggested to explain enhancement. The first

suggestion was that contact with antibody stimulated tumour growth either

directly or by release of stimulatory cell products. The other suggestion

embraces the 'blocking' phenomenon. Antibody binding to the tumour cell

membrane would 'wall off' the tumour antigens so they would be inaccess-

ible to sensitized lymphoid cells capable of tumour cell killing (60),

The Hellstroms (46) demonstrated immunological enhancement in vitro using the colony inhibition assay. Briefly, the method involves the plating of target tumour cells and counting the tumour cell colonies which arise in the presence and absence of specifically sensitized lymphoid cells. Incubation of the target cells with lymphocytes from animals or patients bearing even progressively growing tumours of the same types as the target cells, will frequently result in an inhibition of colony formation. Lymphocytes from animals which have not had a tumour or from animals with a different type of tumour, do not have this effect, thus demonstrating immunologic specificity. If serum from the tumour-bearing donor is added to the target cells, the cytostatic effect of the sensitized lymphocytes may be abrogated. The factor in the serum which results in the inhibition of colony formation is called 'blocking factor' and is specific for the particular tumour-lymphocyte system being used. The cytostatic ability of the lymphocytes was similar for tumour-bearing animals and for animals whose tumours were resected. Serum from animals which had become tumour-free, was frequently capable of inhibiting colony formation of the respective target cells in the presence of complement. This indicates that blocking factor may not be free antibody. Indeed, the relatively simple hypothesis that tumour-specific antibodies on their own may cause enhancement of tumour growth by shielding tumour cells from destruction by sensitized lymphocytes is no longer tenable in the light of recent experiments.

Blocking serum has been shown to exert its effect either upon the target cells or the sensitized lymphocytes. Blocking activity can also be absorbed out of serum by either target cells or lymphocytes and may be eluted from tumour cells at low pH. Blocking factor has been characterized to the extent that it is known to be a high molecular weight moiety. By ultrafiltration at pH 3.1, blocking factor can be dissociated into a large

(MW > 100,000) component and a small (MW < 100,000) component (121).
When mixed with target cells, neither component alone can block lymphocyte
mediated cytostatic effects, but significant blocking can be seen when the
two components are mixed. When mixed with sensitized lymphocytes, the
larger component, which is known to contain antibody, cannot block, but
the smaller component alone or the mixture can block. If blocking factor
were antigen-antibody complexes, it would also explain the disappearance
of blocking factor upon tumour removal and the appearance of complement
dependent cytotoxic antibody (121). When rat hepatoma antigens and specific
antibodies are mixed with excess antibody and incubated with target cells,
lymphocyte cytostatic effects can be blocked. If the mixture contains
antigen excess or if tumour antigen alone is added to the blocking serum,
the blocking effect is not obtained (10). If antigen is pre-incubated
with the lymphocytes however, it can block by itself (11). Soluble tumour
antigen, either free or in immune complexes in antigen excess, have been
detected in the serum of sarcoma bearing rats. Antigen becomes undetectable
48 hours after complete tumour excision, which corresponds to the time of
disappearance of blocking factor and the appearance of cytotoxic antibody
(127). This evidence implies that the primary effect of blocking factor
is on the effector lymphocytes. With hyperimmune anti-tumour serum, it
appears possible to produce efferent blocking, but the central blocking
effect, which acts upon the lymphocytes, is probably more important (45).
In order to differentiate between tumour enhancement mediated by antibody
and that mediated by complexes or antigen, the terms 'blocking' and
'inhibition' have been used respectively (5).

It has been suggested (31) that antibody-mediated and antigen-
mediated immune suppression may act by a similar mechanism. The mechanism
has been postulated to be a concentration or cross-linking of antigen on

the lymphocyte plasma membrane. Thus a large dose of polymerized flagellin

from Salmonella adelaide or small doses of monomeric flagellin plus specific

antibody can produce immunological unresponsiveness (31). By analogy,

tumour-specific antibody could cross-link the antigens on lymphocyte surface

receptors and render them non-functional. It is postulated from this that

the antibody part of blocking antigen-antibody complexes acts by carrying

the antigen to the lymphocyte and cross-linking the antigen to the surface

receptors (45,110). This explanation of immunologic enhancement of tumour

growth is similar to an explanation of allograft tolerance in rats and mice

rendered neonatally tolerant to allogeneic lymphoid cells (110). Lymphoid

cells from the graft recipient were found to be cytotoxic to the tolerated

graft in vitro. Serum from the tolerant animal abrogated the cytotoxicity.

Similar findings were obtained from studies of tetraparental mice, which

had been established as chimeras by joining eight cell stage embryos from

different strains (98). Spleen cells from these mice were cytotoxic to

cultured fibroblasts from either parent strain, and serum from these mice

would block the mixed leukocyte reaction between the parental strains. The

blocking factor could be removed from the serum by absorption with anti-

mouse immunoglobulin. It would thus appear that immunologic enhancement

of tumour growth is probably a complex interaction between sensitized

lymphoid cells and circulating antibody-antigen complexes which possess

the ability to 'paralyze' those lymphoid cells which carry the potential

to destroy the tumour cells.

2.   Manipulation of the Immune Responses by Modified Antigen.

I have already described (Section III:1) findings that immunization

with syngeneic, chemically modified tumour cells, can induce tumour re-

jection. It is thought that modified antigens stimulate cell-mediated immunity but not humoral immunity against the tumour antigens, thus diminishing the possibility of tumour enhancement (101). There are two plausible models for this manipulation of the immune response.

A.   By conjugating fatty acids to proteins, Dailey and Hunter (28) have presented evidence that the properties of an antigen which induce cell-mediated immunity are not in the nature of the antigenic determinants but in the lipophilic nature of the whole immunogen. This mechanism is appealing because it explains the predominance of the cell mediated immune-response against cell membrane antigens. It is possible that the chemical modification (especially of sulfhydryl groups) results in a gross configuration change so that internal hydrophobic regions of the protein molecules are exposed.

The reason why reagents specific for sulfhydryl groups appear to work better than other reagents or modifications such as heat denaturation could be due to denaturation of the antigenic determinants.

In summary, this model suggests a preferential stimulation of cell mediated immunity by modification of parts of the antigen other than the antigenic determinant.

B.   The other model suggests that the antigenic determinants are modified so that the immune responses are directed against these altered determinants. Recognition is different for cell-mediated and humoral immunity, cell mediated immunity having a higher degree of cross reactivity (101). Immunization with chemically modified antigen can induce antibodies against the modified antigenic determinants and which do not cross react with native determinants, while cell-mediated responses do cross-react (113).

This evidence strongly supports the latter model, but the models are not mutually exclusive.

3.   Tumour Rejection Augmented by Antibody

All is not as simple as it may seem: the humoral response is not necessarily harmful in terms of tumour immunity to neoplasms. Sera from some mice and humans with spontaneous tumour remissions or successful tumour removal, have been shown not only to lack blocking factors, but to also contain factors capable of specifically 'unblocking' or abrogating the blocking effect of blocking serum (12). These unblocking antibodies can also be produced by immunization of Mycobacterium bovis (strain BCG) primed animals (even another species) with tumour cells. A possible explanation for unblocking is that the antibodies are directed against both antibody and antigen components of the blocking complexes. It has also been shown that sera from some animals and patients in remission are capable of increasing the cytotoxic effect of lymphoid cells (45). A possible mechanism is the 'arming', by antibody, of non-sensitized cells. Certain immune sera are capable of arming non-sensitized lymphoid cells so that they can specifically kill syngeneic tumour cells. The killing is mediated by B lymphocytes called 'K' (killer) lymphocytes.

Another possible benefit from a humoral immune response against tumours is complement dependent cytotoxic antibody (45). This often appears after tumour removal and is possibly blocking complexes minus tumour antigen. It may facilitate elimination of residual tumour cells by killing directly or by augmenting the cell-mediated response as in bacterial immunity.

We can now see that tumour development may be related to immuno-

suppression or non-responsiveness and/or a relationship between humoral
and cell-mediated immunity. These factors will be determined by properties
of the host, such as its genetic and acquired capacity to respond to certain
antigens, hormone levels and any number of properties that may be associated
with age: and properties of the neoplastic cells, such as location, rate
of growth, invasive potential and quality and quantity of tumour antigens.
Tumour antigens, then, are a likely spot upon which to focus attention in
the study of interactions of host and tumour.

VI.   Experimental Approaches to the Identification of TSTA and TAA

The identification of TSTA and TAA in animal models has been achieved
by a number of experimental approaches and techniques. The major techniques,
and several of their variations will be reviewed here.

.1.   Detection of Cell-Mediated Immunity to Tumour Cells.

The following procedures detect cell-mediated immunity in the tumour
bearer to tumour cells (or extracts) and therefore probably detect the
presence of TSTA.

A.   Animals of an inbred strain can be protected from tumour
development upon challenge with tumour cells if they have previously been
immunized with tumour cells, or certain tumour extracts. Also, animals from
whom small tumours have been removed or ligated are frequently immune to
subsequent challenge with a tumourigenic dose of tumour cells.

B.   Delayed hypersensitivity reactions to tumour antigens can be

measured by skin test or foot pad swelling, with tumour extract in tumour-immune or tumour-bearing animals.

C.   The reactivity of tumour immune lymphoid cells with TSTA can be assayed by several methods:

a. Adoptive transfer, or incubation of sensitized lymphocytes with tumour cells before inoculation into susceptible animals can protect the recipient from tumour development.

b. The blastogenic response can be measured when sensitized lymphoid cells are cultured with mitomycin-treated tumour cells or tumour extracts.

c. The supernatant from this culture (from part b) can be tested for immune lymphocyte mediators such as migration inhibitory factor (MIF) by incubation with guinea pig spleen cells for an indicator system.

d. The sensitized cells can be directly tested for the production of mediators such as MIF when incubated with tumour extract.

e. Sensitized lymphoid cells can be cytotoxic for target tumour cells when they are cultured together. This method is the basis of the colony inhibition and micro-cytotoxicity techniques.

2.   Detection of Antibodies Against TSTA or TAA.

Antigens detected by antibody mediated immunity would not necessarily

be expected to be TSTA since tumour rejection is probably mediated mainly by cellular immunity. However, it may be possible to detect either TAA or TSTA by these techniques.

A.   An Arthus reaction developing at the site of skin testing with tumour extract would detect antigens by complement dependent antibody response.

B.   Tumour-bearer serum may be tested for its ability to block lymphocyte-mediated tumour cell cytotoxicity at the lymphocyte level.

C.   Tumour-bearer or regressor serum, or serum from syngeneic animals immunized with tumour cells, or allogeneic or xenogeneic antisera may be tested for antibody against TAA by several methods. The latter antisera would need to have antibodies against normal tissue antigens removed.

a. Complement dependent cytotoxicity of tumour cells.

b. Fixation of complement upon incubation with tumour antigen.

c. Immune precipitate formation.

d. Immunofluorescence.

e. Autoradiography or radioimmunoassay.

3.   Detection of TSTA and TAA in Human Systems.

In the study of human tumour antigens, many of these methods are not

possible for ethical reasons and some are not practical for other reasons.
It is often difficult to get and store large numbers of human tumour cells
in single cell suspensions and retain their sterility and viability as
required for cytotoxicity reactions. Tumour cells must be continued by
long-term in vitro culture, which for many tumours is very difficult if not
impossible. In experimental animals, the tumour cell lines can be carried
by serial transplants in syngeneic animals. In human cell lines, where
only continuous in vitro cultures is possible, the probability that anti-
genic changes and selective pressures will change the characteristics of
the cell line, makes this approach questionable. With human experiment-
ation it is also very often difficult to provide a good control for many
methods.

Other problems in studying TAA are their apparently low concentrations,
relatively weak antigenicity and the large quantities of contaminating
normal cell antigens (58). Also, it is not known and difficult to ascertain,
whether antigen changes by neoplastic cells are qualitative or quantitative.

4.   The Importance of Detecting Human TSTA and TAA.

There are many possible uses for TAA which make their study and
purification worthwhile, in spite of the problems involved. Although some
of the uses of TAA described here (F,G.) are best applied in the study of
experimental animal tumours, the results of these studies must be evaluated
for correlation with human malignant disease before the cancer patient will
benefit.

A.   With purified TAA, a high titre of specific anti TAA would be
easy to obtain in experimental animals.

B.   Assays for TAA could serve as a diagnostic and prognostic aid

to clinical medicine.   Trace amounts of anti TAA could be detected by radio-

immunoassay.   Such an assay could also be used to screen large numbers of

people for the presence of early malignant disease.

C.   TSTA could be used for specific immunotherapy, or even in the

prevention of neoplastic disease by immunization.   These antigens would

possibly have to be modified to increase antigenicity or to preferentially

stimulate cell-mediated immunity.   Perhaps other TAA could be made immuno-

genic in the cancer patient, and function as TSTA.   Methods which could

augment the immunogenicity of TAA might also make normal tissue components

immunogenic, so purified TAA should be used to eliminate the possibility of

inducing autoimmunity.

D.   TSTA could be used to monitor the effects of immunotherapy on

the patients' immune responses.

E.   The presence of·common or related TAA could provide information

about the etiology of malignant disease.

F.   The relationship between antigen and antibody in blocking

factor could be studied, as could the relative importance of blocking factor

to cell-mediated defenses against cancer.

G.   Information about the biochemistry of TAA could supply a key

to understanding oncogenesis and the basis for the weak antigenicity.

5.   The Uses of Specific Antibodies Against TAA.

A.   Anti TAA could be used in a radioimmunoassay to detect trace amounts of TAA in serum for diagnostic and prognostic purposes. Such methods are already in use for the detection of alpha-feotoprotein and carcino-embryonic antigen although the tumour specificity is not as good as it was earlier hoped (82).

B.   The antibodies might be used for passive immunotherapy either directly if they fix complement and are cytotoxic, or by 'arming' them with cytotoxic drugs or ^{131}I. A better understanding of tumour antigen catabolism and tumour enhancement in humans could greatly facilitate such studies.

C.   If the antibodies were made radiopaque, they could aid radio-logical methods in locating and diagnosing a tumour.

VII.   Objectives of the Present Study

The original goal of this research was to attempt to purify TSTA from different bronchial carcinomas, and to study the immunochemical relationships of these antigens. Although these studies would be much easier to perform in experimental animals, the possible clinical value from studies of TSTA or TAA associated with human malignant disease justifies the concomitant experimental complexity. Studies on syngeneic animal tumours would not give information about naturally occurring immunochemical relationships between tumour antigens. Also, results of experimental animal tumour studies must be evaluated by human experimentation before the clinical significance can be established, and so far, animal experimentation has contributed little to

human immunotherapy.

There are a number of reasons for studying lung cancer. Lung cancer is a very common tumour, has been increasing rapidly, and has a very high mortality. At least in part, the poor prognosis of these patients is related to the late diagnosis, at which time the tumours are often large and metastasized. These factors are suitable for tumour antigen studies since large tumours would mean more tissue to use in the studies and one of the more promising aspects of tumour immunology is early diagnosis by radioimmunoassay for common TAA. The latter point is especially important since there are already high risk individuals, and in heavy cigarette smokers the disease is more difficult to detect. Active immunotherapy is probably more suitable for malignant diseases in which the major method of therapy is surgery (such as lung cancer) rather than irradiation or chemo-therapy which are immunosuppressive. Because the disease is so common and the mortality so high, even a slight improvement by immunotherapy would be significant and detectable.

VIII. Experimental Approaches to the Present Study

1. Extraction of the Tumour Cells

Because of the expected similarities between TSTA and alloantigens, a procedure known to be successful in the extraction of alloantigens was used throughout the present study. Three M KCl extraction is relatively easy to perform, can handle large quantities of tissue, and can produce a good yield of alloantigens from cultured cells (46). Less lipoprotein, which greatly interferes with protein purification, is liberated than by many other extraction methods. It takes several days to complete the extraction and

causes considerable destruction of internal cytoarchitecture of the cell with subsequent release of large amounts of nuclear DNA, nucleoprotein, and protein. However, these materials do not greatly interfere with alloantigen purification (106).

2.   Detection of TAA

A.   Our first attempt to show the presence of TAA in our tumour extracts was by producing an antiserum against the TAA. The method of Gold and Freedman (38) for the demonstration of CEA was chosen. A state of immunological tolerance to the antigens in an extract of normal human lungs, was to be induced in neonatal rabbits so that subsequent immunization with lung tumour extract would induce antibodies only against TAA. Autochthonous normal and tumour tissue extracts could have been used, but if TAA were released from the tumour (like CEA), we could also induce tolerance against these TAA. A pool of extracts from the lungs of six tumour free individuals (at autopsy) was used to minimize any chance of a reaction against histo-compatibility antigens. The antisera were tested by double diffusion in gel against the tumour and normal extracts.

B.   Another test for TAA was an in vitro assay of cell-mediated immunity of lung cancer patients. Peripheral blood leukocytes from cancer patients and healthy donors were tested for MIF production upon incubation with normal lung and tumour extracts. Antigens detected by this method would possibly be TSTA. Unfortunately, during the time that these experiments were being performed, we did not receive any surgical tumour specimens so that autochthonous leukocytes and tumour extract could not be tested.

C.   A direct, non-immunological test for tumour associated

materials was carried out by disc gel electrophoresis.   Normal lung and

tumour extracts were compared in this manner.   To lower the background

protein concentrations and further test for tumour specificity, normal

lung and tumour extracts were fractionated by DEAE cellulose chromatography

followed by Sephadex chromatography and the fractions were analyzed and

compared for the presence of tumour associated bands on disc gel electro-

phoresis.

D.   Instead of eliminating antibodies against normal tissue

components by the induction of tolerance, their removal from the serum of

rabbits hyperimmunized with tumour extract was tried.   Immunoadsorbents

were prepared by insolubilization of the normal lung extracts by the co-

valent coupling to agarose beads and by the polymerization with glutar-

aldehyde.   The absorbed antisera were tested for anti-TAA antibodies to

double diffusion in agar gel and immunoelectrophoresis against normal lung

and tumour extracts.   Some were later tested by indirect immunofluorescence

and immunoautoradiography on tissue sections.   The absorbed antisera were

used to test a wide variety of normal and tumour extracts for TAA by immuno-

diffusion.   These antisera were also used to test patients' serum samples

for TAA by immunodiffusion.

E.   Several cancer patients' sera were tested for the presence

of tumour antibody and/or tumour antigen-antibody complexes.   Sera were

tested against tumour extracts directly by immunodiffusion, for tumour

antibody.   The pH of serum samples was lowered to dissociated immune

complexes, and the serum chromatographed on an acidified Sephadex G-150

column to separate high molecular weight components (antibody?) from low

molecular weight components (antigen?). These components were tested against each other and the high molecular weight components were also tested against several tumour extracts, by immunodiffusion.

F.   The TAA detected in the extracts with the absorbed antisera are probably not TSTA because the immunodiffusion technique is not sensitive enough to detect the expected quantities of TSTA. The possibility that the TAA detected is foetal or embryonic antigen was tested two ways.

a.   Samples of the extracts were assayed for CEA by radioimmunoassay by the Division of Nuclear Medicine at the Vancouver General Hospital (VGH). Several extracts were also tested for α_1-foetoprotein by immunodiffusion against anti-α_1-foetoprotein donated by Dr. S. O. Freedman.

b.   Extracts of foetal lungs were tested by immunodiffusion against the absorbed antisera and compared with tumour extracts by disc gel electrophoresis.

G.   Since the immunodiffusion technique was being used near its limit of sensitivity, attempts were made to establish a more sensitive assay method. Indirect immunofluorescence techniques were tried first because of their widely reported use in the literature. An indirect immunoautoradiography technique was vigorously pursued because of the potential sensitivity of the technique and because it allowed accurate quantitation. It was also hoped to give an indication of the location of antigens within the tissue and cells by using tissue sections. Tissue sections were used instead of cell suspensions for several reasons:

single cell suspensions of normal lung and tumour tissue are very difficult

to produce, store and maintain reproducibility, whereas pieces of tissue

can easily be stored frozen and then made into cryostat microtome sections.

These methods were used to test the absorbed antisera, and cancer patient

sera and acid-dissociated high molecular weight fractions, for antibody

binding to the tissue sections.

H.   Another question raised about the TAA detected in the extracts

by immunodiffusion, pertains to their possible antigenicity in the cancer

patients.   The easiest and most direct way to test this (as well as being

another possible assay for TAA) is skin testing of cancer patients with

extracts shown to contain TAA by the immunodiffusion techniques.   Because

of the possibility of transmitting an infective agent to other patients or

healthy persons, only cancer patients were skin tested, and only with

extracts of their own tumours.   This restriction makes it impossible to

completely control this experiment.   However, the controls included skin

testing with recall antigens and DEAE cellulose fractions of the tumour

extracts.

MATERIALS AND METHODS

I.   Tissue Preparation and Extraction

1.   Preparation of Tumour Tissue

The tumours used in this study were either surgical or post-mortem

specimens.   Surgical specimens were made available through the arrangements

of Drs. P. Coy and J. W. Thomas (B.C.C.I.).   Dr. Coy also supplied the

surgical pathology reports.   Post-mortem specimens and autopsy reports were

supplied by Dr. J. Burton (V.G.H.).   All of the tissues were stored at $4^{o}C$

for less than 5 hours before being minced and extracted.   Macroscopically

normal tissue was trimmed from the tumours which were then cut into small

pieces (1-2 cm diameter), and minced with a tissue grinder at $4^{o}C$.   These

manipulations were carried out in a sterile box equipped with a germicidal

ultraviolet light, as much as possible.

2.   Normal Lung Tissue

Two types of normal lung tissue were used. Initially, macroscopically

tumour-free pieces of normal lung were removed from the lungs of cancer

patients, as distally as possible from the tumour.   Most normal lung tissue

was taken at post mortems of individuals free of malignant disease.   No

attempt was made to wash the tissue free of blood.

3.   Embryonic Lung Tissue

Lungs were removed from embryos at 12 to 20 weeks of gestation.

These specimens, from saline-induced abortions, were supplied by Dr. B. Poland
(V.G.H.). F-48 is a pool of seven embryonic lung extracts and F-49 is a pool
of four extracts.

4.   Tissue Extraction

The tissues were extracted by a method based on an allontigen extract-
ion method of Reisfeld et al (108). The first specimens were minced in
physiological saline, then solid KCl was added to a final concentration of
3.0 M. Later specimens (after C-30) were minced, and then mixed with a volume
of 3.5 M KCl equal to 6 times that of the tissue. Tissues were maintained at
$4^{o}C$ throughout the extraction procedure. Extraction of the tissue was accom-
plished by gentle agitation in the 3.0 M KCl for 18 hrs. The extracted tissue
was then centrifuged for 90 min at 16,000 g and the supernatant was strained
through gauze. The supernatant was dialyzed exhaustively against physiological
saline and centrifuged again. The extract was either sterilized by filtration
through a Seitz filter pad and stored at $4^{o}C$, or stored at $-20^{o}C$. The
extraction procedure was not performed aseptically.

Protein concentration was determined by the standard Lowry method (74)
and/or spectrophotometrically (67) (Appendix A). Because the results by the
Lowry method were usually with 20% of those by spectrophotometric methods
(for 20 samples which were compared), Lowry quantitations were not routinely
performed. Tumour, normal and embryonic tissue extracts were labelled with
the prefixes C-, N-, and F-, respectively.

The 3.0 M KCl extraction method was chosen for a number of reasons. The
method has been successfully applied to alloantigen extraction, is gentle,
reproducible, and can handle large volumes of tissue. The disadvantages are
the long time required and the requirement of a viable single cell suspension

for a high yield of alloantigens from cultured cells. Although much cytoplasmic protein is released besides membrane protein, it does not interfere with alloantigen purification like lipoproteins. Since my study includes TAA besides TSTA, cytoplasmic protein extraction may even be an advantage. It is thought that the chaotropic effects of KCl disrupt hydrogen bonding and break salt linkages (58).

Other common methods of extracting antigens have more serious disadvantages. Tissue homogenization in saline does not extract alloantigens. Even methods used successfully for alloantigen extraction were felt to have drawbacks. Low intensity sonication requires very carefully controlled conditions, is difficult to reproduce, and can only handle small volumes. Antigens extracted with detergents frequently aggregate and are only sparingly soluble in water. Papain can destroy alloantigens and leads to great variability (58).

II.   Neonatal Tolerization of Rabbits.

The method of Gold and Freedman (38) was used to render rabbits tolerant to the normal tissue components. A pool of 6 different normal lung extracts (N pool), was used for the tolerization. These extracts were from malignant disease-free post mortem specimens. Although we could have used an autologous extract from normal lung from cancer patients, we suspected that such normal extracts could contain tumour associated antigens (TAA), and thus induce tolerance to these TAA. Tumour antigens could be present in adjacent normal lung tissue if metastases not visible macroscopically were present, or if TAA were released into the blood, like carcinoembryonic antigen (CEA). This possibility of detecting histocompatibility differences between the heterologous normal extracts and tumour extracts, instead of tumour associated differences, was minimized by using a pool of 6 different heterologous lung

extracts. The use of pooled extracts also allows the preparation of a large volume of extracts and the use of the same normal extract for all of the experiments. Within 12 hrs of birth, 6 rabbits (U.B.C. animal unit) were injected with sterile N-pool (29 mg protein/ml). High protein concentrations were used so that minor components in the extract would be present in greater amounts. The tolerization protocol and the initial immunization series is outlined in Fig. 1.

III.   Immunization of Rabbits

1.   Hyperimmunization

Complete Freund's adjuvant (Difco) was used to hyperimmunize rabbits against tumour extracts. Tumour extracts were diluted 1:1 with adjuvant, emulsified, and 1.0 ml was injected into the rabbits weekly. 0.2 ml was injected intramuscularly (I.M.) into each limb and 0.2 ml intraperitoneally (I.P.). After 4 weeks of immunizations, the tumour extracts were diluted 2:1 with CFA to maximize the amount of antigen and minimize the granulomatous reactions of the rabbits. At this time also, the rabbits were bled from the ear, the serum was removed from the blood, and the complement was inactivated by heating the serum at 56°C for 30 min. The antisera were tested by immuno-diffusion (I.D.) and stored at -20°C. The rabbits were bled weekly and boosted for 5 to 10 weeks or until there was no increase in the antibody detected by I.D. Many of the antisera were also tested for maximum antibody production by precipitin reaction (XIV). This did not correlate with antibody production any better than I.D., and since it required more manipulations, was discontinued.
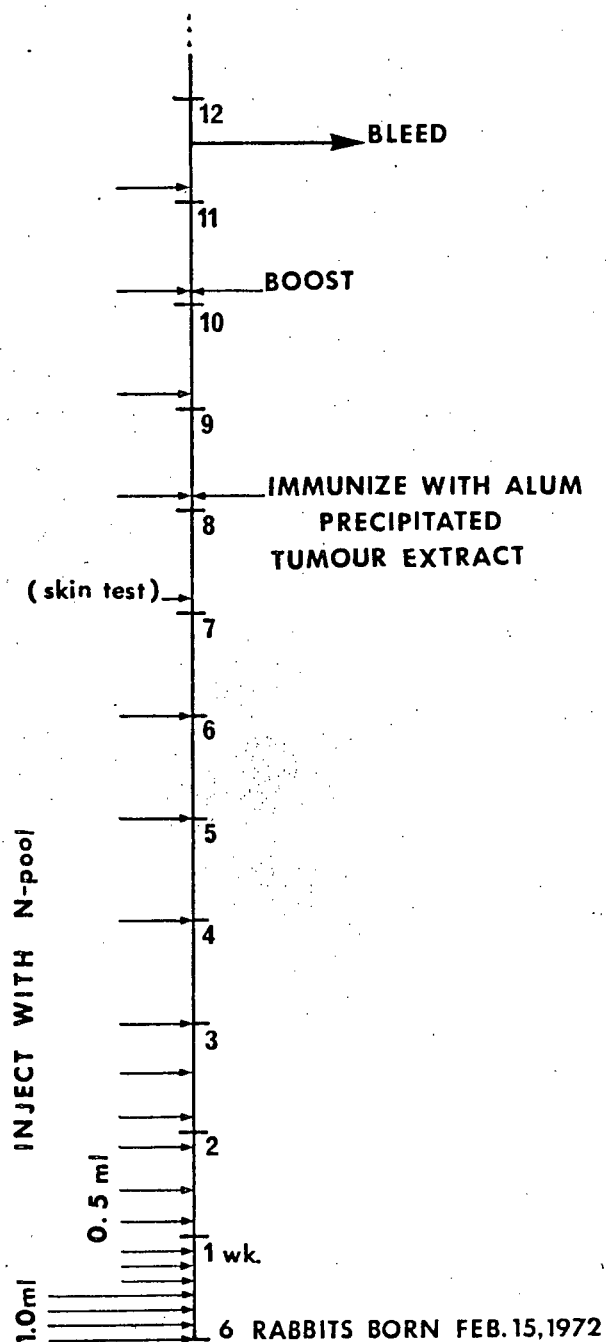
Fig. 1: Procedure for the neonatal tolerization of rabbits to the normal antigens in lung extract.

2.   Alum Precipitate.

There are experimental conditions which preclude the use of CFA as

the adjuvant for immunization. With CFA, it is possible to break immune

tolerance and to induce the production of extraneous antibodies. When these

factors may be important, an alum precipitate of the antigen was used for

immunization instead of a CFA emulsion. The method of Campbell et al (21)

was used. 1.0 ml of a suspension of alum precipitated extract (5.0 mg

protein/ml) was used to immunize the rabbits neonatally tolerant to N-pool.

A similar suspension (100 µg protein/ml) of purified human immunoglobulin

(XV) was used to prepare clean antiserum against human immunoglobulin.

IV.   Immunodiffusion.

1.   Semi-micro.

The method used was similar to that presented by Clausen (22).

In order to facilitate sticking of the gel, the clean 50 x 75 mm micro-

scope slides (Fisher) were precoated with agar by dipping them in 0.2% Ionagar

No.2 (Oxoid) and air drying. 10.0 ml of molten 0.85% Ionagar No. 2 in

barbital buffered saline, was pipetted onto the microscope slide, allowed

to set at room temperature, and allowed to become firm by storage at 4°C for

1 hr. Hole patterns (2 patterns per slide) were punched in the agar with a

no. 2 (0.5 cm diameter) or 3 (0.7 cm diameter) cork borer and the agar plugs

were removed by a Pasteur pipette on an aspirator. The wells were filled

with 500 or 100 µl of antiserum and antigen. The precipitin bands were allowed

to develop for up to one week at 4°C in a humidified chamber.

2.   Micro.

This technique is similar to the semi-micro technique except that only 7.0 mls of Ionagar No. 2 is used per slide and the hole patterns were punched with a template. Six hole patterns (0.2 cm diameter) were used per slide. Each well could hold approximately 7 µl of antigen or antiserum. The reaction times were also shorter.

3.   Staining.

To remove the unbound protein, the slides were soaked in several changes of 0.9% saline at 4°C for 2 to 3 days. Then they were briefly rinsed with distilled water, covered with a piece of filter paper and allowed to dry at room temperature overnight. The filter paper was removed and the slides were soaked in tap water for one minute and in Amido Black stain for 15 min. Destaining was carried out for 2 hrs with several changes of 3% acetic acid, and the slides were air dried. Photographs were taken with the agar side down on white paper, or with transmitted fluorescent light.

4.   Micro Radioimmunodiffusion.

This technique was developed in an attempt to increase the sensitivity of the I.D. technique. After washing micro I.D. slides free of unreacted protein, a dilution (from 1/5 to 1/50) of ^{125}I conjugated anti-immunoglobulin (XIX) was added to either the central (antiserum) wells or all seven of the wells, and the slides were incubated overnight. After a thorough washing in 0.9% saline and drying, the slides were exposed to Kodak Blue Brand -14 X-ray film in the dark at 4°C for 1 to 2 weeks. The film was developed with Kodak

X-ray film developer plus replenisher according to the developer instructions. The slides were then stained with Amido Black stain for comparison of results.

V.   Immunoelectrophoresis

The method used was similar to that described by Campbell et al (21). 4.0 ml of molten Ionagar No. 2 instead of 2.0 ml, was pipetted onto each microscope slide so that larger samples could be applied. The filter paper wick for electrophoresis was not impregnated in the agar but placed on top, at each end of the slide. After adding 100 µl of antiserum to the central trough (Fig. 13c), the slides were treated like immunodiffusion slides.

VI.   DEAE Cellulose Chromatography

DEAE cellulose (Bio-Rad) was suspended in distilled water, allowed to settle and the fines removed by decanting the supernatant. After further washing, and packing in a Buchner funnel, the DEAE cellulose was soaked twice in 0.2 N HCl for 15 min. each time. Several distilled water washes on a Buchner funnel were followed by two soakings in 0.5 N NaOH. After several more distilled water washes, the DEAE cellulose was soaked in 0.1 M phosphate buffer pH 7.5, then washed with starting buffer (0.01 M phosphate buffer pH 7.5).

One ml of packed DEAE cellulose was used for 3 to 7 mg of protein to be fractionated. The slurry was packed into a column and equilibrated with at least 5 times the column volume of starting buffer. The sample, after dialysis against starting buffer, was washed through the column and the bound material was fractionated either by gradient or step elution.

1.   Gradient Elution.

A linear gradient, from starting buffer to starting buffer plus 1.0 M NaCl, was run. Both buffer reservoirs contained 3 times the column volume of their respective buffers. A small volume of 3.0 M NaCl was frequently run after the gradient, to check for complete elution.

2.   Step Elution.

The remaining starting buffer was removed from above the cellulose and 0.05 M phosphate buffer pH 7.5 was flushed through the column until the A_{280} approached zero. The final elution buffer was 0.2 M phosphate buffer pH 7.5 plus 0.2 M NaCl. For our purposes, step elution was found to be superior to gradient elution because the three fractions were convenient to work with and the protein concentrations in each fraction were similar.

3.   Treatment of Fractions.

Fractions were collected on an LKB fraction collector and the elution profile was monitored by reading the absorbance at 280 nm (A_{280}) with a Beckman DB-G spectrophotometer. The fractions in each peak were pooled and concentrated to the original sample volume by dialysis against Ficoll (Pharmacia), vaccuum dialysis, or ultrafiltration on a PM10 ultrafiltration membrane (Amicon). The peaks were labelled I, II and III in the order of elution, and stored at -20°C.

VII.   Gel Chromatography.

Both Sephadex G-75 and G-150 (Pharmacia) were used for gel chromato-
graphy.   2.5 x 90 cm columns were packed and were usually run in 0.15 M NaCl.
The columns were fitted with a flow adaptor and run ascending at room tempera-
ture.   Sample and fraction volumes were 5.0 ml.   The flow rate was maintained
by gravity at 20 ml/hr for G-75 and 15ml/hr for G-150.   The packing quality,
stability, and the void volume of the column bed were determined by chromto-
graphing 2 mg of Blue Dextran 2000 (Pharmacia).   One of the G-75 columns was
further calibrated by the chromatography of 2.0 mg of ovalbumin (M.W. 45,000)
and chymotrypsinogen A (M.W. 25,000).   Fractions were treated as were those
from DEAE cellulose chromatography.

VIII.   Ammonium Sulfate Precipitation.

Enough saturated $(NH_4)_2SO_4$ (Fisher) solution was very slowly added to
protein solutions, at room temperature, with stirring, to make the desired
concentration of $(NH_4)_2SO_4$.   For example, 33%, 40% and 50% saturated solutions
can be achieved by the addition of 1/2, 2/3 and 1 volume of saturated $(NH_4)_2SO_4$
to a volume of protein solution.   All of these concentrations can be used to
precipitate immunoglobulin, depending on the required purity or yield.   The
mixtures were stirred for 1 hr and allowed to settle for 3 hrs.   For 67% and
100% saturation, solid $(NH_4)_2SO_4$ was slowly added, mixed 2 hrs and allowed to
settle several hrs.   The supernatants were decanted after centrifugation at
5,000 g for 15 min. and the pellets were dissolved in a minimum amount of
distilled water.   The dissolved precipitates were dialyzed against 0.15 M NaCl
at 4°C and stored at -20°C.

IX.   Disc Gel Electrophoresis.

The method has been described in detail by Maizel (77). The resolving gels were 7.0% acrylamide (Kodak) and 0.55 x 11 cm. The high pH buffer system was used throughout. A 3.8% acrylamide spacer gel was also used initially, but was not found to improve the results. Sample volumes were from 20 μl to 100 μl, since there was no difference in resolution within this range. Samples were diluted 1:1 with 30% sucrose before application to the gels. 350 to 500 μg protein and 200 to 350 μg protein were found to give optimum resolution for the whole extracts and DEAE cellulose frations, respectively. The gels were stained with Amido Black 10B (Merck) for 2 hrs and destained by overnight diffusion in 7.0% acetic acid at room temperature. Several attempts were made to increase the resolution with the use of Coomassie Brilliant Blue R250 (Colab). The gels were stained for 3 hrs at 37°C or overnight at room temperature in 0.25% Coomassie Blue in 7.0% HOAc, 5.0% MeOH. Destaining by diffusion in 7.0% HOAc, 5.0% MeOH or 7.0% HOAc, 5.0% MeOH required 2 to 3 days. Although the sensitivity may have been increased slightly, the resolution of the bands was not.

X.   Migration Inhibition Factor Test with Human Peripheral Blood Leukocytes.

100 units of heparin (Connaught) was used per ml of blood. Blood was taken from healthy volunteers and patients with bronchial carcinoma and incubated for 1 hr at 37°C with 20% Plasmagel (Laboratoire Roger Bellon, Neuilly, France) in an inverted syringe. Plasma and the buffy coat were transferred to a sterile, siliconized (Siliclad; Canlab) centrifuge tube, through a bent needle. The cells were washed twice with phosphate buffered saline (PBS) plus 10% foetal calf serum (FCS) by centrifugation at 200 g for

10 min. The remaining red blood cells were lysed with 0.85% NH_4Cl, and the leukocytes further washed twice with PBS. The cell pellet was suspended in 7 times its volume of RPMI 1640 tissue culture medium (Gibco) supplemented with 100 units of penicillin and 100 μg of streptomycin per ml. The migration inhibition factor (MIF) test was performed as described by Waterfield et al (134) and all tests were done in quadruplicate.

In order to determine the maximum amount of extract which would be incubated with the leukocytes, healthy donors' leukocytes were incubated with a 1/15, 1/60 and 1/200 dilution of N-pool (29 mg protein/ml). Only the 1/200 dilution (150 μg/ml) did not inhibit the migration of any of the leukocytes tested initially. The finding that the leukocytes from one donor (J.L.), were not inhibited by even a 1/15 dilution of N-pool, strongly suggests that migration inhibition by the extract is not non-specific cytotoxicity. Although we could have diluted the protein concentration of all of the extracts to 150 μg/ml, we chose instead to dilute all of the extracts 1/200. Appendix A shows that all the normal lung extracts have higher protein concentrations than the tumour extracts. Therefore, if all of the extracts were similarly diluted, the normal extract control migration chambers would have more protein than the tumour extract migration chambers. Thus, any inhibition of leukocyte migration by tumour extract is probably not due to non-specific inhibition by high protein concentration, and is more likely to be specific inhibition than if all of the protein concentrations were the same. Also, since the lungs contain a large quantity of blood, and the blood was not removed before extraction, much of the protein in the normal lung extract is serum protein and not lung tissue protein.

During the time these experiments were being done, we received no surgical tumour specimens. Therefore, it was not possible to test autologous tumour extract and leukocytes.

XI.   Guinea Pig Skin Tests

The backs of non-sensitized guinea pigs were clipped, depilated
with Nair, washed, and skin tested with extracts and their DEAE cellulose
fractions.   Undiluted extract (0.1 ml) was injected intracutaneously and
the diameter of erythema was measured at 2, 6, and 24 hours.

XII.   Agarose Immunoadsorbent Columns

The method used was similar to that already outlined (136).   The
normal lung extract to be coupled was dialyzed against borate buffered
saline pH 8.45 (0.2 M borate, 0.3 M NaCl).   One ml of packed Sepharose 4B
(Pharmacia) was used per 6.0 mg of protein.   The Sepharose was washed
with distilled water and the pH was brought to 11.5 with 5.0 M NaOH.   40 mg
of cyanogen bromide (J.T. Baker), dissolved in 1.0 ml distilled water per
25 mg, was added per ml of packed Sepharose.   The pH was maintained at
11.2 to 11.4 for 10 to 15 mins., or until the pH became constant.   After
quickly (less than 2 min.) washing on a Buchner funnel with ice-cold
distilled water and borate buffered saline, the activated Sepharose was
transferred to a tube and the dialyzed normal lung extract was added.   The
tube was sealed and gently agitated on a rocker for 20 hrs at 4°C.   The
coupled Sepharose was transferred to a 1.6 x 80 cm water jacketed Pharm-
acia column, cooled with running tap water, and fitted with a flow adaptor
(Pharmacia) to facilitate buffer changes.   The column was flushed with 3
column volumes of tris buffered saline pH 7.6 (0.1 M tris, 0.4 M NaCl), 3

column volumes of Sorensen's glycine I buffer pH 2.6, and again with tris

buffered saline. Some of the columns were reacted with 0.1 to 1.0 M ethanol-

amine to block any remaining active groups. 2.0 to 4.0 ml of heat inactivated

rabbit anti (tumour extract) serum was added and washed through with either

tris buffered saline or 0. 15 M NaCl. The flow rate was maintained at 15 ml/hr

with an LKB peristaltic pump. 10.0 ml fractions were collected on an LKB

fraction collector and fractions containing A_{280} were pooled. The pooled

fractions were either concentrated by ultrafiltration on an XM100A membrane

(Amicon) or re-absorbed with a glutaraldehyde precipitated normal lung extract.

The concentrated absorbed antisera were stored at -20°C. The anti normal

antibodies could be recovered and the columns regenerated by flushing with

Sorensen's pH 2.6 buffer, and the columns neutralized with tris buffered

saline.

Whether the extracts were reacted at 6 mg or 10 mg protein per ml of

packed Sepharose, 2 to 3 mg protein was bound per ml. Thus for a 70 ml column,

140 to 210 mg of protein was bound. Since the protein concentrations of the

normal extracts used were 30 mg/ml, such a column would be the equivalent of

roughly 6 ml of normal extract. Since 4.0 mls of antiserum was usually

absorbed at a time, it would appear that each volume of antiserum was absorbed

by 1.5 volumes of normal extract. However, it must be remembered that many

of the antigenic determinants will not be accessible to antibody. The absorbed

antisera were not any more specific if only 1.0 or 2.0 mls of antisera were

used.

A problem encountered with these columns was the stripping of materials

from the immunoadsorbents as detected by the loss of their red colour. This

was not observed with any of the several buffers (including low pH) used. It

was observed with ethanolamine, immune rabbit serum and even non-immune rabbit

serum. Efforts were made to prevent this, since it was suspected that many

proteins were being removed. However, the column capacity was not appreciably diminished with each use, although the red colour was completely eliminated. Recent communications with Dr. A. Jackson of Kent Laboratories, Vancouver, have suggested that haemoglobin can be removed from insoluble complexes by the avid binding of haptoglobin.

XIII. Glutaraldehyde Cross-Linked Immunoadsorbents.

1.   Human Immunoglobulin Immunoadsorbent.

150 mg of purified human immunoglobulin (see Section XV.1.) plus 100 mg of bovine serum albumin (Sigma) was insolubilized by polymerization with glutaraldehyde (J.T. Baker) according to the method of Avrameas and Ternynck (4). The protein recovered was 1.5 mg per ml of antiserum (Section III.2.). Much of the antibody activity was recovered and most of the antibody was removed from the antiserum as seen in Fig. 2.

2.   Normal Lung Extract Immunoadsorbent.

The method of Avrameas and Ternynck (4) for the insolubilization of serum proteins was not suitable for the insolubilization of normal lung extracts. Some modifications were required. 50 mg (instead of 10 mg) of glutaraldehyde was reacted per 100 mg of protein. Because the extract concentration was 30 mg protein/ml instead of 50 mg/ml, 25% glutaraldehyde was used instead of 2.5%, to keep volumes and protein dilutions to a minimum. Reaction times were lengthened to 4 hrs of mixing at room temperature and leaving overnight at 4°C. After thorough washing, the precipitate was suspended in PBS equal to the original volume of the extract.
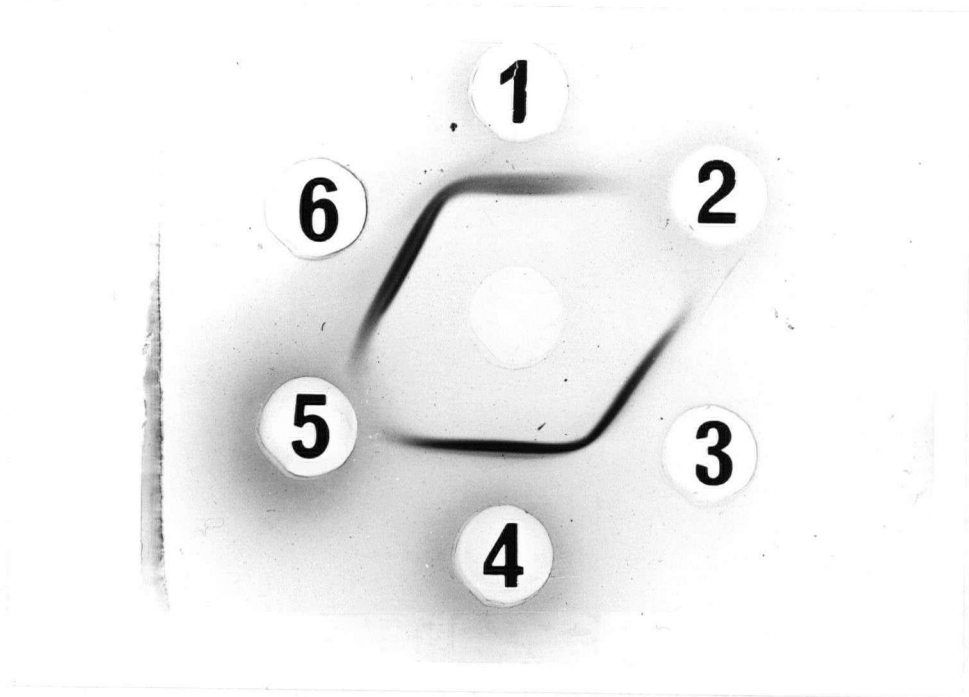
Fig. 2:   Immunodiffusion results of tests for the absorption of rabbit
          anti-human Ig by glutaraldehyde insolubilized human Ig.   The
          control well contained human Ig (40.0 mg) and the outer wells
          contained anti-human Ig preparations as follows:

1. whole rabbit anti-human Ig diluted 1:1, and 4 undiluted

2. absorbed rabbit anti-human Ig diluted 1:1 and 5 undiluted

3. rabbit anti-human Ig eluted from immunoadsorbent diluted
   1:1 and 6 undiluted.

Two volumes of glutaraldehyde immunoadsorbent suspensions were used to further absorb one volume of antiserum already abosrbed by an agarose column immunoadsorbent. After mixing and reacting overnight at $4^{o}C$, the precipitate was removed by centrifugation at 2000 g for 15 min. and the supernatant was concentrated by precipitation with 50% saturated $(NH_4)_2SO_4$ or by ultrafiltration on an XM100 A membrane. Early attempts to regenerate these immunoadsorbents showed that the original capacity was not nearly achieved.

XIV. Other Antiserum Absorption Methods

Even when used in combination, the immunoadsorbent methods of sections XII and XIII.2. frequently could not completely eliminate the anti-normal antibodies from the antisera. Since both of these methods involve reactions with amino groups of proteins, it is possible that both methods could make the same proteins inaccessible to protein, or fail to insolubilize the same proteins. Two methods were used to try to eliminate non-tumour specific antibody.

1. Precipitation of Immune Complexes

Many of the antisera were tested weekly, by precipitin reaction in order to ascertain when the rabbits were maximally producing antibodies. 25 µl of doubling dilutions of N-pool was incubated for 1 hr at $37^{o}C$ with 25 µl of heat inactivated antiserum. The precipitates were washed 3 times with PBS and dissolved in 0.1 N NaOH. The A_{280} was determined for each sample and the dilution at which maximum precipitation occurred was determined (eg. Fig. 3). For the hyperimmune rabbit sera, the maximum precipitates occurred at anti-
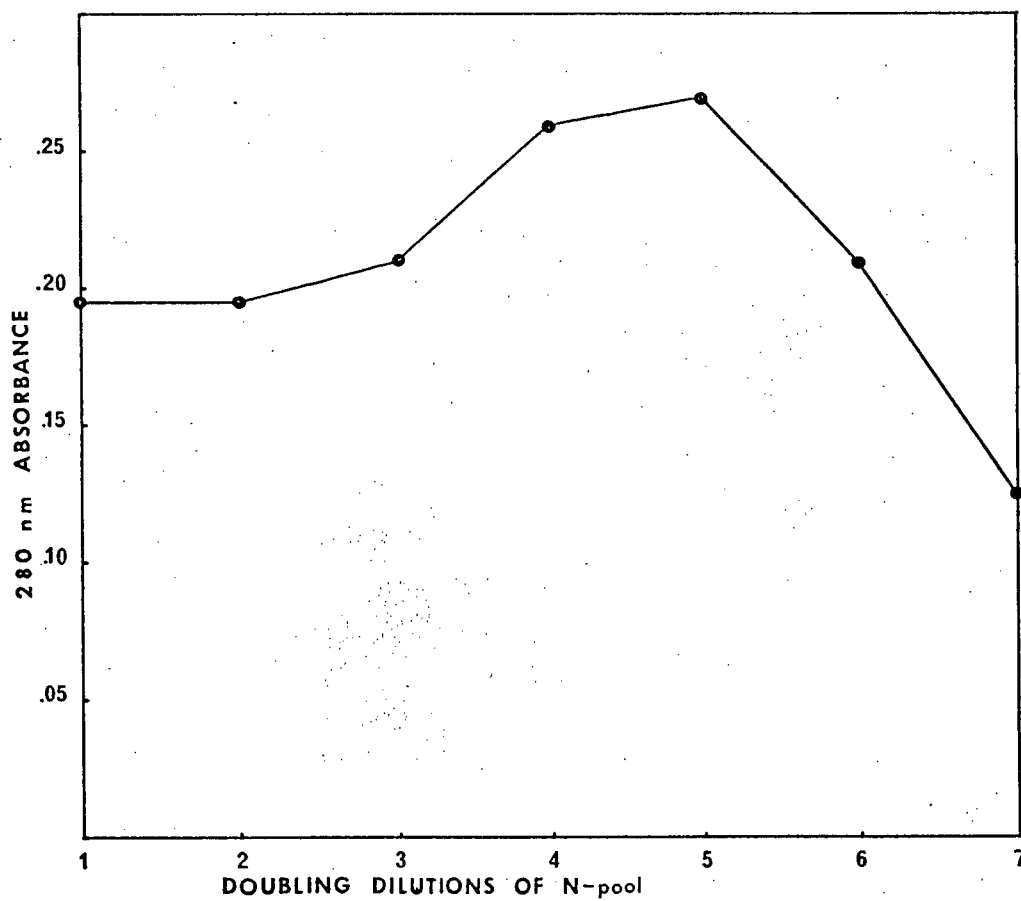
Fig. 3:   Precipitin reaction of rabbit whole antiserum against C-41
           after 8 weeks of hyperimmunization, against doubling dilutions
           of N-pool.

serum:extract ratios ranging from 4:1 to 64:1. No relationship was found between this titer and the ability of the antiserum to produce antibodies against TAA.

With a single antigen, both specific antigen and antibody can be effectively precipitated from solution when incubated at their zone of optimal proportions. However, for a crude protein mixture there is no single zone of optimal proportions, and different antigens are optimally precipitated at different antibody:extract ratios. In order to absorb the antisera by precipitation of immune complexes, the antisera were mixed with N-pool at ratios from 4:1 to 64:1. When these absorbed antisera were subsequently re-absorbed by a solid-phase immunoadsorbent, the antibodies were still not tumour specific.

2.   Absorption with Cells.

This is probably the most commonly reported method for the absorption of antisera. Although we expected our antisera to contain antibodies against internal cell antigens, which would not be absorbed by cells, we tried this method in the hope of complementing our other absorption methods, and for the sake of completion.

Normal lung tissue was minced at 4°C in PBS and the large clumps of tissue were allowed to settle for 20 min. The cells in the supernatant were washed 5 to 7 times with PBS by centrifugation at 800 g. One volume of anti-serum was mixed with one volume of packed cells and diluted 10 fold. This mixture was incubated overnight at 4°C with gentle agitation, the cells were pelleted, and the antibody in the supernatant was concentrated by precipi-tation with 50% saturated $(NH_4)_2SO_4$. This absorption procedure did not result in specific anti tumour antibody, even when combined with the agarose

immunoadsorbent method. Very large amounts of tissue, time and manipulations were also required.

XV.   Purification of Immunoglobulin.

    1.   Human Immunoglobulin.

The immunoglobulin (Ig) fraction was precipitated from 50.0 ml of normal human serum with 33% saturated $(NH_4)_2SO_4$ and further purified by passing the fraction through a DEAE cellulose column in 0.01 M phosphate buffer pH 7.5 (21). After concentrating the material by precipitation with 40% saturated $(NH_4)_2SO_4$, and dialysis against 0.15 M NaCl, the Ig was further purified by chromatographing on a 2.5 x 90 cm Sephadex G-150 column. Fractions in the major peak (A_{280}) were pooled and concentrated on an XM100 A ultrafiltration membrane. Protein was quantitated spectrophotometrically, assuming the molar absorbancy index to be 1.38. The yield was 6.0 mg Ig/ml of serum.

    2.   Rabbit Immunoglobulin.

This was used for the immunization of a sheep, to get anti-rabbit Ig, and was supplied by D. Gregerson. He purified the Ig from rabbit serum by precipitation with 40% saturated $(NH_4)_2SO_4$, passing it through a DEAE cellulose column, and re-precipitating the Ig fraction with 50% saturated $(NH_4)_2SO_4$. The sheep was immunized with 3.0 mg of Ig in 50% complete Freund's adjuvant 3 times, 10 days apart.

XVI.   Human Serum Fractionation.

Whole serum or a 40% saturated $(NH_4)_2SO_4$ cut was tested for free anti-
tumour antibody. To dissociate any immune complexes which may have been
present, the serum (2.0 ml diluted to 5.0 mls) or the $(NH_4)_2SO_4$ precipitate
cut was chromatographed on Sephadex G-150 equilibrated in 0.1 M HOAc, 0.15 M
NaCl. The effluent was immediately neutralized by dialysis against 0.2 M
phosphate, 0.15 M NaCl pH 7.5. Fractions in the Ig peak (Fig. 4) were pooled
and concentrated by either precipitation with 50% saturated $(NH_4)_2SO_4$ or
ultrafiltration on an XM100 A membrane. The remaining fractions of the column
volume were concentrated by either precipitation with saturated $(NH_4)_2SO_4$ or
ultrafiltration on a PM10 membrane (Amicon). Although the separation (Fig. 4)
is not good, the results of Fig. 5 show that most of the Ig was in peak 2.
Both pre-operative and post-operative sera were tested.

XVII.   Indirect Immunofluorescence.

The method used was described by Culling (26). Instructions, including
the operation of a cryostat microtome (International, I.E.C.) were arranged
by C.F.A. Culling (Department of Pathology, U.B.C.). Pieces of tissue were
snap frozen in liquid nitrogen and stored at $-70^{\circ}C$. 6 μm sections were fixed
to clean microscope slides by air drying.

1.   Absorbed Anti(Tumour Extract) Serum.

Either 10 or 25 μl of absorbed antisera or normal rabbit serum, was
incubated with tumour and normal lung tissue sections in a humidified chamber
at room temperature for 30 min. The slides were rinsed with PBS and up to 20
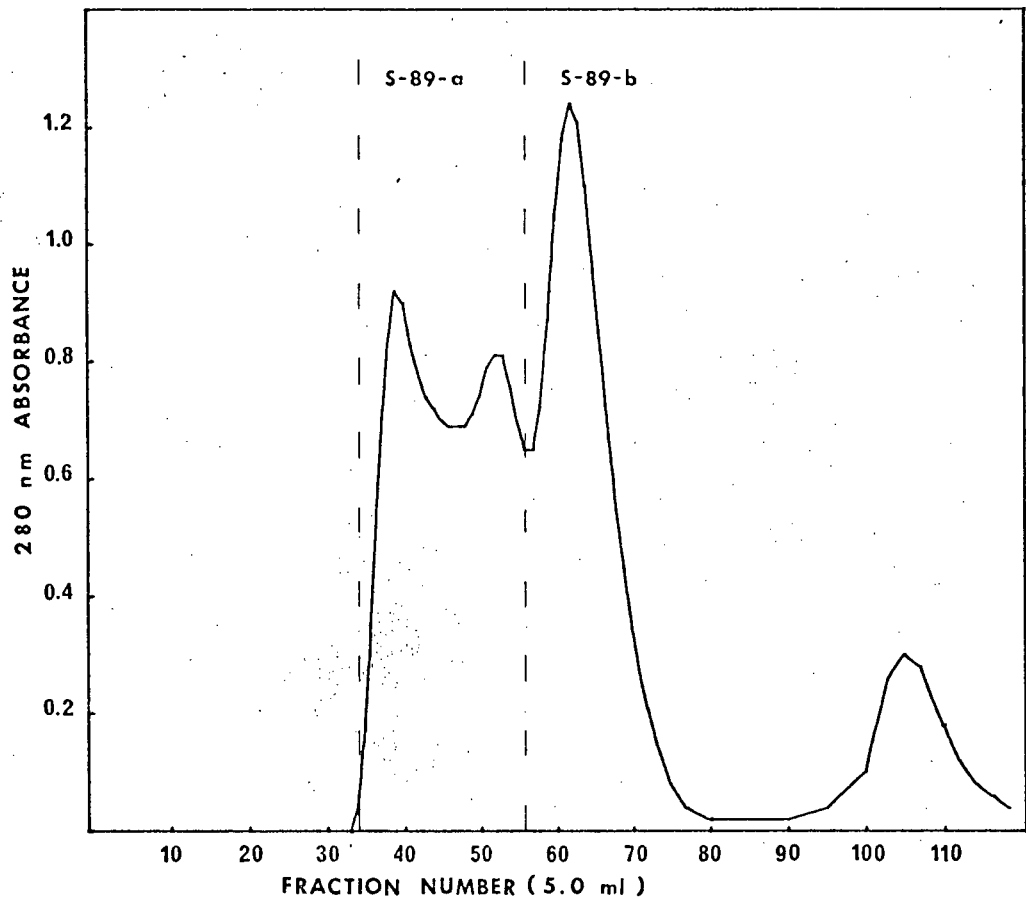
Fig. 4: 2.0 ml of serum from patient 89 (S-89) fractionated on a
Sephadex G-150 column (2.5 x 90 cm) equilibrated in 0.1 M
HOAc, 0.15 M NaCl. The fractions in S-89-a were concentrated
by ultrafiltration on an Amicon X M100A membrane and in
S-89-b on a PM10 membrane.
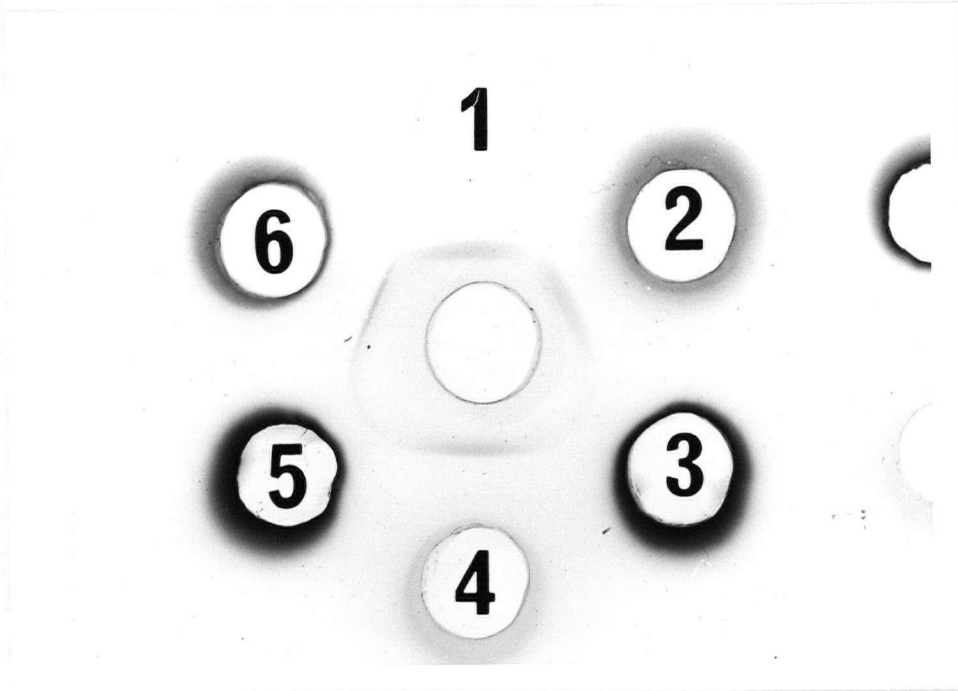
<u>Fig. 5</u>:   Immunodiffusion results using purified antibodies to human Ig
          (diluted 1:5) against human serum and fractions designated as
          follows:

          1. Human Ig (200 µg)   2. S-68-a diluted 1:5   3. S-68-b diluted 1:5

          4. S-89 diluted 1:5   5. S-89-b diluted 1:5   6. S-89-a diluted 1:5

          The presence of a precipitin band in wells 3 and 5 shows that
          the Sephadex G-150 fractionation of the serum Ig was incomplete
          The heavy staining around wells 2,3,5 and 6 suggests that the
          Sephadex fractions contained poorly soluble components.

slides at a time were washed with 250 ml of PBS, 5 times with agitation for

a total of 30 min. The tissue sections were then incubated with fluorescein

conjugated goat anti(rabbit IgG) IgG (Microbiological Associates), washed

again, mounted in buffered glycerine mountant, and sealed with a coverslip.

The sections were examined by a microscope with a dark ground condenser.

Several dilutions of the reactants were also tried, but because of the great

difficulty in quantitation and the more promising results by indirect immuno-

autoradiography (being done concurrently), the dilutions of the reactants,

required for optimum sensitivity were not determined.

2.   Human Serum.

Healthy donor and cancer patients' sera and globulin fractions were

tested similarly. The labelled antibody was fluorescein conjugated rabbit

anti (human globulin) globulin.

XVIII. Indirect Immunoautoradiography.

1.   Experimental Techniques.

The method initially used was analogous to the immunofluorescence

method. 8 μm tissue sections were used because it was easier and faster to

prepare large numbers of good sections. Instead of a fluorescein label, the

anti Ig was conjugated with ^{125}I (Section XIX). Bound radioactive label was

assayed by the method described by Kelly et al (61). Slides were fixed by

soaking in absolute MeOH for 15 min., then soaked in water for 30 min. and

allowed to dry. The following steps were carried out in a darkroom equipped

with a No. 2 red safelight (Kodak). NTB-2 nuclear track emulsion (Kodak)

was heated to 43°C and transferred to a coplin jar. Two slides at a time (back to back) were slowly dipped 3 times for a total of 10 sec. The slides were drained and air dried vertically for 1.0 hr. Exposure to the emulsion was at 4°C in tape-sealed microscope slide boxes (containing a few grams of dessicant) and ranged from 36 hr to one week. Initially, the developing was done as described by Kelly et al (61).

However, problems were encountered: some tissue sections and the nuclear track emulsion would often detach from the slide and the background grains were extremely high. Thoroughly cleaning the slides by washing with acid-alcohol or detergent had no noticeable effect. Precoating the slides by dipping them in 0.2% gelatin helped the emulsion stick more firmly but interfered with the sticking of the tissue sections. By fixing the tissue sections in MeOH for one min., coating the slides with 0.2% gelatin, and keeping the developing times to a minimum and the temperature to 12°C, there were no further problems with the slipping of emulsion or tissue sections. (The problem with the emulsion varied with each batch). The emulsion was developed for 2.5 min. in full strength Kodak Dektol Developer, rinsed in H_2O for 10 sec., fixed in Edwal High-Speed Fixer with Hardener for 3.0 min., rinsed with H_2O for 5.0 min., and dried for 1.0 min. in MeOH. The cells were stained with 6% Geimsa solution for 2.5 min. and washed with H_2O for 2.5 min. After air drying, the slides were examined under oil immersion.

The changes in methodology had no noticeable effect on the high background grains which were never eliminated in spite of several attempts. These attempts included: fixing the tissue sections to plastic instead of glass, adding FCS or adult bovine serum to the PBS used for rinsing the slides, preincubating of the tissue sections with non-conjugated anti-Ig, and adding activated charcoal to the ^{125}I-conjugated anti-Ig. The labelled anti-Ig would bind to the glass and directly to the tissue sections even

if there was no incubation with the intermediate antiserum. To determine which component of the conjugated anti-Ig was responsible for this stickiness, it was mixed with beef serum or globulin, ^{127}I-conjugated beef globulin, or KI. The KI produced a significant inhibition of binding of the ^{125}I conjugated anti-Ig to the tissue sections, but iodide is a chaotropic agent, and besides increasing the solubility of proteins, can also dissociate antibody-antigen complexes (105). When the other materials were added, only a slight decrease in background was observed. The ^{125}I conjugated anti-Ig subsequently had 20% bovine serum added to it. Dr. I. Berczi (Department of Immunology, University of Manitoba) has reported similar problems with an analogous technique (personal communication).

The problem with the high background was circumvented by incubating dilutions of the ^{125}I-conjugated anti-Ig with the tissue sections. The dilution which resulted in the maximum tolerable background grain level was used for subsequent experiments. This dilution was determined for each labelled antisera and could be estimated afterwards according to the ^{125}I halflife. This dilution ranged from 1/200 to 1/20.

2.   Quantitation

Two methods were used to quantitate the binding of labelled antibody. The first was for a rough comparison in order to determine the effects of techniques to lower background grain counts and to determine the dilutions that optimize tumour specific differences. The whole tissue sections were quickly scanned and 3 or 4 representative areas were visually compared. The other method was sensitive but was very tedious and time consuming to perform, and had to be blind controlled because of its subjective nature. The whole tissue sections were carefully scanned, noting any areas of exceptionally

high background to be avoided later. The numbers of grains on one represent-
ative cell was counted for 50 microscope fields. This large number was required
because of the high background.

3.   Testing Human Serum and Fractions

10 µl of cancer patient or healthy donor serum or fractions (Section XVI)
were incubated on each tissue section for 30 min. in a humidified chamber at
room temperature. The slides were rinsed and washed as in Section XVII), dried
in MeOH for 1 min. and incubated with a pre-determined dilution of ^{125}I conju-
gated rabbit anti (human Ig) globulin for 30 min. The slides were again rinsed,
washed, and dried, and then treated as in part 1.

A further attempt to minimize background grains was done by iodinating
immunoadsorbent purified antibody (Section XIII.1.) instead of the ammonium
sulfate globulin fraction of the rabbit antiserum.

4.   Testing Absorbed Rabbit Anti (Tumour Extract) Serum

10 µl of absorbed antiserum and a pre-determined dilution of ^{125}I
conjugated sheep anti (rabbit Ig) globulin were reacted with tissue sections
as in part 1. The grain counts were far too high to count on both normal and
tumour sections, indicating that antibodies against normal tissue antigens
were still present. Several antisera were re-absorbed (Section XII, XIII.2.)
and tested again. One particular antiserum (C-66) was re-absorbed several
times until no antibodies were detectable by immunodiffusion, and still
required a 1/50 dilution before the grain count was low enough to be con-
veniently counted. A convenient dilution for all of the absorbed antisera
was determined for subsequent experiments.

XIX. Iodination.

The Ig fraction of the antiserum was partially purified by precipitation with 33% saturated ammonium sulfate and dialyzed against 0.1 M phosphate buffer pH 7.5. Protein concentration was determined spectrophotometrically and diluted to 50 mg protein per ml with 0.1 M phosphate. The iodination method was carried out by the chloramine T method (56). 100 μl of protein solution (5 mg) was added to a shell vial and transferred to a fume hood at room temperature. One to 2.0 mCi of carrier-free Na^{125}I in 0.1 N NaOH (New England Nuclear) was added with a 20 μl capillary pipette fitted on a disposable 1.0 cc syringe. 200 μg (20 μl) of chloramine T (Eastman) freshly dissolved in 0.1 M phosphate buffer pH 7.5 was added and the solution was mixed by bubbling air through it. The reaction was terminated after 3.0 min. by the addition of 200 μg (20 μl) of sodium metabisulfite (Fisher). 50 μl of 1% KI was added and the solution was applied to a 1.5 x 8 cm Sephadex G-25 column to separate the iodinated protein from the iodine. The column had been pretreated with 5.0 mls of FCS and rinsed with PBS to minimize non-specific binding of the ^{125}I to the column. The sample was washed through the column with PBS and 8 drop fractions were collected in disposable plastic tubes. The radioactivity in each fraction was measured with a Geiger-Müller counter (Fig. 6), the samples in the voided peak pooled, and the volume measured.

Triplicate 10 μl samples were dried on glass fiber filter discs (Reeve Angel), which were suspended in 8.0 ml of scintillation fluid. Gamma radiation was counted with a Isocap 300 scintillation counter for 0.1 min.

The specific activity was calculated as follows:

The average counts per min. was 1.29×10^6 for 10 μl = 1.29×10^8 cpm/ml.

$$1.29 \times 10^8 \text{ cpm} \times 1.25 = 1.615 \times 10^8 \text{ DPM}$$

$$1.615 \times 10^8 \text{ DPM} \times \frac{\mu Ci}{2.6 \times 10^8 \text{ DPM}} = 62.1 \ \mu Ci = 0.062 \text{ mCi/ml}$$
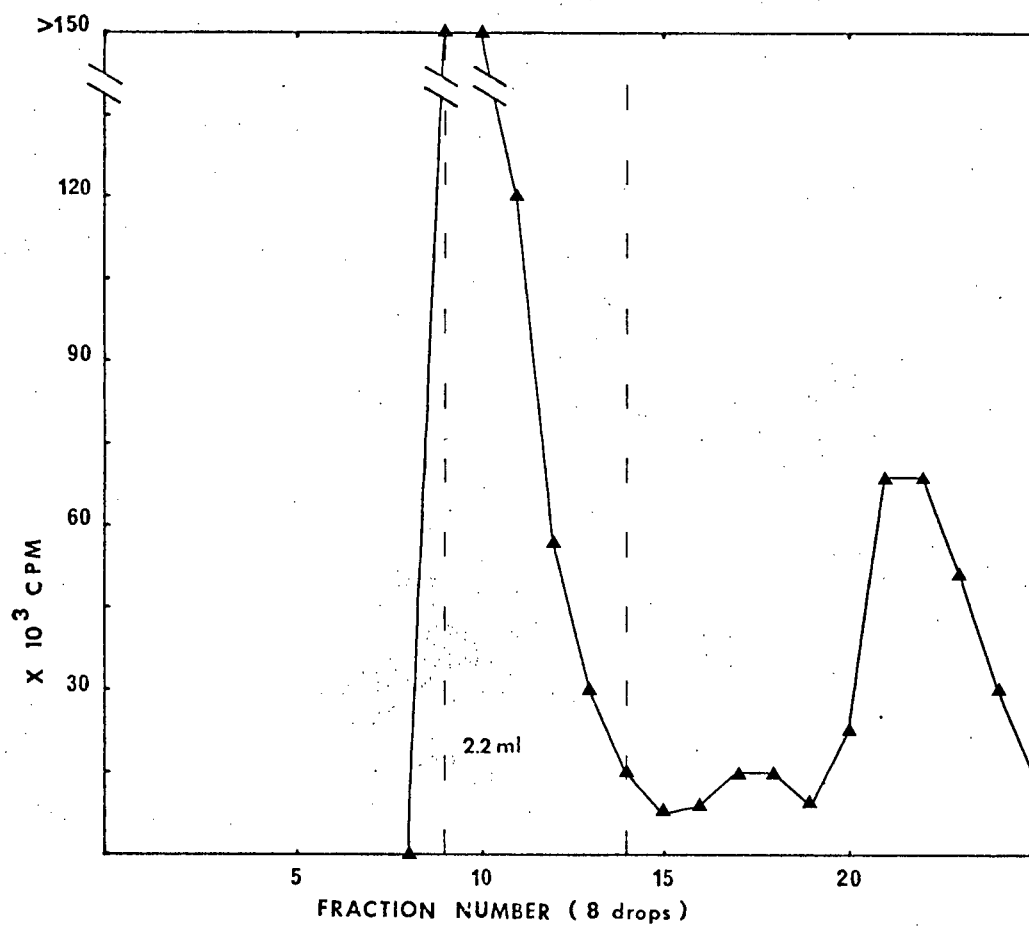
Fig. 6: The removal of free ^{125}I from 5.0 mg of Ig conjugated with ^{125}I by a 1.5 x 8 cm Sephadex G-25 column.

If no protein was lost, we have 5.0 mg/2.2 mls

$$= 2.27 \text{ mg protein/ml}$$

$$\text{specific activity} = \frac{0.062 \text{ mCi}}{2.27 \text{ mg protein}} = 0.027$$

XX.   Statistical Analyses

Gel statistical analyses were performed with a programmable Hewlett Packard 9810 A calculator. For most results, the significance was determined with a programme for the Student's t test. When the means of several groups were being compared, such as in the indirect immunoautoradiography surveys (Section XVIII.3.), significance was determined by the method of 'least significant difference' for a probability of 0.05 and 0.01 according to Li (71). I would like to thank Dr. J. Yensen for advice on statistical analysis.

XXI.   Skin Testing of Cancer Patients

Whole extracts and DEAE cellulose fractions were sterilized by filtration through a 0.22 μm membrane filter (Millipore) and were stored at -20°C. All skin testing was done by intradermal injection of 0.1 ml of material. Several extracts and fractions were skin tested in non-immune guinea pigs for the presence of skin reactive factors. I would like to thank Dr. P. Coy, B.C.C.I. for arranging patient cooperation and performing the tests. Patients were injected with extracts and fractions of their own tumours, either diluted or undiluted. The diameter of erythema (but not always of induration) was read by Dr. Coy if possible, otherwise by the patients themselves at home. Most patients were tested for immunological energy by the ability to respond to recall antigens such as a 1:100 dilution of Candida (Hollister), 5 T.U. of

tuberculin (Connaught), and Mumps Skin Test Antigen (Eli Lilly).

XXII. Analysis of Extracts for CEA

CEA analysis was performed by the Hansen method (73) (Hoffman-La Roche) on undiluted and on a 1:5 dilution of the extracts. I would like to thank Dr. E. Mincey and Ms. B. L. Archibald at the Division of Nuclear Medicine, V.G.H. for performing these assays.

<u>RESULTS AND DISCUSSION</u>

I.   <u>Neonatal Tolerization of Rabbits to Antigens of Normal Lung Extracts</u>

The rationale of this technique is to render a neonatal rabbit

tolerant to the antigenic components of the malignant tissue, so that when

the rabbit is subsequently immunized with tumour extract, the predominant

response will be directed towards tumour specific antigens (38). A pool of

six normal lung extracts was used as the tolerogen in order to minimize any

histocompatibility differences. Normal lung from the cancer patient was not

used because it was suspected that microscopic foci of metastases might be

present or the TAA produced by lung tumours might be free to circulate as

does CEA from colonic tumours. Six rabbits were used for induction of neo-

natal tolerance and all survived. Sera from these rabbits one week after

immunization with alum precipitated tumour extract, when tested by immuno-

diffusion against both normal and tumour extracts, produced a very faint

precipitin band against tumour extract but not normal extract. It could

not be determined whether the difference detected was qualitative or quan-

titative. To increase the titre of these antisera so that these questions

could be answered, the rabbits were given booster injections of the same

alum precipitated tumour extracts. Subsequent testing of the antisera

showed multiple heavy precipitin bands against both normal and tumour

extracts. Apparently the tolerant state is not long lasting and is easily

reversed. This appears to be a common problem since no successes with this

technique have recently been reported.

Although the 3.0 M KCl method of extraction was originally used for

single cell suspensions (108), it was shown to be efficient even when finely

ground tissue was used. Attempts to further extract tissue pellets from

KC1 extracts by treatment in a Waring blender with saline indicated that

very little material could be liberated this way. Disc gel electrophoresis

of these samples showed that no new protein bands could be liberated from

the tissue pellets by this method. Other workers (18,72,83) have reported

successful tumour antigen solubilization for several tumours using hypertonic

KC1. Studies on guinea pig tumours (84) have shown that it is possible to

recover 15 to 40% of the tumour antigen activity from tumours extracted in

this manner. Immunologic specificity was maintained and the antigens were

stable at -70°C for several months. While it is probable that antigens may

be extracted by other agents such as detergents, it was decided to use KC1

exclusively in this study since other laboratories were starting to use this

method so extensively. It was felt that standardization of a single pro-

cedure would simplify data analysis at later stages.

II.   Migration Inhibition of Allogeneic Human Leukocytes by Extracts of

       Patients' Tumours

Tests for the production of factors by sensitized lymphocytes upon

reaction with specific antigen are among the best in vitro correlates of

cell-mediated immunity (16). Tests for the production of migration inhibition

factor (MIF) are possibly the most sensitive of these techniques and are

certainly the most intensively studied. Since cell-mediated immunity may

have a major role in tumour rejection, antigens detected by the MIF assay

with cancer patients' leukocytes would likely correlate with tumour specific

transplantation antigens (TSTA). This method has been used successfully to

detect solubilized guinea pig tumour antigens (72,124) and was found to be

more sensitive than delayed cutaneous hypersensitivity by requiring 1/4 to

1/25 of the antigen for detection. Tumour immunity was specific for the

particular tumour being studied and stronger in animals immunized with tumour than in tumour bearers. This method has also been used in hamster (15) and virus-induced mouse tumours (15, 131).

MIF experiments were performed using allogeneic leukocytes and extracts to standardize the technique while waiting for surgical specimens so that an autochthorous system could be tested. Migration index (MI) can be defined as:

$$\frac{\text{area of migration in test culture}}{\text{area of migration in control (no antigen) culture}} \times 100\% = MI$$

The results (Fig. 7) show a non-tumour specific inhibition of migration for all of the leukocytes tested against some of the tumours and normal tissue extracts tested. The ability of the extracts to elicit different responses with different leukocyte populations also made it impossible to determine the protein concentration which minimizes non-specific reactions. The protein concentrations used ranged from 47 µg/ml to 200 µg/ml. Other workers (115) used a protein concentration of human tumour extract of 100 µg/ml and claimed that non-specific inhibition may occur at concentrations greater than 200 µg/ml. For KCl extracts of breast carcinoma (75), concentrations greater than 1000 µg/ml were toxic to leukocytes whereas inhibition of migration occurred in the narrow range of 500 to 750 µg protein/ml.

Maluish and Halliday (78) report a method analogous to the MIF technique for the detection of cell-mediated immunity to human tumours: the leukocyte adherence inhibition test. Leukocytes from patients with a wide variety of tumours were tested against different tumour extracts. Inhibition of leuko-cyte adherence was induced by extracts of tumours of the same type as the cell donor, even at protein concentrations from 50 µg to 4000 µg/ml. Extracts of
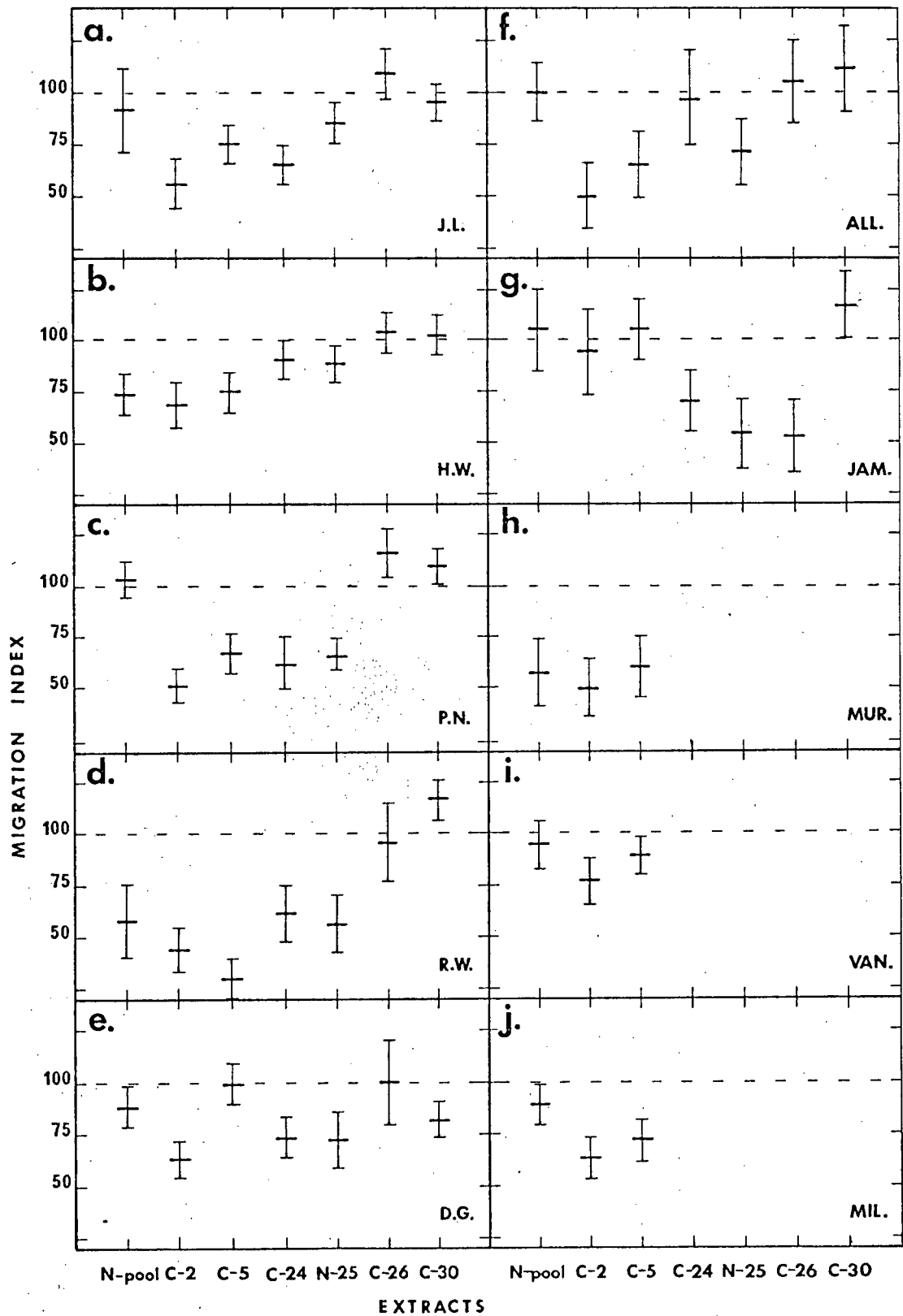
Fig. 7:   The effects of normal lung extracts (N-pool and N-25) and
          tumour extracts (C-2, C-5, C-24, C-26 and C-30) on the
          migration of leukocytes (including the standard deviation)
          from healthy donors (a-e) and heterologous lung cancer
          patients (f-j).

other tumour types did not cross react. The data in Fig.7 suggest that a normal extract control should be included in such studies.

The results of the normal extract controls in Fig.7 have been supported by the results of McIllmurray et al (76). They report that extracts of colonic tumours inhibit the migration of leukocytes from cancer patients slightly but significantly more than leukocytes from sex and age matched control donors. However, there was a considerable amount of overlap. in responses, and extract of normal mucosa from the same surgical specimen also produces similar results. Since their extracts were used at a final concentration of 1.0 to 2.0 μg./protein/ml, it is possible that the results obtained were due to non-specific cytotoxicity if healthy donor leukocytes are healthier and better able to handle the stress. Tumour antigens could be released from the tumour and trapped by the nearby normal tissue, although this is an unlikely explanation since migration inhibitions induced by both extracts were minor.

The occasional enhancement of leukocyte migration which has been observed (Fig. 7) has also been reported by others (23). Their results also frequently showed migration inhibition of healthy donor leukocytes by several extracts, and migration inhibition induced by normal tissue extracts.

Wolberg (141) suggests that at least part of the migration inhibition may be due to killing of the migratory cells by factors in the tumour extracts. This does not explain the results of Fig. 7 because: normal lung extracts also could inhibit migration, and tumour extracts did not induce migration inhibition with all of the leukocyte preparations. It has been suggested (75) that the unexplained results may be due to histocompatibility differences, but this is not likely the case with the results in Fig. 7 since the cell cultures were incubated for only 20 hr which should exclude any in vitro sensitization to histocompatibility antigens (75).

MIF tests using homologous tumour extracts and leukocytes were not
performed because we did not receive any surgical specimens during this time.
Even though statistical analysis might demonstrate significantly greater
reactivity with tumour extracts and cancer patients' leukocytes than with
normal controls, the frequent reactivity by the normal controls suggests that
at least some of the antigens detected were not tumour specific. Until such
antigens are eliminated from the extracts, MIF tests are not considered
suitable as an immunochemical assay for TAA. Further discussion of the
reactivity by the controls is included with the skin test results (section
VIII).

III.   Disc Gel Electrophoresis of the Extracts and their Fractions

The initial disc gel electrophoresis studies were performed on one of
the tumour extracts (C-26) and fractions separated by DEAE cellulose chroma-
tography with gradient elution (Fig. 8). The control extract (N-pool) was
separated into two fractions by step DEAE cellulose chromatography. One of
the fractions of C-26 (C-26-I) was further fractionated by chromatography on
Sephadex G-75 (Fig. 9).

By careful examination of the protein banding patterns, a band with an
Rf of 0.28 to 0.30 could be located in the tumour extract (C-26) which was
not present in the normal extract (N-pool) (Fig.10). This band was found in
the first fraction collected from DEAE cellulose (C-26-I) and the second peak
from Sephadex G-75 (C-26-Ib). This column was calibrated with Blue Dextran,
ovalbumin, chymotrypsinogen A, and ribonuclease, and the molecular weight of
C-26-Ib was determined to be between 21,000 and 60,000.

Further evidence that this component was tumour specific was obtained
by hyperimmunizing a rabbit with C-26-I. The antiserum was absorbed free of
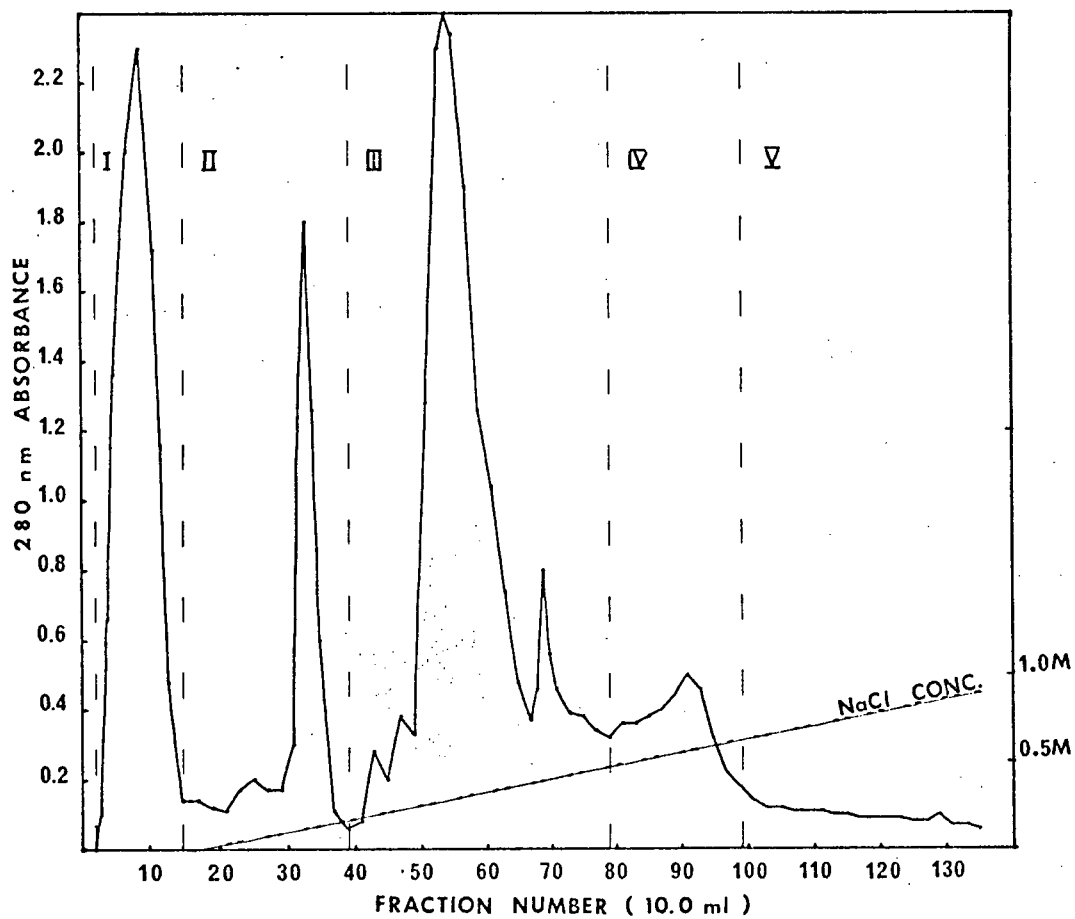
Fig. 8:   C-26 extract (40.0 ml) in 0.01 M phosphate buffer
          pH 7.5 was fractionated on a 2.5 x 20 cm DEAE cellulose
          column by a linear NaCl gradient.
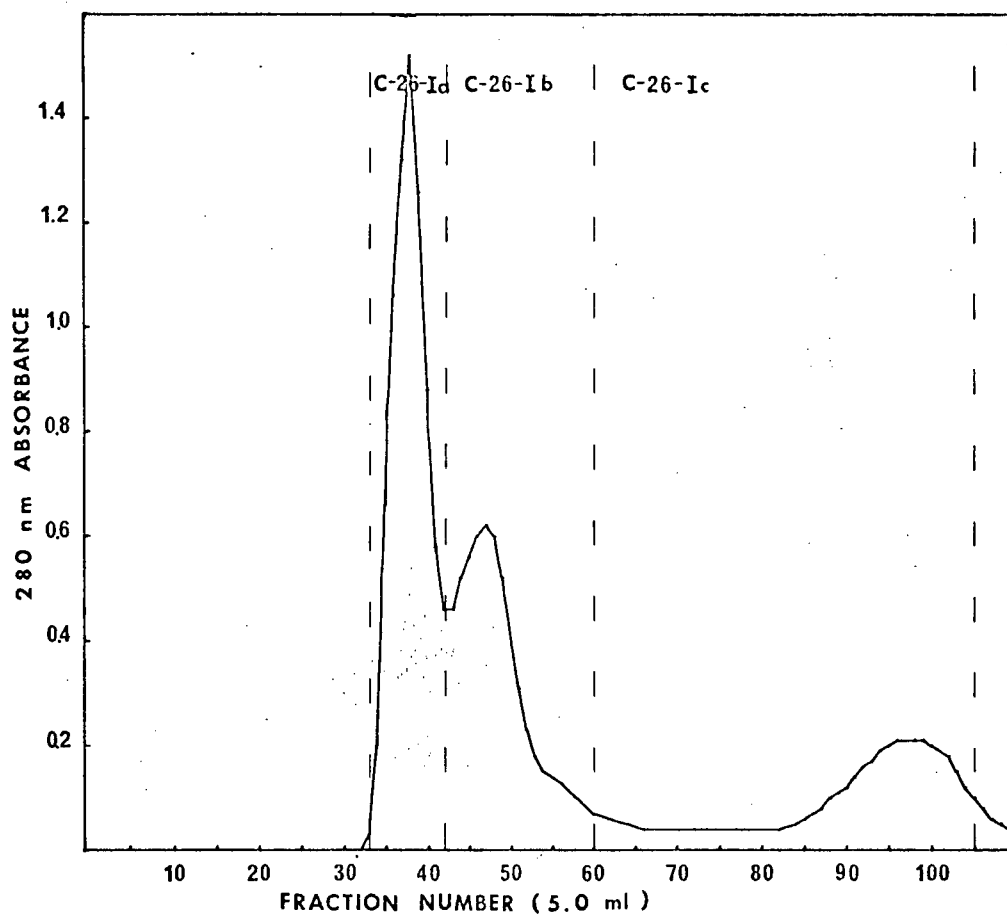
<u>Fig. 9:</u>   5.0 ml of C-26-I (10.0 mg/ml) fractionated on a
2.5 x 80 cm Sephadex G-75 column.
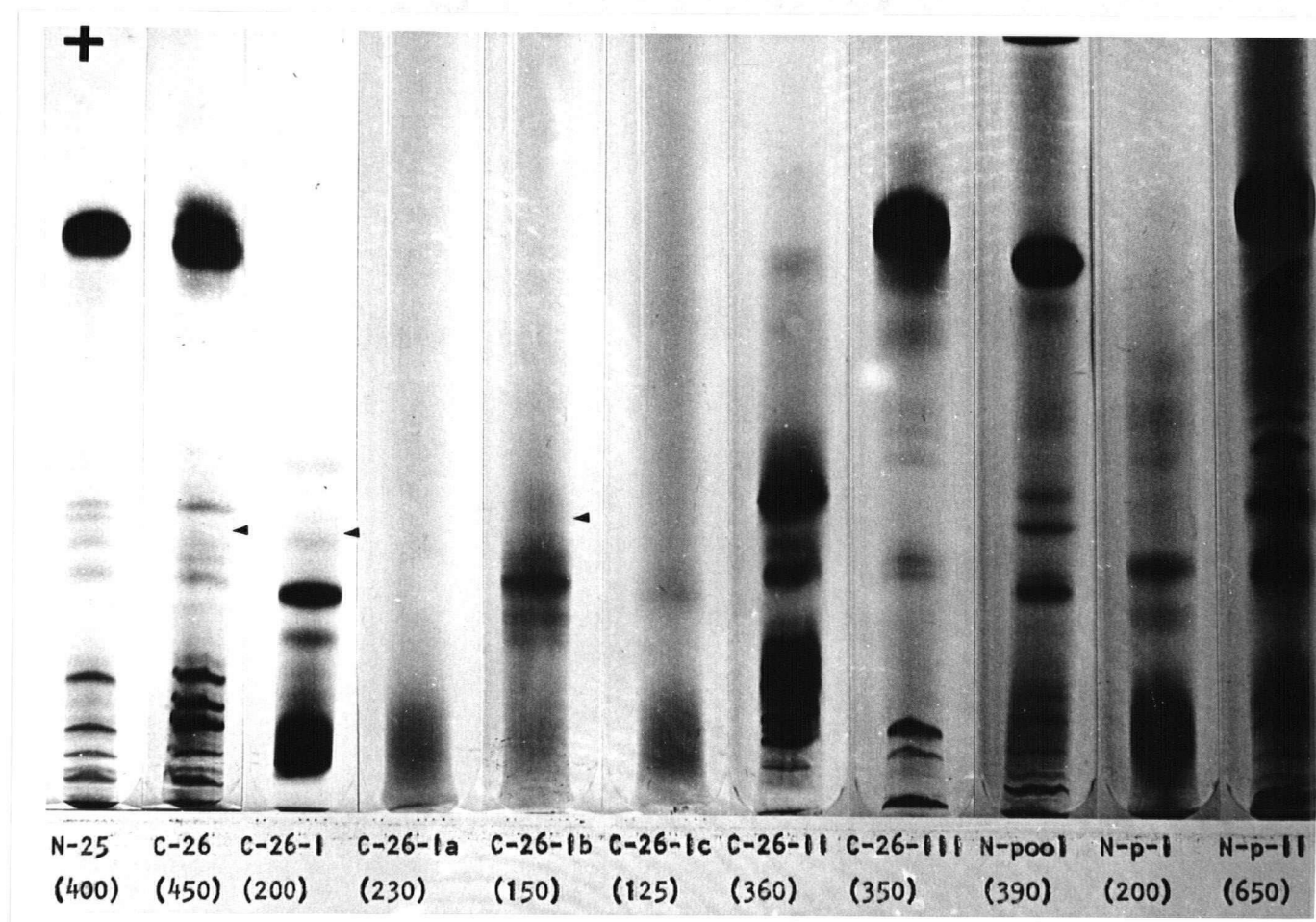
Fig. 10: Disc gel electrophoresis of fractions of C-26 tumour extract and normal extracts. The DEAE cellulose fractions of C-26 (I, II and III) were prepared by gradient elution and the fractions of C-26-I (a, b and c) were prepared by chromatography on Sephadex G-75. The fractions of N-pool (I and II) were prepared by step elution from DEAE cellulose. The figures in parentheses are the amounts of protein added to the gels (μg). The arrows point to bands not present in the normal extracts or fractions but present in C-26 and fractions.

anti (normal antigen) antibodies with an immunoadsorbent prepared by co-
valently coupling normal lung extract to Sepharose 4B. When tested by immuno-
diffusion, this absorbed antiserum did not react to give a precipitin band
against normal lung extract, but did against C-26, C-26-I and C-26-Ib (Fig.
11). This antigen was also precipitated by 50% saturated $(NH_4)_2SO_4$.

The properties of this TAA differ from the TAA detected in broncho-
genic carcinoma extracts by other workers. Both antigens detected by Yachi
et al (142) were larger than IgG and both antigens detected by Okada and Ikeda
(93) are not eluted from DEAE cellulose until the phosphate concentration
is greater than 0.05 M at pH 7.5. Tumour antigens from experimental animal
tumours have been reported with a wide range of properties. For example,
Baldwin et al (9) report an antigen with a molecular weight of 55,000, and
Meltzer et al (84) report an antigen of 75,000 to 150,000 molecular weight.

In order to test whether the TAA detected here was an embryonic anti-
gen, extracts of embryonic lungs were analyzed by disc gel electrophoresis
(Fig.12b). The tumour associated material detected in C-26 was not detected
in the embryonic extracts F-48 or F-49. However, this could be due to
enzymatic digestion in vivo or by previous extraction by the salt used to
induce the abortions.

Because the initial disc gel electrophoresis results looked so promis-
ing, several more extracts and fractions were also analyzed (Fig.12) in the
hope of finding a common TAA or other 'unique' antigens. From these photo-
graphs, it is obvious that the extracts display a wide variety of electro-
phoretic patterns. Similar results have been reported for guinea pig tumour
extracts (59). The difficulty of comparing grossly dissimilar electrophoretic
patterns, and the inability to detect the TAA of C-26 in other extracts,
limit the usefulness of the technique. (The antigen in C-26 was detected in
a few other tumour extracts, but only after a large number were tested). The
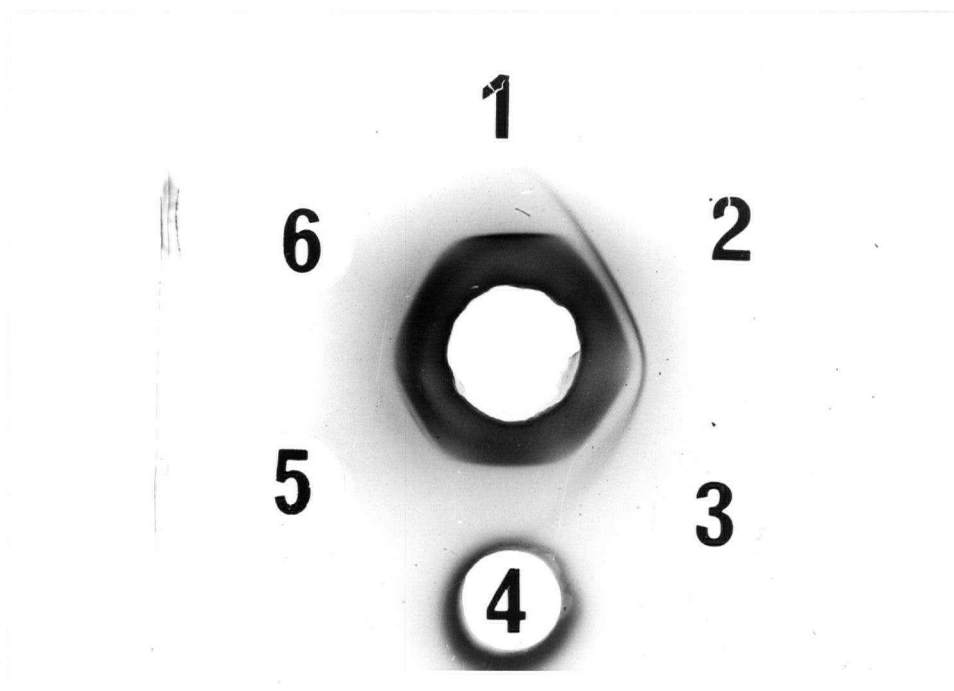
Immunodiffusion results using abosrbed antiserum to C-26-I
against extracts and fractions designated as follows:

1. C-26-Ib   2. C-26-I   3. C-26   4. N-pool

5. C-26 fraction insoluble in 50% saturated $(NH_4)_2SO_4$

6. C-26 fraction   soluble in 50% saturated $(NH_4)_2SO_4$

Note that the precipitin band which is obvious against C-26
and C-26-I can also be seen near the C-26-Ib well.

Fig. 12a:

C-24 C-24-I C-24-II C-24-III C-30 C-30-I C-30-II C-30-III C-46 C-46-I C-46-III
(425) (175) (200) (325) (600) (340) (400) (750) (460) (475) (700)
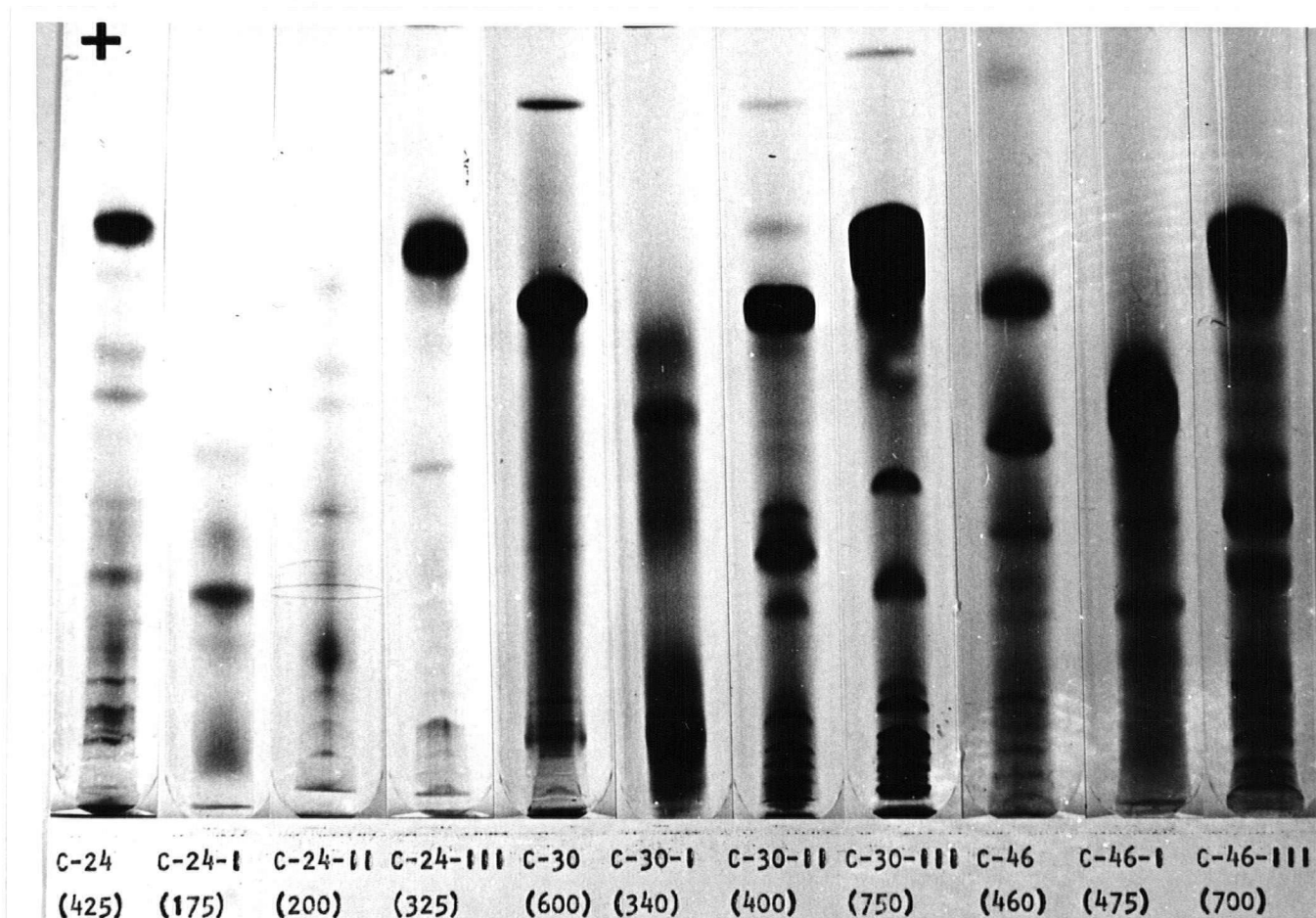
Fig. 12: Disc gel electrophoresis results of: a. tumour extracts and DEAE cellulose fractions and b. foetal extracts (pooled) and DEAE cellulose fractions and other tumour extracts. The tumour specific component in C-26 (arrow) could not be demonstrated clearly in any other extracts or fractions. Note the wide variation of band patterns and the large number of bands. The figures in parentheses are the amounts of protein (μg) added to the gels.
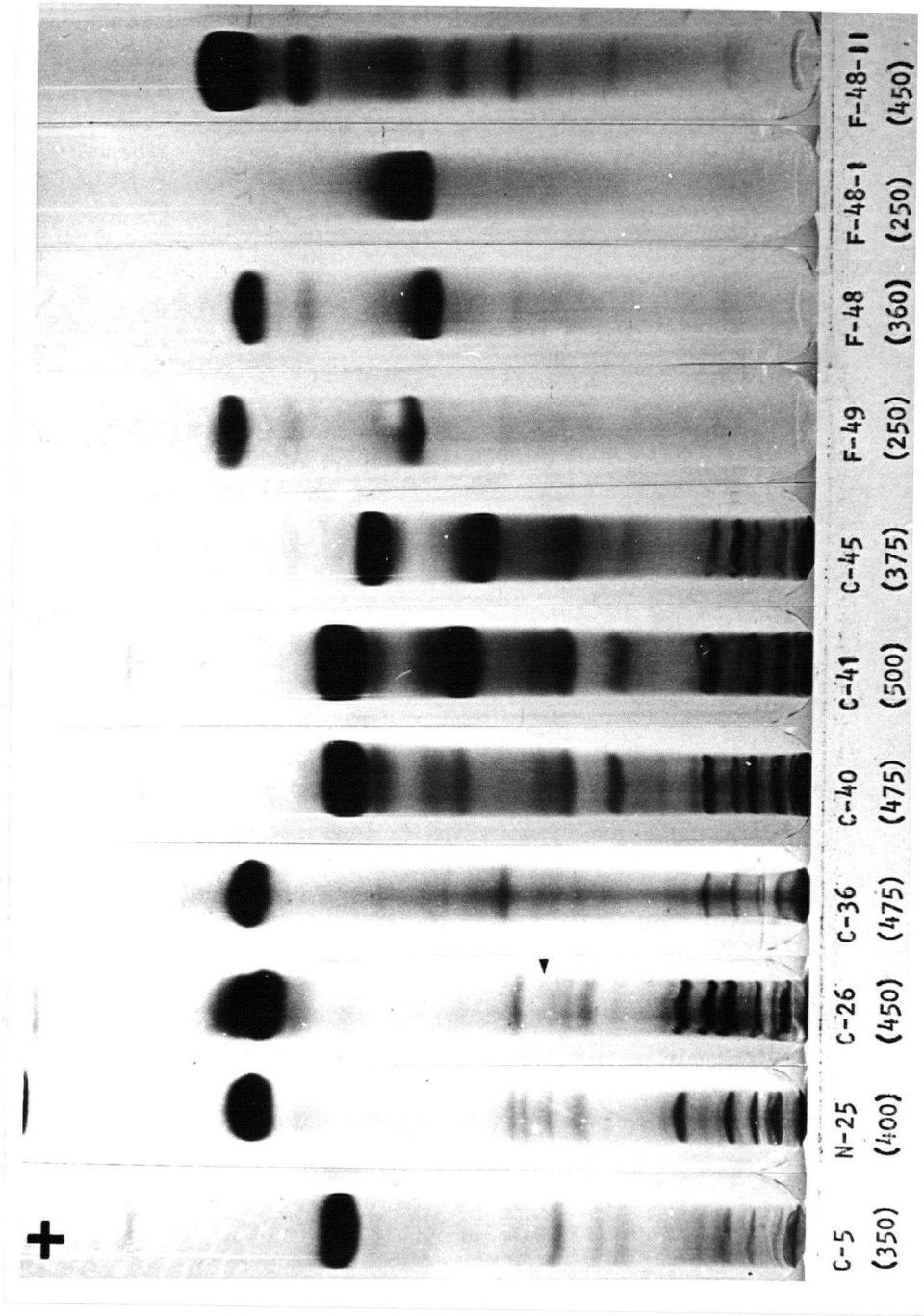
Fig. 12b:

minor differences between the extracts make it difficult to decide which

band to focus attention upon in determining antigenic properties. Neverthe-

less, on the basis of these results it is clear that TAA were present in some

tumour extracts.

Using doubling dilutions of bovine serum albumin (Sigma), it was

determined that 2.5 μg of protein could be detected by the disc gel electro-

phoresis conditions used. Although the sensitivity of disc gel electro-

phoresis can be increased by staining with Coomassie Blue instead of amido

black (77), sensitivity was generally not the main problem. The large quantity

of other materials present could obscure tumour specific bands, making

comparison difficult.

Disc gel electrophoresis would be of more use at a later stage of the

studies as a step in purification or a test for purity, but at this time was

not pursued. Further studies on the biochemistry of the TAA detected in C-26

were not done because the apparent uniqueness of this antigen limited its

usefulness.

IV.   Immunodiffusion Studies

1.   The Detection of TAA in Extracts Using Absorbed Rabbit Antisera

One of the more common methods of demonstrating the presence of human

TAA is with the use of antisera from animals hyperimmunized with tumour

extract or tissue. The animals must be hyperimmunized in order to induce a

detectable antibody response against the minor antigens present. Antigenic

competition by the large quantities and number of normal antigens could

inhibit the antibody response to the very low concentration of TAA. It has

been suggested (55) that HL-A antigens comprise at most 1% of the total

membrane protein extracted by 3.0 M KC1.   TSTA is probably much less than 1%

of the tumour extract because there is probably much less TSTA than HL-A

antigen.   For a tumour extract with 10.0 μg protein/ml, the maximum amount

of TSTA would be less than 100 μg/ml.   This quantity would be within the limit

of sensitivity for the immunodiffusion technique of 1.0 μg protein/ml (35)

only under optimum conditions.   Since it is unlikely that a high titre anti

(TSTA) serum would be produced by immunization with a crude extract, any TAA

detected by this technique would probably not be TSTA.

A.   The Use of Immunoadsorbents

The large quantities of antibodies to normal antigens must be removed

from the antisera.   This is usually done by absorbing the antisera with

normal cells and tissues.   Large volumes of tissue and antisera would be

required to absorb these antibodies.   It can also be difficult to reproduce

the absorption when studying human tumours, as the tissues do not store well

and different tissues can vary considerably in their expression of antigenic

components.   Another disadvantage to the use of cells for absorbing antisera

is that internal cell components, against which antibodies may be developed,

are not necessarily present on the cell surface, and will therefore not be

removed by such a procedure.   Extracts, however, store well as measured by

the reproducibility of immunodiffusion and disc gel electrophoresis results.

A technique was developed whereby anti (normal) antibodies in the rabbit

antisera were removed on a solid phase immunoadsorbent prepared by the

 coupling of normal lung extract proteins to cyanogen bromide activated

Sepharose 4B.   The antisera were then concentrated and tested for antibodies

against tumour and normal extracts by semi-micro immunodiffusion.   Examples

of whole antiserum compared to absorbed antiserum are shown in Fig.13a and
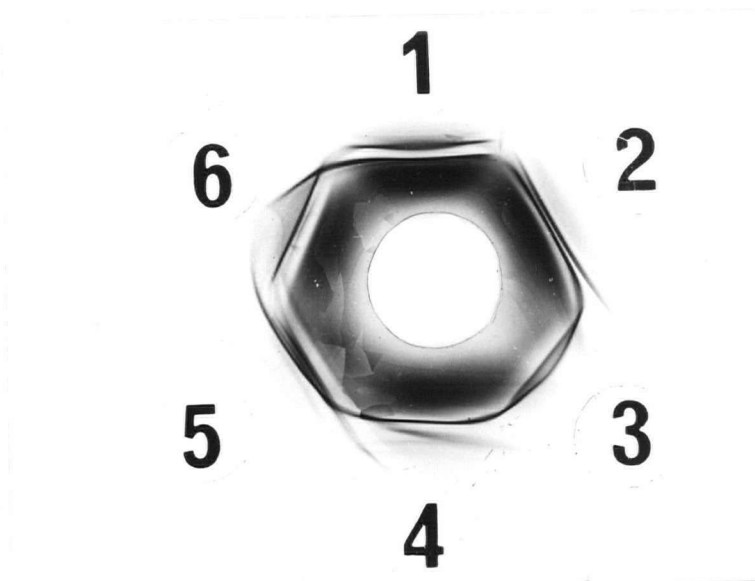
Fig. 13a:
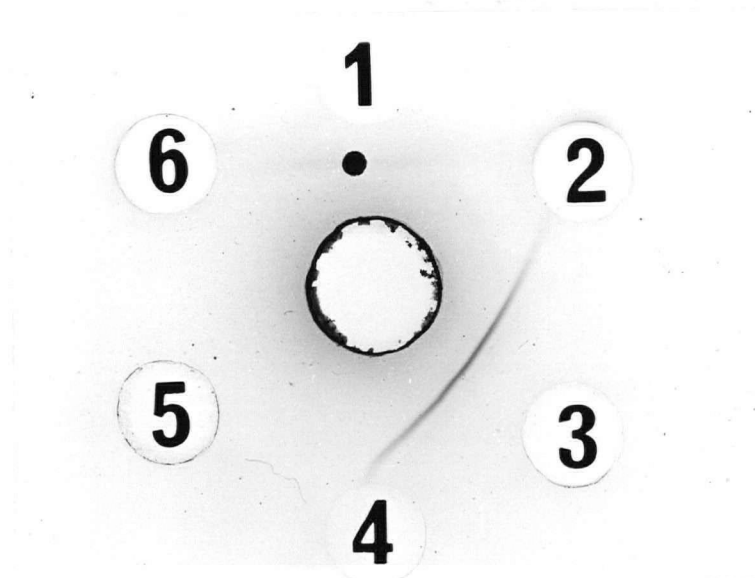
Fig. 13b:
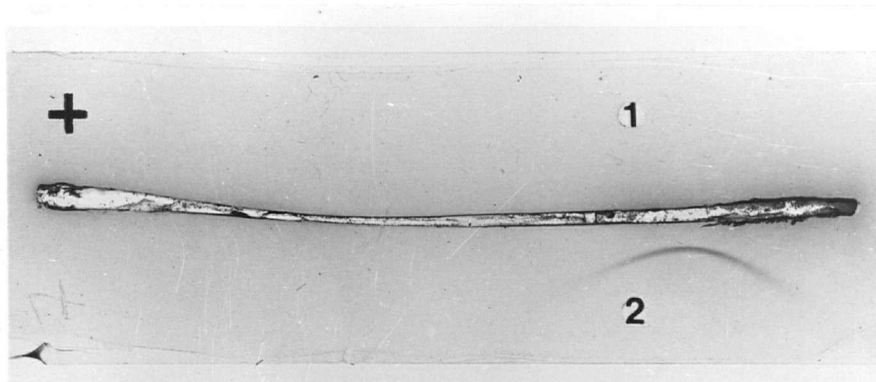
Fig. 13c:
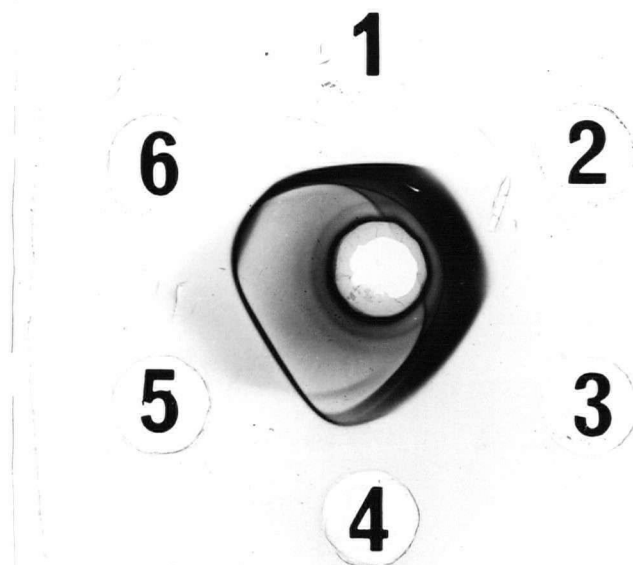
Fig. 13d:

Fig. 13:   Immunodiffusion and immunoelectrophoresis results using
                  rabbit anti-C-26.

a.   Immunodiffusion of whole anti-C-26 against:

        1. C-26   2. N-pool   3. C-30
        4. C-40   5. C-24   6. C-41

b.   Immunodiffusion of absorbed anti-C-26 against:

        1. C-26   2. N-50   3. C-26-I (DEAE cellulose fractions)
        4. C-40   5. C-24   6. N-pool

c.   Immunoelectrophoresis of absorbed anti-C-26 against:

        1. N-pool and 2. C-26

        Note that this absorbed antiserum appears to be thoroughly absorbed
        and that only one tumour antigen is resolved.

d.   Immunodiffusion of eluted 'anti-normal' antibodies from anti-C-26
        serum against:

        1. C-26   2. C-41   3. C-24
        4. C-40   5. C-30   6. N-pool

13b respectively. The antiserum in Fig.13b was absorbed by one pass through the column. This can also be demonstrated by immunoelectrophoresis (Fig.13c). That these results were not strictly due to inactivation of antibodies was demonstrated by eluting the adsorbed antibodies and testing by immunodiffusion as in Fig.13d. By eluting the antibody and re-equilibrating in starting buffer, the columns were used for up to 10 times.

Most of the antisera could not be absorbed as completely as that in Fig. 13b,c. More representative results are demonstrated in Fig. 14. With many of the antisera, none of the methods tried could remove all of the anti (normal) antibodies. A range of volumes (0.5, 1.0, 2.0 and 4.0 ml) of 4 antisera were absorbed by a 25.0 ml immunoadsorbent column. Up to 2.0 ml could usually be absorbed with equal effectiveness, but even the small volume (0.5 ml) of some antisera were not completely absorbed. Larger immunoadsorbent columns (70.0 ml) could handle 4.0 ml of antiserum but 0.5 ml of some antisera were still not completely absorbed. Pre-absorbing the antisera with normal lung extract by precipitation of immune complexes, and pre-absorbing the antisera with normal lung tissue were also not effective at eliminating all of the anti (normal) antibodies. (see Materials and Methods, section XIV). The best results were obtained by a combination of the Sepharose immunoadsorbent followed by a batch immunoadsorbent using normal extract insolubilized by polymerization with glutaraldehyde (Fig. 15). Although the anti (normal) antibodies were often not completely eliminated even by this combination of methods, the diminished intensity of the reaction made visualization of tumour-specific precipitin bands clearer and the interpretation easier. If tumour associated bands could not be detected or if the normal component bands were prevalent the antisera were re-absorbed a third or more times. The adsorption capacity of the glutaraldehyde conjugated immunoadsorbents was considerably decreased after use,
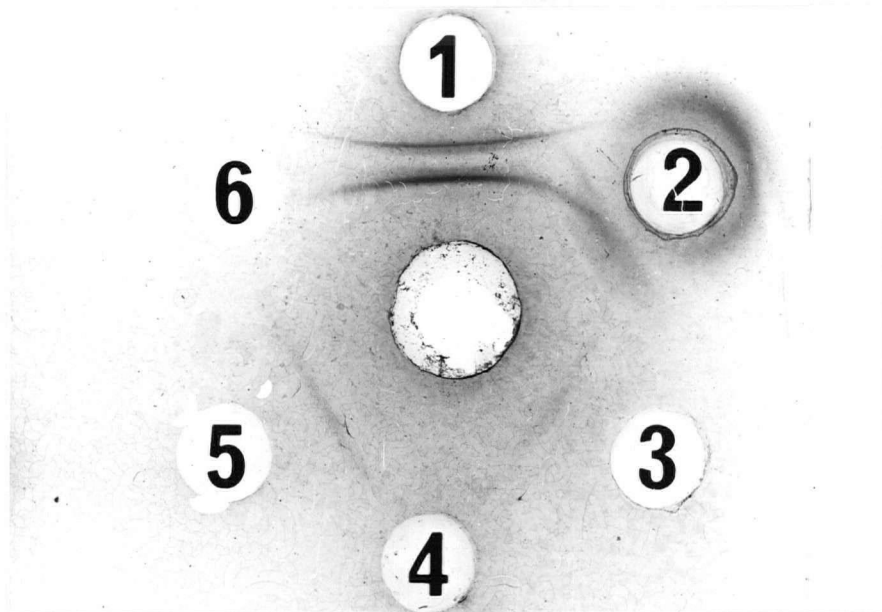
Fig. 14a:
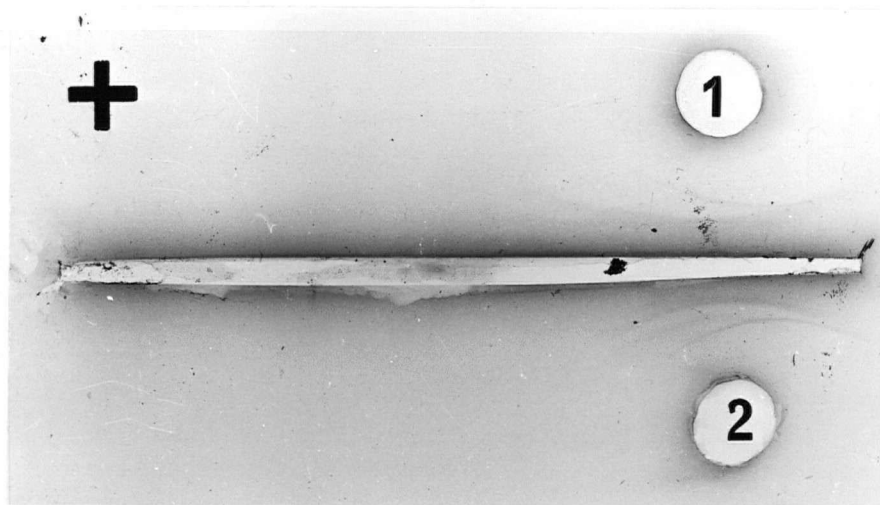
Fig. 14b:

Fig. 14:   Immunodiffusion results using absorbed antiserum to
C-40 against: a. extracts designated as follows:

1. C-40   2. N-50   3. C-24
4. C-6   5. C-26   6. PBS

and immunoelectrophoresis results against: b.

1. N-pool   2. C-40

Note that a tumour associated precipitin band can
be resolved by counting the bands.

Fig. 15a:
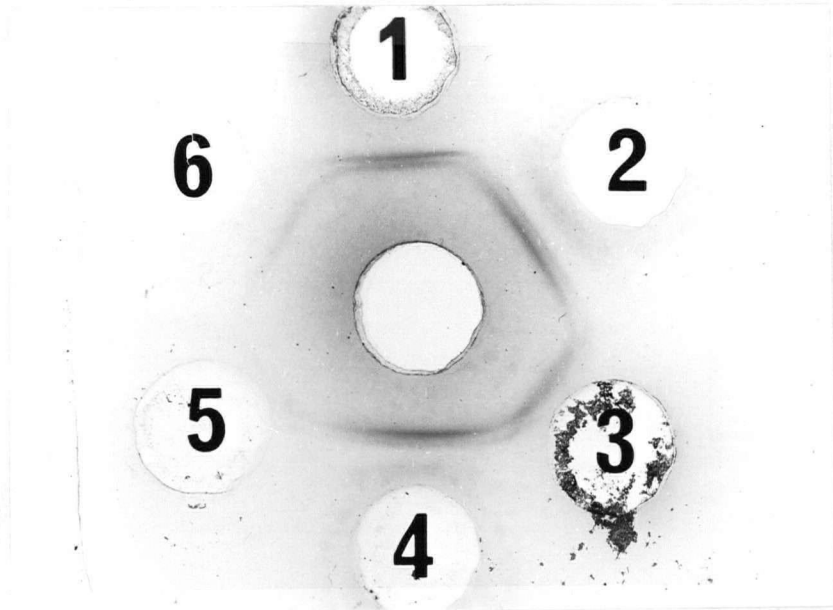
Fig. 15b:
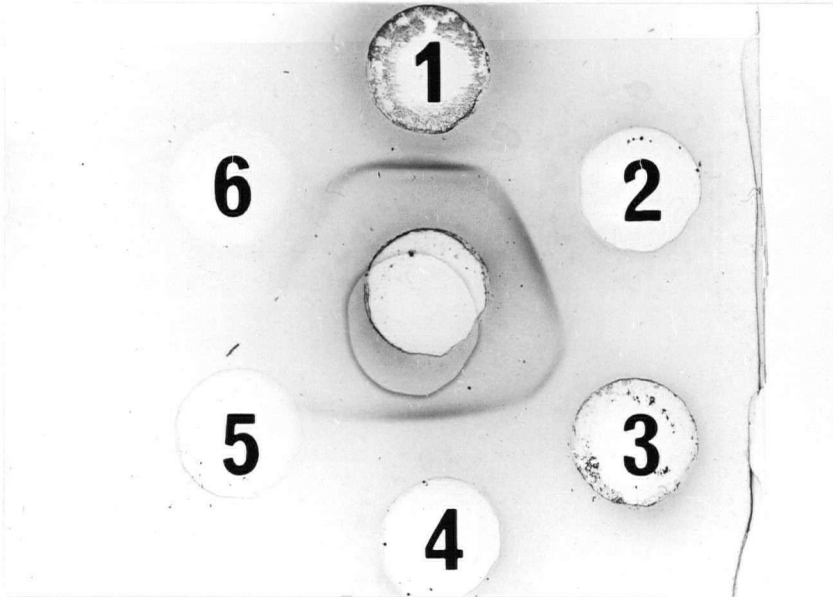
Fig. 15:   Immunodiffusion results using absorbed antiserum to C-67
            against six extracts designated as follows:

            1. C-67   2. N-pool   3. C-67 diluted 1:1
            4. N-pool 5. C-67 diluted 1:9 6. N-pool diluted 1:9

            The antiserum in part a, was absorbed once through an
            immunoadsorbent of Sepharose 4B insolubilized N-pool. The
            antiserum in part b was re-absorbed with glutaraldehyde
            insolubilized N-pool. Note that the number and intensity
            of precipitin bands is less after re-absorption of the
            antiserum.

so were used only once.

B.   The Detection of Tumour Associated Antigens

The results of a survey of 15 absorbed antisera tested against a panel of extracts by immunodiffusion are presented in Table I, according to tumour pathology.   Each series of 6 extracts included one sample of homologous extract and at least one normal or foetal lung extract.   The normal lung extracts tested were N-pool, N-25, N-50 and N-83.   The extracts were used at the protein concentration attained by the original extraction.   The majority of the extracts had protein concentrations between 5.0 and 15.0 μg/ml with the exception of normal extracts which were used at higher protein concentrations (see Appendix A).   The reason for using normal extracts at protein concentrations higher than the tumour extracts in this study was that it was hoped that simple quantitative differences in antigenic components between normal and tumour extracts might be resolved by this approach.   It is recognized that such a precaution would only eliminate false positives of this nature if quantitative differences were in the range of 5 fold (the difference in protein concentration between normal and tumour extracts).   Greater differences would not be detected.   However, if consistant quantitative differences of this magnitude were found and could be shown to be tumour related, such an observation would still have potential diagnostic value, even though the component might not be unique to the tumour.   Several extracts and antisera were also tested at dilutions ranging from 1:1 to 1:4, but in no case could previously undetectable bands be seen, although some bands became sharper.

The results from this extensive study (Table I) were not easy to interpret in all instances.   After all of the absorbed antisera were tested,

Table I:

Immunodiffusion results using absorbed antisera to extracts from tumours
of 5 main types of pathology tested against individual extracts of tumours
of a variety of pathologies, all thought to arise from the lung. A
positive result means that the precipitin band was not detected in any of
the normal lung extracts (N-pool, N-25, N-50 and N-83). A '±' was used if
there was any reason to doubt a positive result. A 'i' indicated that the
precipitin band was closer to the central than the outer wells.

    a.   Absorbed antisera vs. squamous cell carcinoma extracts

    b,   Absorbed antisera vs. adenocarcinoma and anaplastic carcinoma
        extracts

    c.   Absorbed antisera vs. oat cell and alveolar cell carcinoma, a
        variety of less common lung tumour, and foetal lung extracts

Table Ia:

Absorbed Antisera	Antigen Extract Squamous Cell Carcinoma																
Squamous Cell Carcinoma	C-1	C-6	C-53	C-60	C-61	C-63	C-66	C-69	C-71	C-74	C-75	C-76	C-79	C-87	C-90	C-93	C-94
C-6	+	+	+	±	+	-	+	-	+	+	-	+					
C-53	-	±	+	+	+	+	±	±	-	+	+	+	-	+	+	+	+
C-57	-	±	+i	+i	+i	+i	++i	+i	+i	+i	+i	+i	+i	+i	+i	+i	+i
C-63	+i	+	+i			+i	+i	+i	+i	-	-	-	+i	-	-	-	-
C-66	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-
C-71	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	±
Adeno-Carcinoma																	
C-24	±	-	-	-	-												
C-26	-	-	-	-	-		±	-	-	-	-	+	-	-	-	-	-
Anaplastic Carcinoma																	
C-41	+i	+i	+i	+i	+i	+i	+i	+i	-	+i	+i	+i	+i	+i	+i	+i	+i
C-46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C-62	-	+	+i	+i	+i	-	+	+i	+i	-	-	-	+	-	+	-	+
C-67	-	-	+i	-	+i	+i	±	±	-				-	-	-		
Oat Cell Carcinoma																	
C-40	±	±	-	±	+	±	-	-	-	+	-	+	-	-	-		
C-78	±	+	±	-	-	-	-	+	++i	-	-	+	+	+	-	+i	+
Alveolar Cell Carcinoma																	
C-30	-	+	+	±	±	+		-	-	±	±	±	-	-	-	-	-

Table Ib:

Absorbed Antisera	Antigen Extract														
	Adenocarcinoma									Anaplastic Carcinoma					
Squamous Cell Carcinoma	C-3	C-5	C-24	C-26	C-36	C-45	C-58	C-85	C-88	C-41	C-46	C-56	C-62	C-67	C-92
C-6	−	−	−	−	−	+	−			+	+	+	+	±	
C-53	−	±	−	−	−	±	−	−	±	±	±	+	+	+	−
C-57	−	−	−	−	−	+i	−	+i	+i	+i	+i	+i	+i	+i	+i
C-63	−	−	−	±	+i	−	−	+i	−	+	−	+i		+i	−
C-66	−	−	−	−	±	+	−	−	−	−	−	−	−	±	−
C-71	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adeno-Carcinoma															
C-24	−	−	+	−	+	−	−			+	±	−			
C-26	−		−	+	+	−	−	−	−	−	−	−		−	−
Anaplastic Carcinoma															
C-41	−	+i	−		+i	±	+i	+i	+i	+i	+i	−	+i	+	−
C-46	−	−	−	−	−	±	−	−	−	±	±	−	−	−	−
C-62	−	−	−	±	−	+i	−	−	+i	++i	+i	+i	++i	+	−
C-67	−	−	+i	+	+	+	−	+i	−	+i	+	+i	−	+i	−
Oat Cell Carcinoma															
C-40	−	−	−	−	−	±	−	−	−	±	−	±	±	−	
C-78	−	−	−	−	−	±	−	±	−	−	±	−	+	±	−
Alveolar Cell Carcinoma															
C-30	−	−	+	+	+	−	−	−	−	−	−	−	−	+	−

Table Ic:

Absorbed Antisera	Antigen Extract												
	Oat Cell Carcinoma						Alveolar Cell Carcinoma		Carcinoid	Metastatic squamous *	Mesothelioma	Mixed squamous-Adenocarcinoma	Rhabdomyo-sarcoma	Feotal Lung
Squamous Cell Carcinoma	C-40	C-64	C-65	C-70	C-72	C-73	C-30	C-81	C-4	C-57	C-54	C-82	C-77	F-48
C-6	-	-	-	±	+	+	-		-	+	-		+	-
C-53	±	+	±	-	+	+	±	+	-	-	-	-	+	-
C-57	-	-	+i	+i	+i	+i	+i	+i	+i	++i	-	+i	+i	-
C-63	-		+i	+i	+i	-	±	-	-	+i	+i	+i	-	-
C-66	-	-	-	-	±	-	±	-	-	-	÷	-	-	-
C-71	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adeno-Carcinoma														
C-24	-						±			-		-		-
C-26	-	-	±	-	-	-	+	-	±	-	-	±	-	-
Anaplastic Carcinoma														
C-41	+i	+i	+i	+i	-	-	-	+i	-	+i	+i	+i	+i	+i
C-46	-	-	-	-	-	-	±	-	-	-	-	-	-	-
C-62	+i	-	+	+i	+i	-	+	-	-	-	-	-	+	-
C-67	-	-	+	-	-		+	-	-	+i	+i	±		-
Oat Cell Carcinoma														
C-40	+	-	-	+	-	+	+	-	-	±	-	-	-	-
C-78	-	-	-	-	-	+i	-	-	-	-	-	-	+	-
Alveolar Cell Carcinoma														
C-30	-	+	-	+	±	-	+	-		-	-	-	-	-

* metastasized from the lung to the mouth

the immunodiffusion slides were evaluated independently by myself, Anajane

Smith and Professor Julia Levy. Positive results which were not definite

were labelled "±" in order to minimize the possibility of including false

positives in the study. It should be emphasized that false negative results

are expected because of the limited sensitivity of the technique. (The

pathology of the tumours was not known at this time).

There was considerable variation from one antiserum to another in

terms of the degree of cross-reactivity, the presence of any tumour specific

bands and the number of apparently tumour specific antigens detected.

Because of the marked differences between individual antisera in Table I

each one will be discussed briefly here.

C-6. This antiserum was absorbed only by the column procedure and

did not present any major problem in terms of anti-normal activity. It was

a relatively weak antiserum and anti-tumour bands were faint (Fig. 16). A

considerable degree of cross-reactivity with other squamous cell carcinoma

and anaplastic carcinoma extracts was noted (Table I).

C-53. This antiserum retained considerable anti-normal antibody

even after multiple absorptions, as seen in Fig.17. The immunodiffusion

results were difficult to interpret, but anti-tumour bands which cross-reacted

extensively with other types of tumour pathology were found (Table I).

C-57. Even after both column and batch absorption, this antiserum

still had considerable anti-normal activity (as did most of the antisera).

Further absorption decreased the intensity of all of the bands, not just the

anti-normal bands. A tumour associated inner band ("i") could be observed

towards the centre well (Fig.18). This antibody was very cross-reactive

with all tumour types except adenocarcinomas, against which there was only

moderate(3 of 9) cross-reactivity (Table I). Another tumour associated

band was observed occasionally (C-57 and C-69 in Fig.18).

Fig. 16:   Immunodiffusion results using absorbed antiserum to
            C-6 against six extracts designated as follows:

1. C-6   2. C-1   3. C-2

4. N-pool   5. C-3   6. C-5

Note the weak positive reaction against C-6 and C-1.

<u>Fig. 17:</u> Immunodiffusion results using absorbed antiserum to C-53 against six extracts designated as follows:

1. C-53   2. C-57   3. C-58

4. N-pool   5. C-60   6. C-61
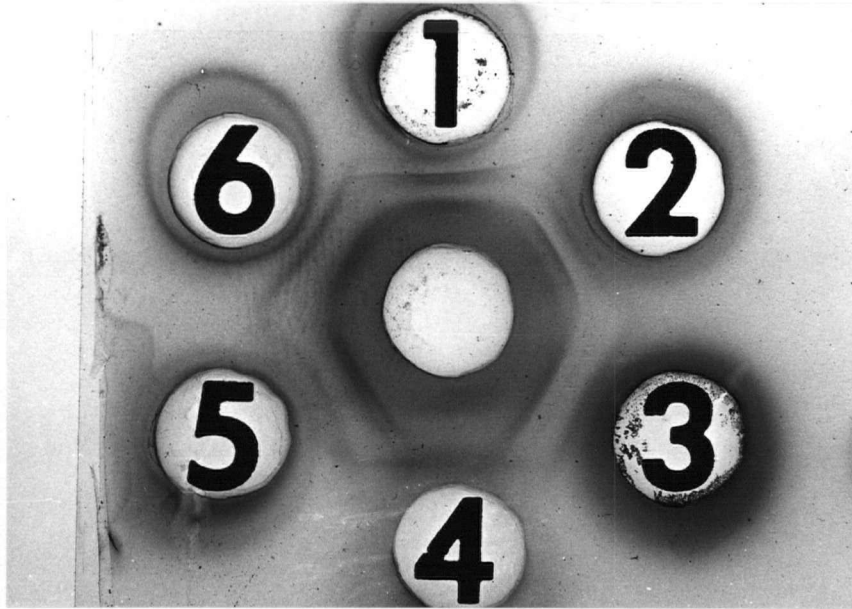
Fig. 18:   Immunodiffusion results using absorbed antiserum to
             C-57 against six extracts designated as follows:

1. C-57   2. C-66   3. C-67

4. N-pool   5. C-53   6. C-69

Note the inner band which shows a line of identity
with all of the extracts but N-pool.

C-63. This antiserum was similar to anti-C-57, only there were more anti-normal bands and weaker tumour bands, making analysis more difficult (Fig.19). TAA were not detected as often with this antiserum as with anti-C-57 (Table I).

C-66. This antiserum was very weak (in spite of hyperimmunization of the rabbit). A single absorption could remove almost all of the antibody. However, antigen was detected in one extract (C-45) and suspected in 5 others (Table I).

C-71. Anti-C-71 was stronger than anti-C-66, but was still too weak for a good analysis of the extracts. When the precipitin bands are faint and not long enough to meet the bands of the adjacent wells, it is difficult to determine identity or non-identity of the components, and the bands cannot be compared (Fig.20). Nevertheless, TAA were detected in 3 of the squamous cell carcinoma extracts (Table I). A possible explanation for the inability to detect TAA in the homologous extracts is given in the discussion of anti-C-62.

C-24. This was a very weak antiserum from a rabbit not as intensively hyperimmunized as later rabbits. Absorption was easy and efficient (Fig.21), but TAA were detected in only 6 of the extracts tested (Table I).

C-26. This antiserum could be easily and efficiently absorbed, and was much stronger than anti-C-24. More than one TAA could be detected with homologous extract (Fig.22), but these could only be detected with certainty in 4 of the extracts (Table I), which limits the usefulness of the TAA. The infrequent cross-reactivity was in agreement with the disc gel electrophoresis results (section III), where the tumour associated materials could not be detected in any of the other extracts tested. The antigen(s) was stable for several months whether stored at -20°C and freeze-thawed often, or stored at 4°C (sterile).

Fig. 19:   Immunodiffusion results using absorbed antiserum to
            C-63 against six extracts designated as follows:

            1. C-63   2. C-1   3. C-2

            4. N-pool   5. C-3   6. C-4

            Note the presence of precipitin band with C-63 and
            C-1, slightly inside (i) the heavier outer bands.

Fig. 20: Immunodiffusion results using absorbed antiserum to C-71 against six extracts designated as follows:

1. C-71   2. C-79   3. C-81

4. N-83   5. C-82   6. C-85
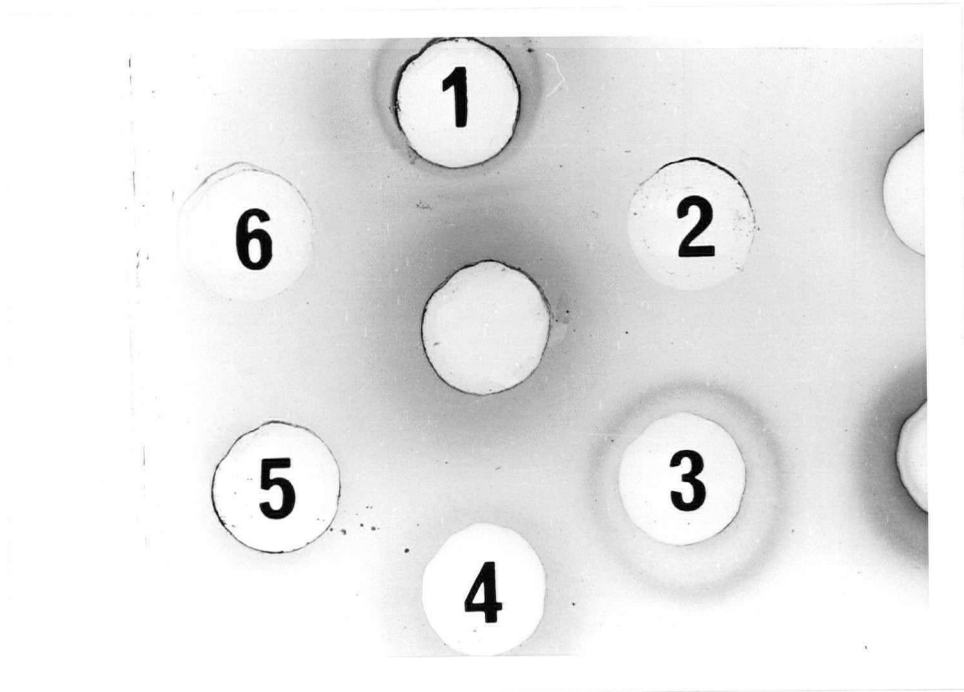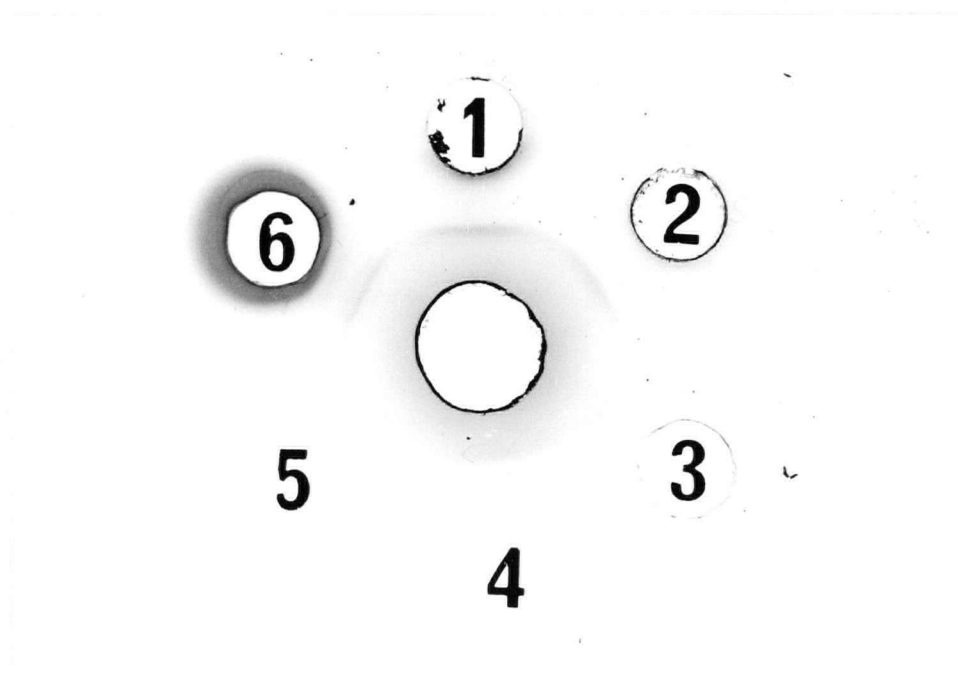
<u>Fig. 21:</u>   Immunodiffusion results using absorbed antiserum to
              C-24 against six extracts designated as follows:

1. C-24   2. C-26   3. F-48

4. N-50   5. C-2   6. C-5

This was a particularly weak antiserum which could
be effectively absorbed, resulting in a single,
faint precipitin band with C-24.

Fig. 22: Immunodiffusion results using absorbed antiserum to C-26 against six extracts designated as follows:

1. C-26   2. N-pool   3. C-6
4. C-24   5. C-40   6. C-30

Note the presence of more than one precipitin band with C-26, one of which is also found with C-30.

C-41. This antiserum was very similar to anti-C-57 in terms of the

wide cross-reactivity of an inner precipitin band (Table I). This anti-

serum detected the presence of an antigen in foetal lung extract which showed

partial identity (by spur formation) with an antigen in C-63 (Fig.23). No

other antisera showed any tumour associated reactivity with foetal extracts.

Tests were done later to determine if the TAA was carcinoembryonic antigen

(CEA) or α_1-foetoprotein (AFP). The results (section V and IV.2.) demon-

strate clearly that the antigen detected by anti-C-41 was neither of these

antigens.

C-46. This was a fairly strong antiserum (Fig.24), but it could not

irrevocably detect TAA. Frequent reactions of C-46 extract with other anti-

sera (Table I) suggest that a TAA was present in the extract. Several

different immunoabsorbents (N-50, N-pool and a pool of all of the normal

extracts prepared with both Sepharose 4B and glutaraldehyde) were used without

success. These factors suggest that properties of the immunological system

of the individual rabbits may be important in the production of anti-TAA.

C-62. This was one of the more interesting antisera studied. Anti-

normal reactivity could be removed effectively if the antisera were absorbed

more than twice. Inner bands were detected in 12 of the tumour extracts,

but the strong outer band (Fig.25) was of far more interest. (Similar

precipitin bands were observed with anti-C-78). Finding such an antigen in

an appreciable number (ie. 11, Table I) of extracts suggests that this

antigen might be of use for diagnostic purposes. Recent results have shown

that the antigen of Fig.25 was not detectable in extracts which had been

freeze-thawed repeatedly, even though it was readily detectable in extracts

which had been stored at -20°C (Fig.26). Therefore, it is probable that

this antigen is even more common than indicated by Table I . (Thermal

denaturation or enzymatic degradation could be responsible for the loss of
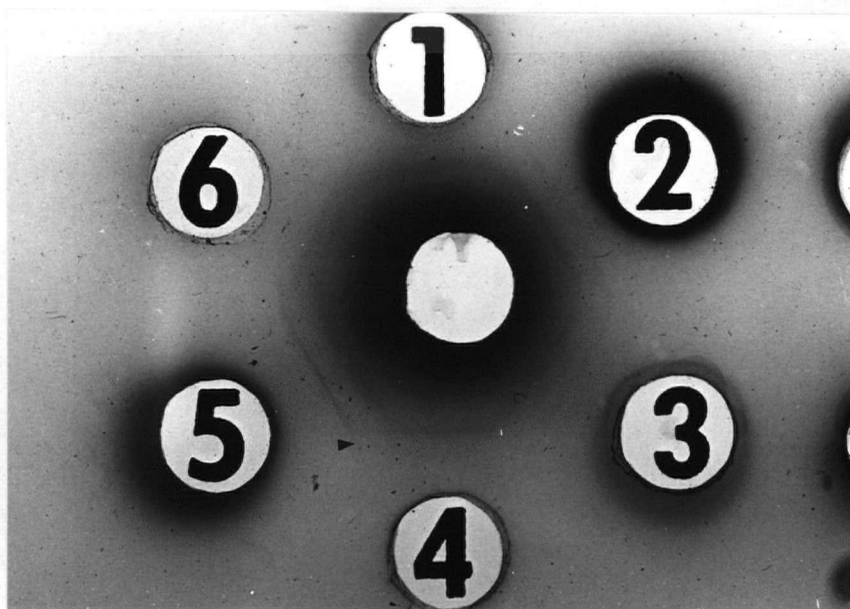
Fig. 23:   Immunodiffusion results using absorbed antiserum to
            C-41 against six extracts designated as follows:

1. C-41   2. C-65   3. C-70
4. F-48   5. C-63   6. C-85

The precipitin band against C-63 shows partial
identity with an antigen in F-48. None of the
normal lung extracts demonstrated such a reaction.
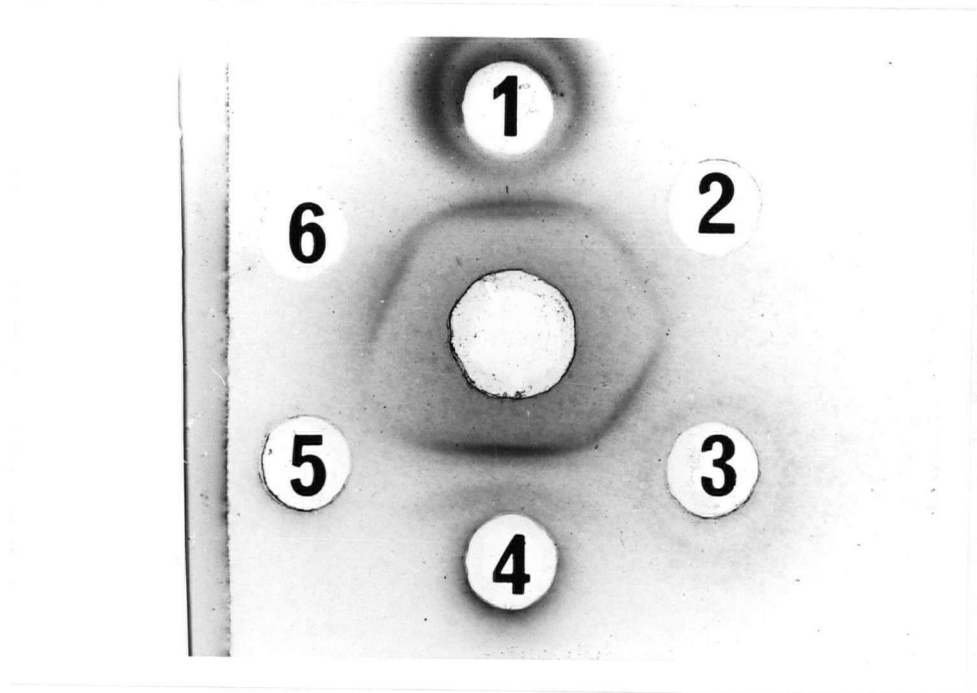
Fig. 24:   Immunodiffusion results using absorbed antiserum to
            C-46 against six extracts designated as follows:

1. C-46   2. C-1   3. C-2

4. N-pool   5. C-3   6. C-5
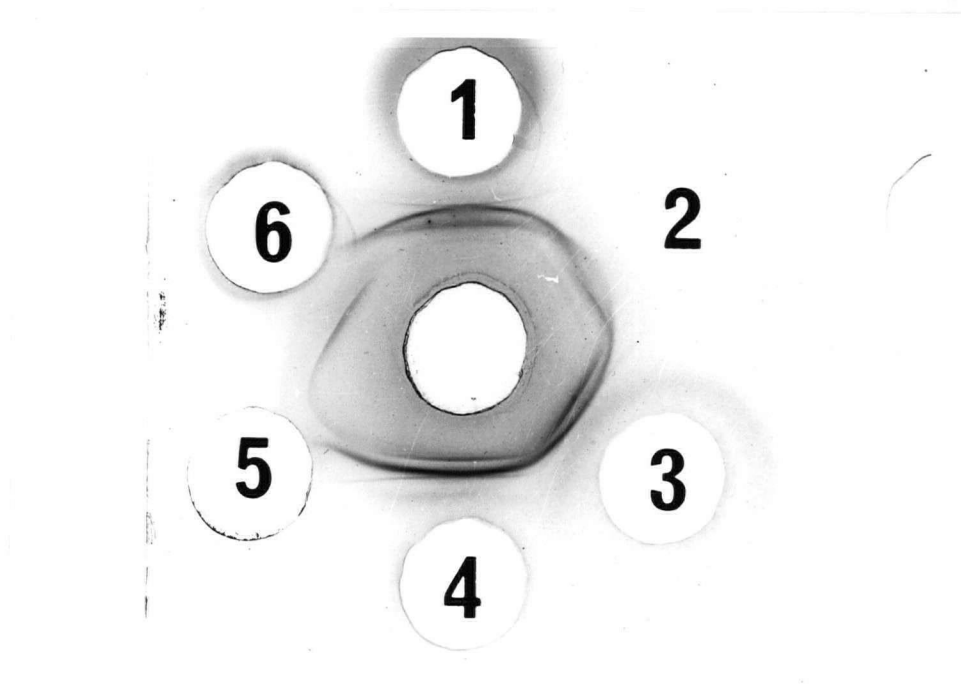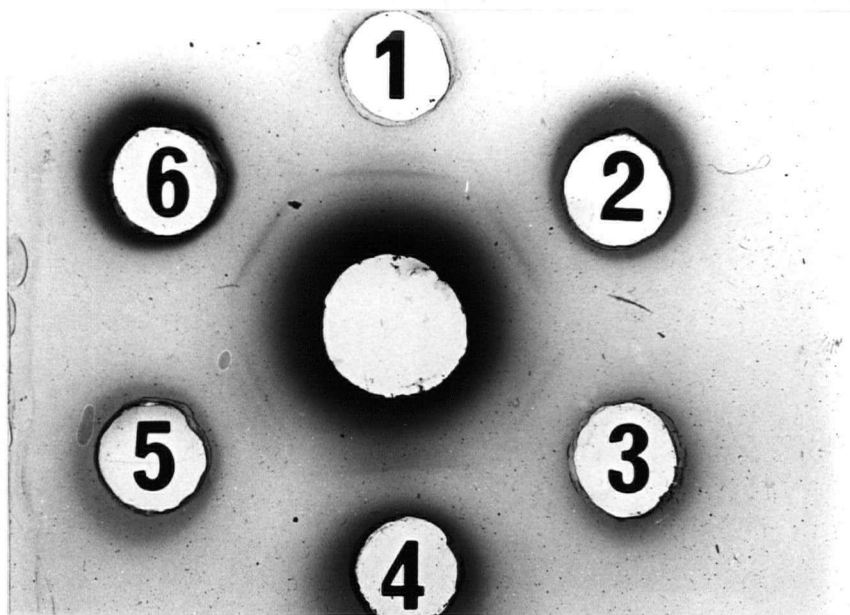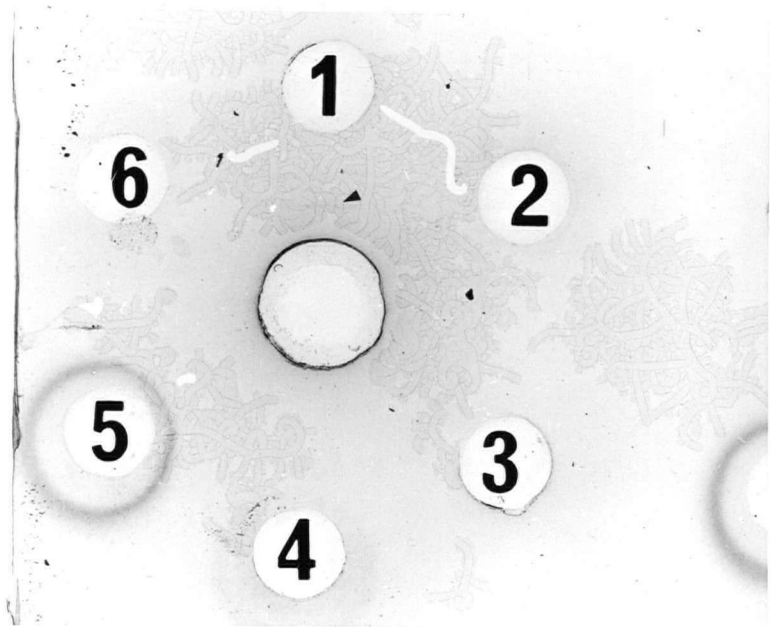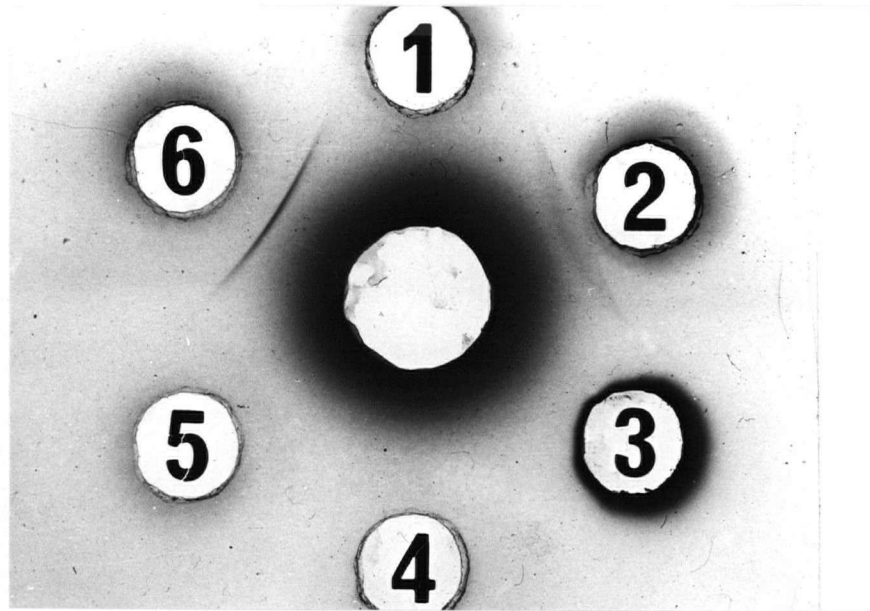
Fig. 25:   Immunodiffusion results using absorbed antiserum to
            C-62 against six extracts designated as follows:

            1. C-62   2. C-77   3. C-78

            4. N-pool   5. C-93   6. C-94

            While strong precipitin bands developed against C-77
            and C-94, no bands formed against homologous extract.
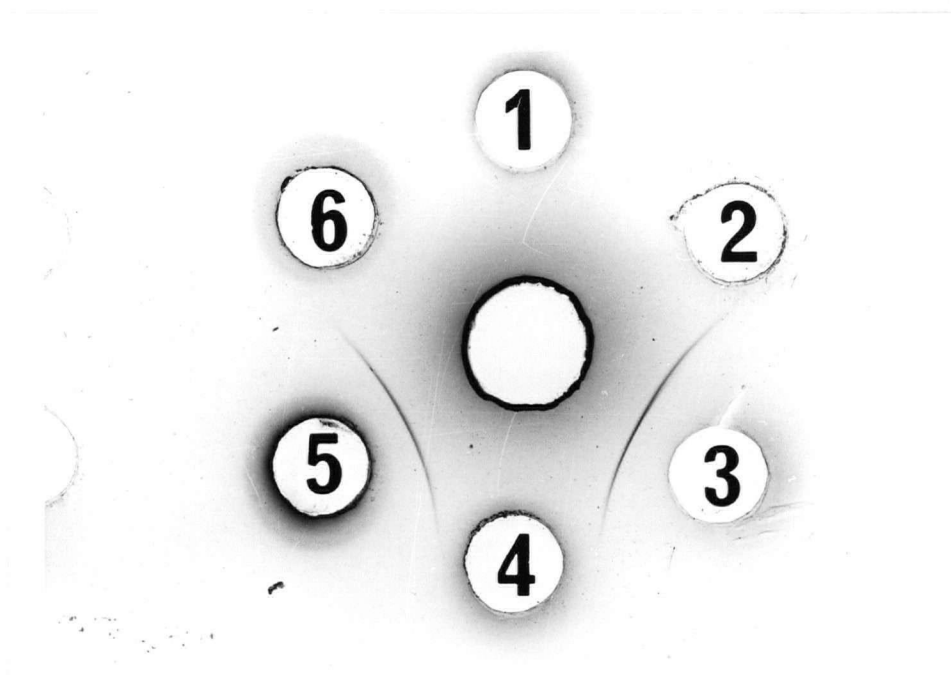
<u>Fig. 26</u>:   Immunodiffusion results using absorbed antiserum to C-62
against six extracts disignated as follows:

1. N-pool

2. repeatedly freeze-thawed C-94

3. stock preparation of C-94, stored at $-20^{o}C$

4. N-pool

5. stock preparation of C-77, stored at $-20^{o}C$

6. repeatedly freeze-thawed C-77

Note the complete absence of detectable antigen in the
freeze-thawed extracts.

antigen activity). The loss of antigen activity by freeze thawing could also explain the frequent non-reactivity of antisera with homologous extracts (Fig. 25), because they were freeze-thawed at least 5 times for the hyperimmunization of the rabbits. (The bulk of each extract was stored at $-20^{\circ}C$ and thawed only when a 10 ml aliquot had been used. This small sample was stored at $-20^{\circ}C$ but was frequently freeze-thawed). Because of the possibility of a change in antigenicity upon storage, fresh normal lung extracts were regularly added to this study.

C-67. Anti-C-67 was another antiserum which was difficult to absorb. Anti-normal activity persisted and anti-tumour activity was very weak, making analysis difficult (Fig.27). Both inner and outer bands were detected, and they cross reacted widely (Table I).

C-40. This antiserum was much like anti-C-67 in terms of the moderate cross reactivity, but inner bands were not observed (Table I). Fig.28, which shows the tumour associated band for C-40, also clearly demonstrates the importance of using different normal lung extracts for controls. Some apparently lack antigens found in other normal extracts.

C-78. This antiserum was very much like anti-C-62 except that inner bands were only detected in 3 extracts (Table I). The loss of activity of antigens in the outer precipitin bands detected by this antiserum (as described for anti-C-62) could explain the inability to detect TAA in the homologous extract (Fig.29a), and the infrequent occurrence of outer band antigens in other extracts (Table I). However, when the absorbed anti-C-78 was tested against the stock solutions of C-78 and C-62, antigenic activity was only detected in C-62. From the two sharp antigen bands in C-94, it is apparent that two similar antigens were present (Fig.29a). A later test of absorbed anti-C-78 against doubling dilutions of C-94 extract from the stock solution (Fig.29b) detected antigen at a 1/4 dilution of C-94, although only
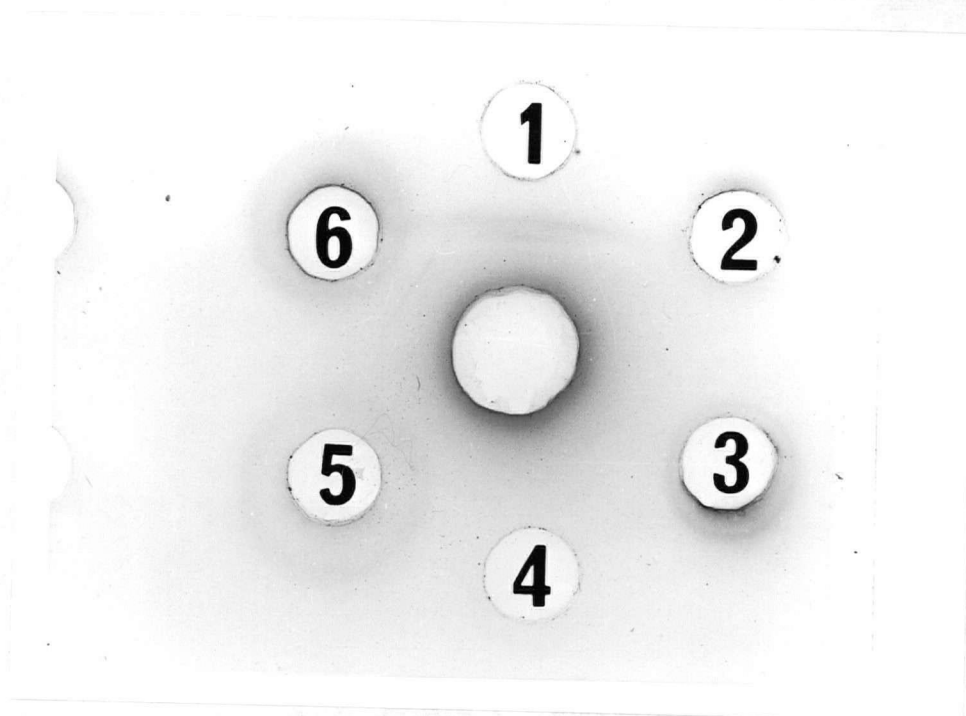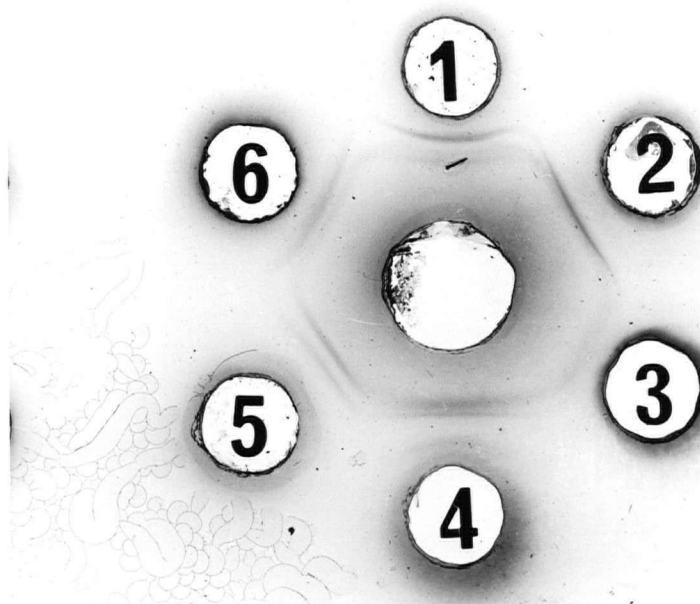
Fig. 27: Immunodiffusion results using absorbed antiserum to
          C-67 against six extracts designated as follows:

          1. C-67   2. C-87   3. C-88

          4. N-pool   5. C-90   6. C-92
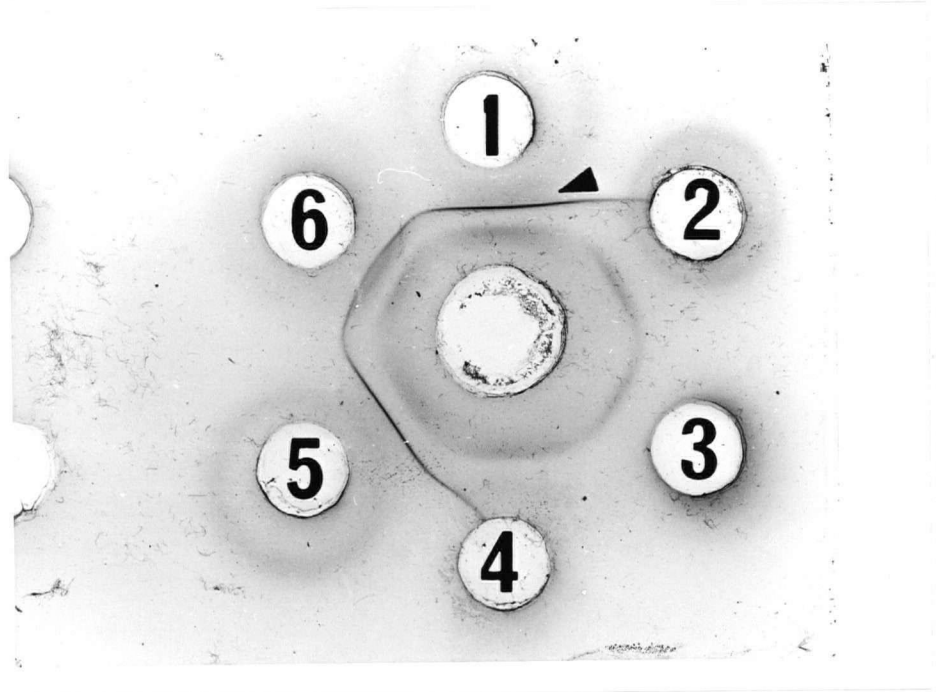
Fig. 28:   Immunodiffusion results using absorbed antiserum
           to C-40 against six extracts designated as follows:

1. C-40   2. C-53   3. C-54

4. N-pool   5. C-56   6. N-50

The arrow points to the tumour associated band.
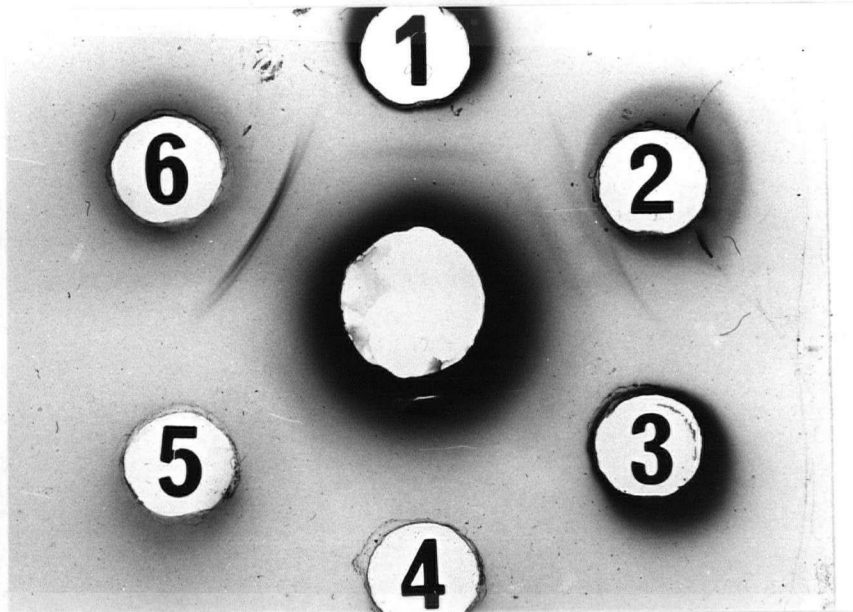
Fig. 29a:
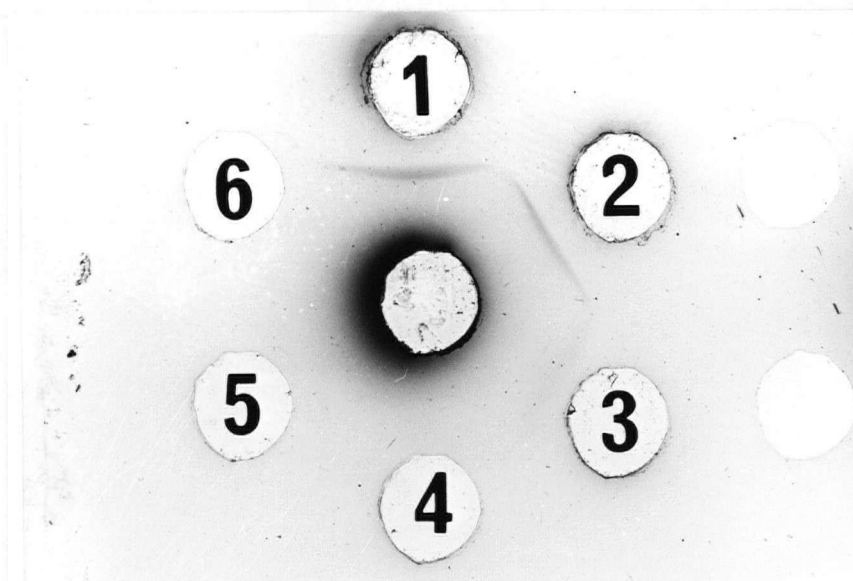
Fig. 29b:

Fig. 29:   Immunodiffusion results using abosrbed antiserum to C-78
            against: a. six extracts designated as follows:

1. C-78   2. C-77   3. C-78
4. N-pool   5. C-93   6. C-94

            b. six dilutions of C-94 in saline as follows:

1. C-94 undiluted   2. diluted 1/2   3. diluted 1/4
4. diluted 1/8   5. diluted 1/16   6. diluted 1/32

In part a. note the occurrence of two heavy precipitin
bands against C-94.

one antigen was detected. Since this antigen was not detected in any normal

extract even at full strength (including the stock solution of N-83, the most

recent normal lung extract), C-94 contains at least 4 times as much of this

antigen as the normal extracts.

C-30. Anti-C-30 demonstrated a strong precipitin band with homologous

extract and a faint reaction with 15 other tumour extracts as in Fig.30a.

Again, analysis was difficult. Further absorption could clear the antiserum

of all detectable anti-normal activity, but this antiserum could not detect

antigen in any extracts but the homologous (Fig.30b.

The results of Table I suggest not only that TAA exist and are common

to many tumour extracts, but also that at least two TAA may be present in

several extracts. The percentage of positive results from Table I is

summarized in Table II according to tumour pathology. It should be noticed

that these results can only be compared within each antisera column because

of the wide variations in the antisera.

There appears to be a correlation between tumour pathology and the

degree of cross-reactivity as demonstrated by this technique, and that this

correlation was better after the questionable positives were removed (Table

II). The results with the antisera against the squamous cell, anaplastic

and oat cell carcinoma extracts were generally similar; the percentage of

positive reactions was highest for extracts of these pathologies and lowest

for adenocarcinoma extracts (Table II). The antisera against the adeno-

carcinoma (and similarly the alveolar cell carcinoma although the number of

samples was small) extracts reacted maximally (27%) with extracts of similar

pathology and lower (0,5,and 13%) with extracts of squamous cell, anaplastic

and oat cell carcinomas. The differentiation between these 2 groups of

lung tumour pathology is of particular interest because there is little or

no relationship between the incidence of alveolar cell carcinoma and adeno-
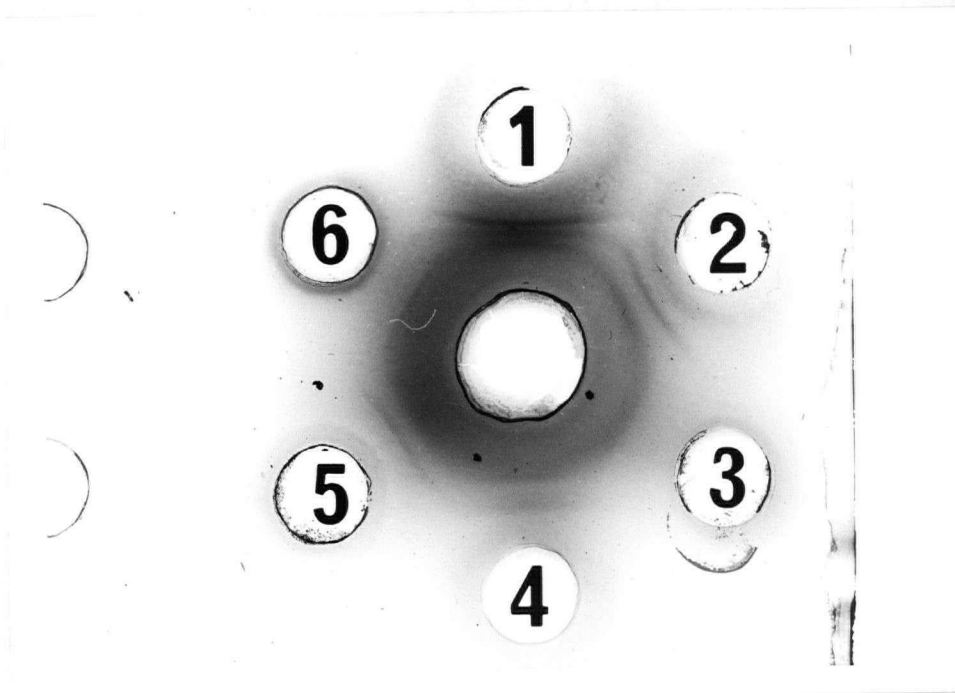
Fig. 30a: Immunodiffusion results using antiserum to C-30 absorbed
with N-pool insolubilized by both Sepharose 4B and glutar-
aldehyde and tested against six extracts as follows:

1. C-30   2. C-53   3. C-54
4. N-pool   5. C-55   6. C-56

Note the outer band with C-30 which shows a line of
identity with a faint band near the C-53 well.
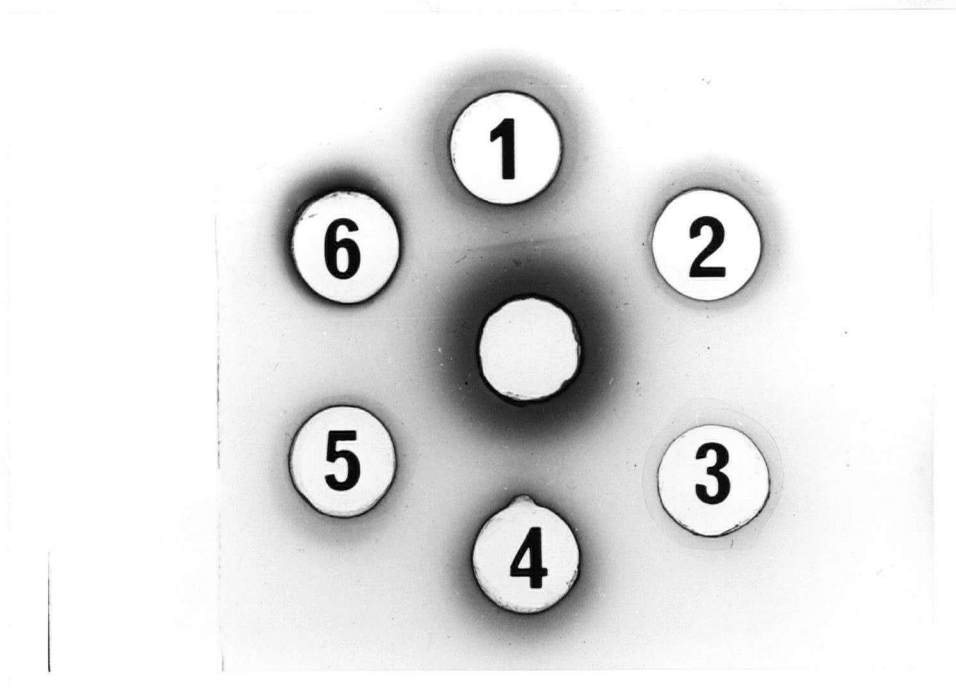
<u>Fig. 30b</u>:   Immunodiffusion results using antiserum to C-30 absorbed
twice as in part a. and re-absorbed with N-83 insolubilized
by Sepharose 4B, and tested against six extracts as follows:

1. C-30   2. C-79   3. C-81
4. N-83   5. C-82   6. C-85

Absorbed Antisera (number)	EXTRACTS									
	squamous cell carcinoma (17)		adenocarcinoma (9)		anaplastic carcinoma (6)		oat cell carcinoma (6)		alveolar cell carcinoma (2)	
	total	$+$only	total	$+$only	total	$+$only	total	$+$only	total	$+$only
anti-squamous cell carcinoma (6)	55	47	23	13	59	44	46	34	55	27
anti-adenocarcinoma (2)	14	5	27	27	25	13	14	0	67	33
anti-anaplastic carcinoma (4)	50	46	42	33	63	54	39	39	50	38
anti-oat cell carcinoma (2)	53	34	17	0	55	9	33	33	25	25
anti-alveolar cell carcinoma (1)	50	19	33	33	17	0	50	33	50	50

Table II: A summary of the data presented in Table I showing the percentage of positive results (+) and positive plus equivocal (±) results. Because of the wide variations in the antisera, these results should only be compared within each row of antisera results.

carcinoma and known carcinogens (ie. as in tobacco), while as much as a 25

fold increase in the incidence of squamous cell, oat cell and anaplastic

carcinomas (although this group may be heterogeneous) has been shown for

smokers (66). Thus there would appear to be a relationship between TAA and

aetiological factors in lung cancer. On other factors which may be associated

with the aetiology of lung cancer, Watson (135) has suggested that viruses

may be related to alveolar cell carcinoma on the basis of an increased

incidence of viral lung infections in the history of these patients. Further,

Mohr et al (57), using an antiserum against a pellet containing virus-like

particles from human alveolar cell carcinomas, could detect (by immuno-

diffusion) common TAA in the serum of patients with both alveolar cell

carcinoma and primary adenocarcinoma of the lung.

Because of the detection method used, negative results do not

necessarily mean that TAA were not present in the extracts. The vast quan-

tities of normal antigens could have impaired the anti-tumour response of

the rabbits or the normal bands could have obscured the TAA precipitin bands.

Also, the TAA concentration in the extract could have been too low (either

intrinsically or by degradation) to form a visible immune precipitation or to

stimulate antibody production. In order to resolve the negative results

and verify the questionable positive results, two approaches need to be

taken. One approach requires the development of a more sensitive detection

method and the other is either eliminating some of the normal antigen load

in the tumour extract or minimizing the antibody response to normal antigens.

The latter approaches should decrease the antigenic competition and allow a

better antibody response to the TAA. The concommitant decrease in the amount

of anti (normal antigen) antibodies should allow the immunoadsorbent tech-

niques to absorb the antisera more efficiently.

Poor sensitivity is indicated by the lack of reactivity of some of

of the absorbed antisera and the general weakness of the anti-TAA reactions. This is because the rabbits, the immunoadsorbents and/or the immunodiffusion techniques were probably being taxed to near their limits. Many of the anti-TAA reactions were diminished if not eliminated even by a 1:1 dilution of either component. The faintness of the precipitin bands and the interference by normal components made it difficult to determine whether the antigens we have called TAA are a qualitative or quantitative difference between tumour and normal extracts. If the antigens are common, a significantly different quantity between non-malignant and malignant diseases could be useful clinically in a manner described in part VI of the introduction. If the TAA are unique or only rarely cross-reacting, their clinical use is not nearly as great.

Immunodiffusion studies have been used to show the presence of tumour antigens in a variety of tumours. Baldwin et al (9) have used immuno-diffusion to confirm other assays for TAA in rat hepatomas. Bhattacharya et al (13) have shown the presence of at least two TAA with some cross-reactivity and common antigens in carcinoma of the ovary (13). Lewis et al (68) claim to be able to detect cancer in general by a simple immunodiffusion technique, although 'some serum factors which occur in abnormal but non-neoplastic conditions may have molecular spatial configurations stereoiso-merically similar to the cancer antigen'.

Several workers have used immunodiffusion techniques to assay human lung cancer antigens with a variety of results. Okada and Ikeda (93) reported the detection of two TAA, one of which could also be detected at lower levels in normal tissue extracts. These antigens were chemically different from the two lung cancer associated antigens detected by Yachi et al (142). The latter antigens were found widespread in extracts of tumours of different pathology and tissue of origin. One of the antigens (which was more frequently

associated with lung cancer than the other) gave a reaction of partial

identity with an antigen in foetal tissue extracts which is comparable to

the results with anti-C-41 (Fig.23). All three of these reports used pools

of tumour extracts for antiserum preparation, which has a great advantage

in the ease of preparation and absorption of the antiserum. This study was

not done with antiserum against pooled tumour extracts because it was felt

that a more complete study could be done with individual antisera against

extracts of different tumour types. It was also felt that the length of

time it took to receive less common types (ie. alveolar carcinoma) of lung

cancer (and their pathology reports) might have allowed considerable anti-

genic changes in the first extracts collected. Mohr et al (87), in an

immunodiffusion study using absorbed antiserum against alveolar cell-culture

supernatant, could detect antigen in the supernatants of cultures of tumour

cells but not normal lung cells.

2. α_1-foetoprotein

The finding that a TAA detected by absorbed anti-C-41 showed partial

identity (Fig.31) with an antigen in a foetal extract suggested that one (or

more) of the TAA might be foetal associated. One of the possibilities was

α_1-foetoprotein (AFP). Extracts were tested against 3 dilutions of absorbed

goat anti (AFP) by micro immunodiffusion. Large quantities of AFP were

detected in the foetal extract but none in the tumour or normal lung extracts.

These extracts were tested further for AFP by semi-micro immunodiffusion

against a 1:2 dilution of the anti-AFP. The results in Fig.31 show that AFP

could not be detected in the tumour extracts by this method and on the basis

of the large quantity of AFP in the foetal extract, the TAA detected in the

tumour extracts with the absorbed antiserum were not AFP.
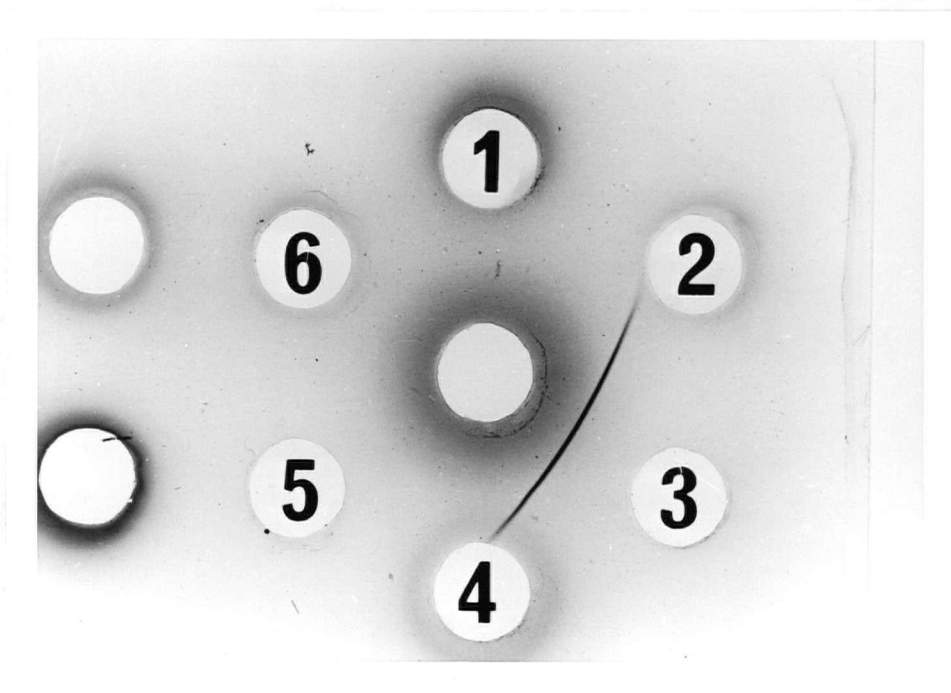
Fig. 31:   Immunodiffusion results using purified goat antiserum
           to α_1-foetoprotein (diluted 1:2) against extracts
           designated as follows:

           1. C-53   2. N-pool   3. F-48
           4. C-40   5. C-3   6. C-57

3. Studies on Patients' Sera for Detection of TAA Using Absorbed
    Rabbit Antiserum

Six of the absorbed antisera were tested against serum samples from
ten lung cancer patients and two healthy donors. Patients' sera included
pre-operative, post-operative and post-mortem samples. It was hoped that
circulating TAA might be detected in this way, if its concentration were
high enough. No TAA were detected in any of the sera. This observation
was not surprising since the immunodiffusion technique used would be unlikely
to detect antigen levels lower than about 2.0 μg/ml (section IV.5.).

4. Studies on Patients' Sera for Detection of Antibodies to TAA

Other immunodiffusion experiments tested human serum as a possible
source of anti-tumour antibody. Antibodies in serum which can precipitate
antigen from homologous tumour extract would not be expected to be against
self antigens, although the existence of anti-self antibodies has been
suggested (110) to be required for the maintenance of immunologic tolerance
to self antigens. If such antibodies were present in human serum, detection
would be unlikely because of their expected low concentrations. Hall (40)
suggests that autoimmunity may be associated with neoplastic diseases.
Such antibodies against self would be of great interest both clinically and
in the study of neoplastic disease processes. If antibodies were detected,
it should not have been difficult to differentiate between autoimmune-anti-
bodies and TAA-antibodies by testing against autologous normal lung extract.

Serum samples from 14 cancer patients (appendix C) and 2 healthy
donors were each tested against all of the homologous tumour extracts and 4
normal lung extract controls by micro-immunodiffusion. No precipitin bands
were detected.

Anti-TAA antibodies in serum can be either freely circulating (6) or

in the form of antibody-antigen complexes (10). In order to dissociate

complexes which might be present and to separate the antibodies from the

tumour antigens, the patients' sera (or the $(NH_4)_2SO_4$ precipitate of) were

acidified and chromatographed on a Sephadex G-150 column equilibrated in

0.1 M HOAc, 0.15 M NaCl pH 2.8 (Fig. 4). Separation depends upon the antigen

being included in the column volume. Although Fig. 4 demonstrates that

included and excluded fractions were not well separated, testing them against

anti-human immunoglobulin (Ig) (Fig. 5) shows that the included fractions

contained only small amounts of Ig. The excluded and included fractions

were immediately neutralized and concentrated to the starting volume by

ultrafiltration. Seven cancer patients' sera were fractionated in this way.

Semi-micro immunodiffusion tests were done with included vs. excluded

fractions, excluded fractions vs. tissue extracts, included fractions vs.

absorbent-tumour-sera, and included and excluded fractions vs. whole sera

according to Fig. 32. No tumour specific reactions were observed.

5.   Studies on Radioimmunodiffusion for Improvement of Resolution

In an attempt to improve the sensitivity of immunodiffusion, a radio-

immunodiffusion technique was developed. Instead of detecting immune pre-

cipitates by a protein stain, which also stains protein not removed by the

washing procedure, the micro-immunodiffusion plates were reacted with [125]I-

conjugated anti-Ig after the original immunodiffusion had been run. Purified

human Ig at serial 1:4 dilutions was used as the test antigen and was run

against purified rabbit anti (human Ig) antibody. The rabbit antibody had

been previously purified by elution from a human Ig immunoadsorbent (pre-

pared by insolubilization of human Ig with glutaraldehyde). Dilutions of
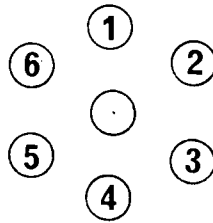
<u>Fig. 32:</u> Testing patient whole serum, serum fractions, tumour extract and absorbed antisera for antibody and antigen by immunodiffusion. Centre well - patient serum.

1. homologous tumour extract

2. S-a; high mw serum fraction from Sephadex G-150 in HOAc

3. S-b; low mw serum fraction from Sephadex G-150 in HOAc

4. absorbed antiserum (homologous for C-66 and C-71, anti-C-57 for others)

5. normal human serum

6. heterologous cancer patient serum

The sera and fractions tested in this way were: S-53, S-66, S-68, S-71, S-89, S-90, and S-92 (see Appendix C).

the rabbit antibody were also tested. After thoroughly washing the plates

to remove unreacted components, a dilution of ^{125}I-conjugated sheep anti

(rabbit Ig) globulin was added to the central well only or all 7 wells, and

incubated. After the washed and dried plates were exposed to X-ray film,

they were stained with amido black for comparison (Fig. 33). Using

purified components, this radioimmunodiffusion technique was not able to

detect any precipitin bands not detected by the protein stain. In fact, the

addition of the ^{125}I-conjugate made analysis more difficult because of the

heavy background from the remaining ^{125}I-conjugate and the formation of new

precipitin bands with residual rabbit antibody and the ^{125}I-conjugated sheep

anti (rabbit Ig) (Fig. 33a). The latter finding suggests that this method

could be very sensitive if the whole plates were soaked in ^{125}I-conjugated

anti-Ig. However, this would require the preparation and handling of large

volumes of ^{125}I-conjugate, which we were not prepared to do at the time.

When the results of Fig. 33 were compared with identical dilutions of anti-

body and antigen by semi-micro immunodiffusion (without reaction with ^{125}I-

conjugate), the semi-micro technique (Fig. 34) was able to detect 2 µg protein

per ml and the micro technique (Fig. 33b), only 50 µg/ml. Thus, the semi-

micro technique was more sensitive both qualitatively and quantitatively,

and was used for all immunodiffusion experiments unless otherwise indicated.

The micro technique was frequently used for pre-testing dilutions of antigens

and antisera.

V. Carcinoembryonic Antigen Assay

Although early reports almost exclusively found carcinoembryonic

antigen (CEA) in the serum of patients with digestive tract cancers, later

reports showed that CEA could be detected in the serum of patients with

Fig. 33:   Micro-radioimmunodiffusion using the following serial
                           1:4 dilutions of human Ig as antigen in the outer wells:

a.   6.0 mg Ig/ml   b.   1.2 mg Ig/ml   c.   0.25 mg Ig/ml

d.   0.05 mg Ig/ml   e.   0.01 mg Ig/ml   f.   0.002 mg Ig/ml

The rabbit anti-human Ig was added to the central wells of the patterns as
follows:

Patterns 1 and 4, undiluted;   2 and 5, 1:9 diluted;   3 and 6 1:100 diluted.

The ^{125}I conjugated sheep anti-rabbit Ig was diluted 1:4 and 5.0 μl (20,000
DPM) was added to all of the wells in patterns 1, 2, and 3.   A 1:19 dilution
(5,000 DPM) was added to the wells in patterns 4, 5, and 6.

Photograph a.   The resulting X-ray film shows a very high background and
                  human Ig was detected only in wells a, b, and c of pattern
                  1 and well a of pattern 4.

Photograph b.   After staining the original slide the backgrounds were low
                  and human Ig was detected down to 0.05 mg/ml.

The bands detected near the antigen wells are probably due to a reaction
between the ^{125}I-conjugate and rabbit antibody which was not removed by the
washing.
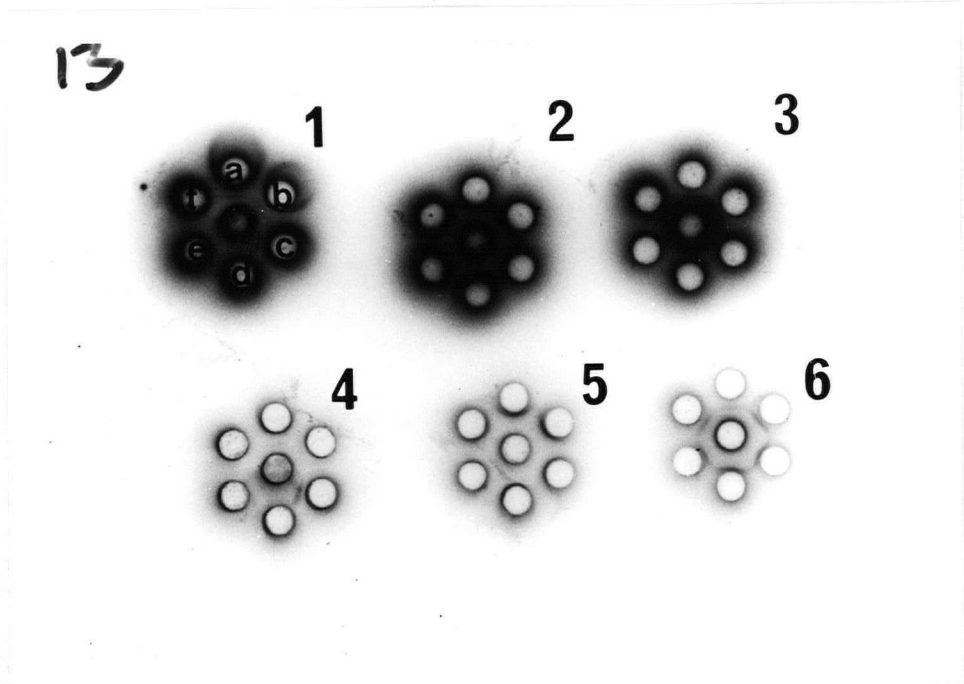
Fig. 33a:
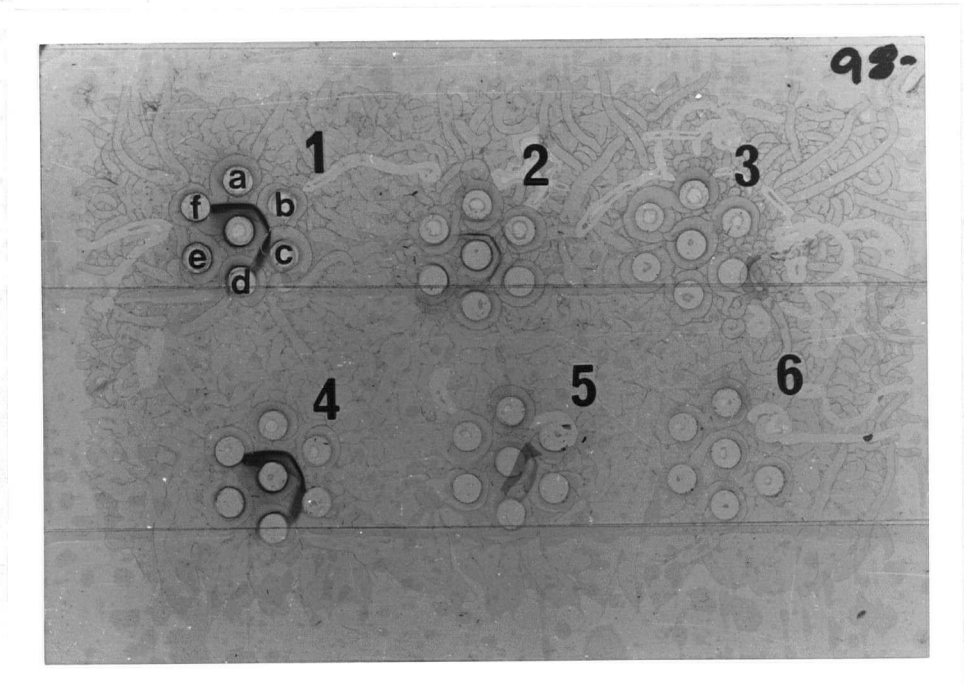
Fig. 33b:
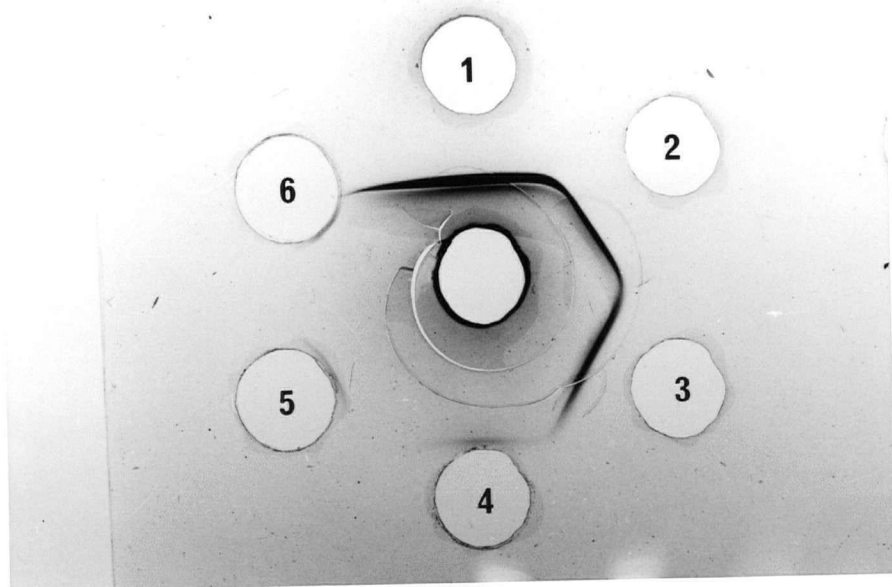
Fig. 34:   Immunodiffusion results using rabbit antibodies to
            human Ig against serial 1:4 dilutions of human Ig
            as follows:

1. 6.0 mg Ig/ml   2. 1.2 mg Ig/ml   3. 0.25 mg Ig/ml
4. 0.05 mg Ig/ml   5. 0.01 mg Ig/ml   6. 0.002 mg Ig/ml

Note that a precipitin band can be seen adjacent to
well 6.

other forms of malignancies and certain non-malignant diseases (1). Since many lung cancer patients have detectable circulating CEA (1), it was decided to have the tumour extracts assayed for CEA by radioimmunoassay.

The extracts first tested were of tumour and normal lung tissues from the same specimen. Normal lung tissues for extraction were taken as far as possible from the site of the tumour and were macroscopically free of tumour. The results of Table III show high CEA levels in both tumour and normal extracts. (Normal plasma levels of CEA are 0 to 2.5 µg/ml (128)). Although this was originally interpreted to suggest that CEA was released from the tumour into the surrounding tissue (possibly via the serum), later experiments showed that high levels of CEA could also be detected in extracts of lung not associated with any malignant disease (Table IV).

Later CEA analyses were carried out to determine if the TAA detected by other methods were CEA, and if there was a relationship between the tumour pathology and CEA level. The CEA levels (Table IV) of the fractions of C-26 (described in section III) prove that the antigen(s) detected in C-26-I were not CEA. High levels of CEA in normal lung extracts (Table IV) are also reported by Tillack et al (128). This finding, and the low levels of CEA in several tumour extracts prove that the common TAA(i) detected by immunodiffusion (Table I) were not CEA. However, it is possible that some of the other TAA were CEA.

The low levels of CEA in F-48 could be due to the method of induction of the abortions as discussed in section III.

There was no apparent correlation between CEA level and lung tumour pathology (Table IV) which also is in agreement with Tillack et al (128). The osteogenic sarcoma extracts were included as negative controls because of the high CEA levels in normal extracts.

Tumour Extract	Protein Concentration (mg/ml)	CEA ng/ml	Lung Extract	Protein Concentration (ng/ml)	CEA ng/ml
C-1	6.9	>25	N-1	22.4	21.8
C-2	17.6	>25	N-2	9.8	>25
C-5	13.8	>25	N-5	17.2	>25
C-6	15.4	9.0	N-6	25.7	16.8

Table III: Quantitation of CEA in undiluted extracts by radio-
immunoassay. The normal lung was from the same
individual as the tumour of the same number, and
was microscopically free of malignant disease.
Normal plasma levels of CEA are 0 to 2.5 ng/ml.

Squamous Cell Carcinoma		Adeno-carcinoma		Anaplastic Carcinoma		Oat Cell Carcinoma		Alveolar Cell Carcinoma		Mixed Squamous Adeno-carcinoma		Mesothelioma		Rhabdomyo-sarcoma		Squamous Metastasis		Foetal Lung		Normal Lung		Osteogenic Sarcoma	
	CEA		CEA		CEA		CEA		CEA		CEA		CEA		CEA		CEA		CEA		CEA		CEA
C-1	82	C-3	57	C-41	38	C-40	50	C-30	>150	C-82	>150	C-54	>150	C-77	26	C-57	11	F-48	3	H-pool	120	C-68	0
C-6	9	C-5	>150	C-46	24	C-64	1	C-81	43													C-73	1
C-53	90	C-24	>150			C-65	90															C-91	0
C-60	150	C-26	>150			C-70	150													N-25	44	C-96	0
C-61	0			C-56	150	C-72	120													N-50	98		
C-63	41			C-62	150	C-78	150													N-83	36		
C-66	1			C-67	18																		
C-69	150			C-92	1																		
C-71	150																						
C-74	64																						
C-75	57	C-56	120																				
C-76	>150	C-45	42																				
C-79	150	C-85	58																				
C-87	17	C-88	58																				
C-90	53																						
C-93	40																						
C-94	15																						

Table IV: CEA content of extracts according to tumour pathology. CEA was measured by radioimmunoassay of undiluted and 1:5 diluted extracts. The CEA values are given in ng/ml and values greater than 100 ng/ml are approximations. Protein concentrations of the extracts are in appendix A.

VI.   Immunofluorescence

The studies reported previously on the detection of TAA by immuno-
diffusion were interesting and potentially important. However, several

questions regarding the nature of these observations must be considered. In

the first place, these antigens were detected in tissue extracts, and there

was no information on their location on or in the tumour cell. While tumour

specific materials within tumour cells may have significance in diagnosis

and/or treatment, those antigens located on the cell surface would appear to

have greater potential in terms of tumour immunology. Therefore, some probe

into the location of these materials would be important. Secondly, these

antigens were detected by immunization of rabbits with tumour extracts, and

therefore no knowledge of their antigenicity in humans was achieved. Attempts

to identify either these antigens, or antibodies to them in patients' sera

by immunodiffusion, were unsuccessful: this is not surprising because of

the relative insensitivity of this technique and because of the low levels

of either antibody or antigen one would expect to find in the patients' sera.

For these reasons, further experiments were designed to develop

techniques which would both probe the locations of these antigens and increase

the sensitivity of the detection methods so that some conclusions could be

drawn regarding the immunodiffusion data. The sections on immunofluorescence

and autoradiography (VII) techniques address themselves to these issues.

The rationale of the indirect immunofluorescence (IF) technique is

similar to that described for the indirect autoradiography technique in

section VII. The indicator was anti-Ig conjugated with fluorescein instead

of ^{125}I. Many problems were common to both techniques which were being used

concurrently and they were similarly approached.

When the absorbed antisera (anti-C-66, C-71 and C-78) were used, anti-

body was bound to the normal lung and tumour tissue sections, but could not be shown to be tumour specific even if the antisera were further re-absorbed. The poor quantitative nature of this technique meant that in order to detect differences in the binding of fluorescent label to the tissue sections, an antiserum with a higher titre of anti-TAA and/or better absorbed would be required. Experiments using cancer patient and healthy donor sera as a possible source of antibody also did not allow the resolution of tumour associated differences in fluorescence. Two sources of background fluorescence were found to contribute to the problem of quantitation. There was background from the binding of the conjugated anti-Ig to the tissue and from autofluorescence of the tissue (possibly the connective tissue). The IF technique was pursued no further because the autoradiographic technique appeared more promising.

IF techniques are commonly used for the demonstration of TAA. Baldwin and Glaves (7) and Borsos et al (17) report quantitation of the IF technique by the inhibition of the fluorescent labelling of experimental animal tumour cell suspensions by the pre-incubation of antiserum with tumour extract.

Studies on human tumours (89, 91) report the detection of TAA or cell smears or suspensions by patients' sera, although positive fluorescence by the normal controls implies that the results may be equivocable. Other workers (29) could not detect anti-TAA in patients' sera by IF techniques. Yachi et al (142) reported preliminary results of positive fluorescent staining of lung tumour tissue sections (cryostat) and negative staining of normal tissue sections by absorbed xenogeneic anti-tumour serum. I have not been able to obtain this kind of result using a similar technique, although the incomplete absorption of the antisera was known to be a problem. Also, the work of Yachi et al (142) was reported in 1968, and they have not published any information on the subject since then.

VII. Indirect Autoradiography

The indirect autoradiography (IA) technique was expected to be very
sensitive, versatile and readily quantitated. Most studies of cellular
antigens by indirect, labelled anti-Ig techniques (7, 17, 43) use cell
suspensions, either fresh or from cell cultures. This was not considered
practicable for the studies we wished to undertake for several reasons. Dead
cells and debris bind much higher levels of ^{125}I-conjugate than viable cells.
This suggests that if fresh tumour cells are used, they should be used
immediately, as viability decreases greatly with storage. This is not suit-
able for comparing samples over a period of time. Tumour cell cultures are
not suitable because: it is difficult to maintain large numbers of different
cultures, the cultures generally cannot be prolonged indefinitely, they are
frequently overgrown by fibroblasts, and surface antigen properties can
change during culture (89). Cells in culture may also contain various con-
taminants, including mycoplasma and extraneous viruses (ie. from the foetal
calf serum). Frozen sections were used because they could be stored easily,
minimum antigen denaturation or loss was expected, and it was hoped to be
able to locate tumour antigens within the tissues and cells. Although it
was realized that the non-viability may lead to an increased background, it
was expected to be similar for normal control tissue sections and therefore
could be eliminated in the quantitation.

1. Studies with Absorbed Rabbit Antiserum

Tumour and normal lung tissue sections were incubated with a pre-
determined dilution of absorbed rabbit anti (tumour extract) serum, then
tested for bound antibody with a pre-determined dilution of ^{125}I-conjugated

sheep anti (rabbit Ig) globulin. After reacting with nuclear track emulsion and developing, the tissue sections were observed for grains. The grains were not only on the tissue sections, but also on the microscope slide glass itself (similar effects were also observed with plastic microscope slides). This was shown to be non-specific binding to the glass by the observation of grains when microscope slides with no tissue sections were incubated with ^{125}I-conjugate. So far it has not been possible to demonstrate specific binding with any particular part of the tissues or cells. Welsh et al (138) have recently reported a technique which may be of use for this type of study. Instead of using radio-iodinated anti-Ig preparations, they used ^{125}I-conjugated protein A from Staphylococcus aureus, which has a high affinity for the Fc portion of most mammalian IgG subclasses.

In the attempts to minimize background grains, one of the absorbed antisera (anti-C-66) was re-absorbed until no more antibody was detectable by immunodiffusion. When this re-absorbed antiserum was tested undiluted by IA, the grains were far too numerous to count. A 1/50 dilution was required to lower the grain count to less than 10 grains/cell for both normal and tumour tissues. Thus the technique is at least 50 times more sensitive in antibody detection than is immunodiffusion. Also, the immunoadsorbents did not remove all of the antibodies against normal antigens.

The initial results with the dilutions of the absorbed antisera against C-66 usually showed significantly higher grain counts/cell on the tumour than on the normal lung sections. The specificity of the sheep anti (rabbit Ig) serum was demonstrated by immunoelectrophoresis against rabbit antisera and human tissue extracts as shown in Fig. 35 . To further test the specificity of the binding of ^{125}I-conjugate, experiments were carried out to test the inhibition of binding by pre-incubation of 10 μl absorbed antisera overnight at 4°C with 50 μl normal lung and tumour extracts.
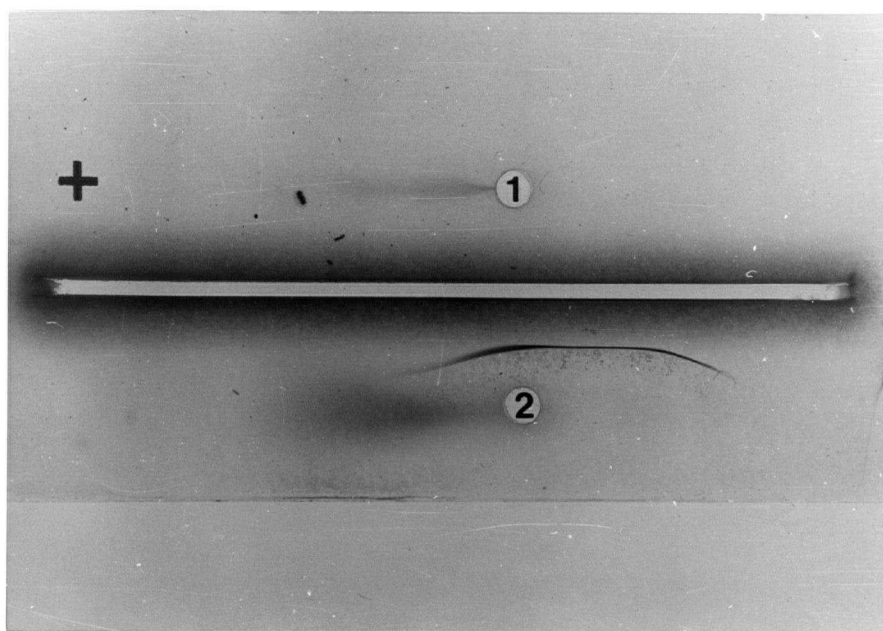
Fig. 35:   Immunoelectrophoresis results on the specificity of
             the sheep anti-rabbit Ig against:

1. N-pool

2. rabbit anti-C-71

Note that no antigen was detected in the human lung
extracts.

From Figure 36 it can be seen that when the absorbed antisera were pre-incubated with homologous extract there was no significant difference between the number of grains/cell of tumour and normal tissue sections. Pre-incubation with PBS or normal lung extracts generally resulted in significantly more binding to the tumour section cells than to normal section cells (Fig. 36). These findings are consistent with the idea that TAA were present on the tumour sections and that the anti (TAA) antibody activity could be absorbed from the antisera with homologous extract but not with normal lung extracts. When the tissue sections were incubated with diluted (Fig. 36a,b) or undiluted (Fig. 36c) normal rabbit serum, no significant difference was detected between tumour and normal sections.

On the basis of the promising results of Fig. 36, a larger experiment was carried out testing 4 diluted, absorbed antisera and normal rabbit serum against 8 different tissue sections. Data were analyzed by the method of Least Significant Difference. No significant binding of the label to the tumour section cells was observed. The grain counts varied from 1.5 to 3.5 grains/cell compared to 2.5 to 9.5 grains/cell in Fig. 36. Using a similar technique, Dr. I. Berczi (personal communication) and his associates found that the dilutions of the reagents are critical for maximizing the tumour-normal differences and that many tumour cells may have antibody bound to their surface antigens. They used a technique (for counting radioactivity bound to a known number of fixed cells from a cell suspension) which could be quickly and easily quantitated. Their technique may be applicable to the studies reported here, although two observations we have made with the tissue sections suggest that serious problems could arise: certain cells (possibly macrophages or mast cells) in the tissue sections bound very high amounts of label (Fig. 37a) even if only the ^{125}I-conjugate was added, and patches of the tissue sections occasionally had higher backgrounds than
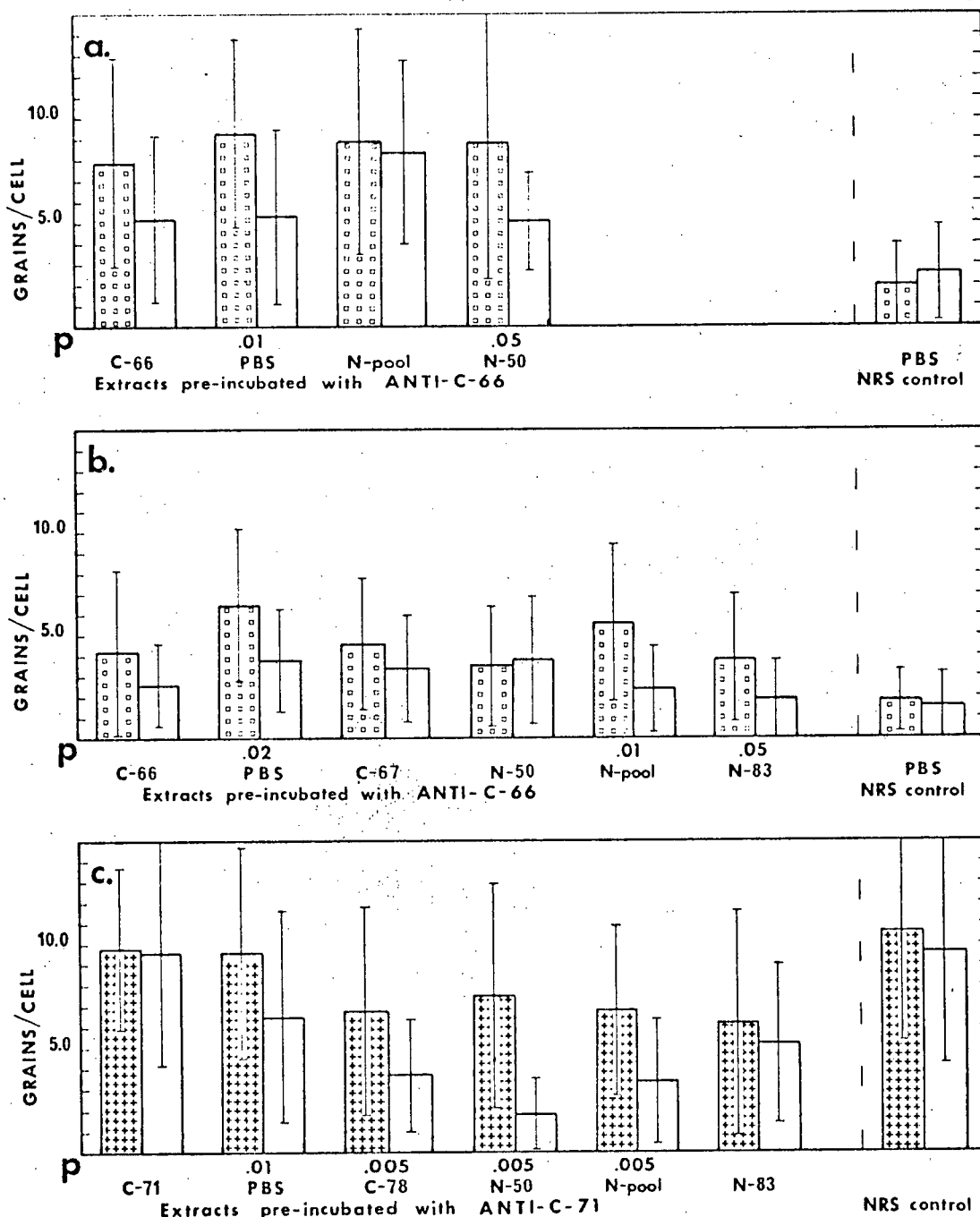
Fig. 36: Absorbed antisera tested for the binding of antibody to tumour and normal lung tissue sections by indirect autoradiography. The absorbed antisera were pre-incubated with PBS on the extracts indicated below each figure. The standard deviation (S.D.) for each grain count is given and the probability (p) is given where the difference in grain counts between tumour and normal tissue sections was significant by the Student's t test.

☐ N-86 tissue section   ▦ C-66 tissue section
▦ C-71 tissue section

a,b. Absorbed anti-C-66 and normal rabbit serum (NRS) diluted 1:55

c.   Absorbed anti-C-71 diluted 1:100 and NRS undiluted.

Fig.37a
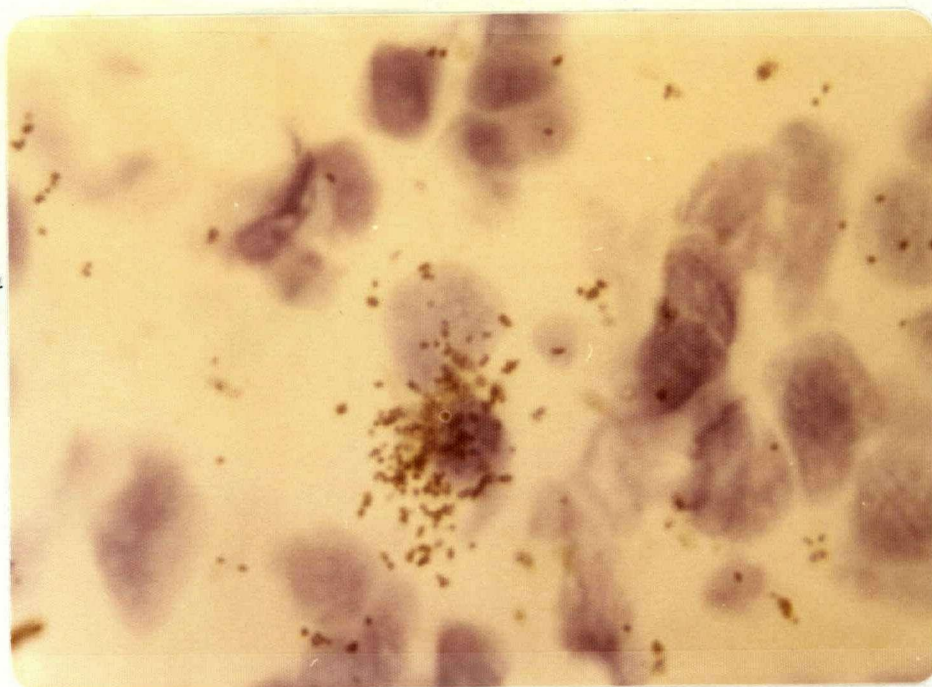
Fig.37b:

Fig. 37: Photographs of uneven indirect autoradiography results.

a. This photograph was from a N-86 normal tissue section incubated with a 1/100 dilution of absorbed anti-C-78, but similar heavily grained cells were also found in some tumour sections and whether ^{125}I conjugated anti-rabbit Ig or anti-human Ig were used.

b. This photograph was from a C-74 tumour tissue section incubated with PBS and ^{125}I conjugated anti-rabbit Ig. Note the higher grain counts in the top half. The cells in such areas were not counted.

surrounding areas (Fig. 37b). It was not practicable to determine the optimum experimental conditions and the experiment was not repeated because with the technique we used it was very time-consuming and difficult to do accurate quantitation.

It would appear that antiserum raised in rabbits to human tumour extracts, no matter how carefully and thoroughly it is absorbed, will continue to have levels of anti-normal components detectable by this technique and any experiments utilizing this type of antiserum would be self defeating. However, because of its sensitivity, it was thought that the IA technique might be amenable to the detection of specific anti-tumour antibodies in the serum of patients.

2.   Studies with Human Serum

The tissue sections were incubated with lung cancer patients' sera undiluted, followed by ^{125}I-conjugated rabbit anti (human Ig) globulin, or later, by purified anti (human Ig) antibody conjugated with ^{125}I. However, the background counts remained high and a dilution of the conjugate was required so that the grains could be counted. To test whether the high grain count was due to human antibody already present in the tissue sections, they were incubated with non-conjugated anti-Ig and washed, then incubated with the ^{125}I-conjugate. No difference was detected whether or not the non-conjugated anti-Ig was added, suggesting that pre-existing antibodies were not responsible for the high backgrounds.

A major difference was observed between the absorbed rabbit antisera experiments and the human sera experiments: the human sera could be incubated with the tissue sections undiluted and the grain counts remained within a countable range. If this result is compared to the 1/50 dilution required of the re-absorbed anti C-66 serum which had all of the detectable precipi-

tating antibody absorbed, human serum contains less than 1/50 of the auto-antibody required for detection by immunodiffusion against the extracts.

This technique was further studied in spite of the serious problems encountered because of its potential sensitivity and the small amounts of materials required suggested that it might be useful clinically. Anti-tumour antibody may be circulating freely in the blood, in which case it would be present in whole serum and the $(NH_4)_2SO_4$ precipitated globulin fraction or it might circulate as a complex with tumour antigen. To dissociated immune complexes, the serum was chromatographed on an acidified Sephadex G-150 column as discussed in section IV.4. It has been shown that blocking factors (immune complexes?) may be present in tumour bearer serum, but when the tumour was removed, anti-tumour antibodies could be detected (43,120). Thus, an assay for this antibody could be a prognostic aid by determining complete tumour removal.

A series of experiments was carried out to test patient whole serum, the serum globulin fraction, the G-150 excluded fraction and normal human serum for antibody which would bind to tumour and normal tissue sections. Each serum sample and its components, and normal human serum were tested against homologous tumour, heterologous normal lung (N-86), and another tumour as in Fig. 38. (Not all of the other tumour sections were counted). The grain counts/cell for each serum sample were analyzed by the Student's t test for the significance of counts on the tumour sections, compared to the normal lung sections. The results of Fig. 38 suggest that anti-tumour antibody (or immune complexes in antibody excess) were present in the serum samples S-71 and S-68, as well as in the fractions, although it could not be determined if more antibody were present in the fractions. The increase in label bound to normal tissue sections with the serum fractions (Fig. 38) could be due to the decreased protein solubility observed in immunodiffusion studies (Fig.
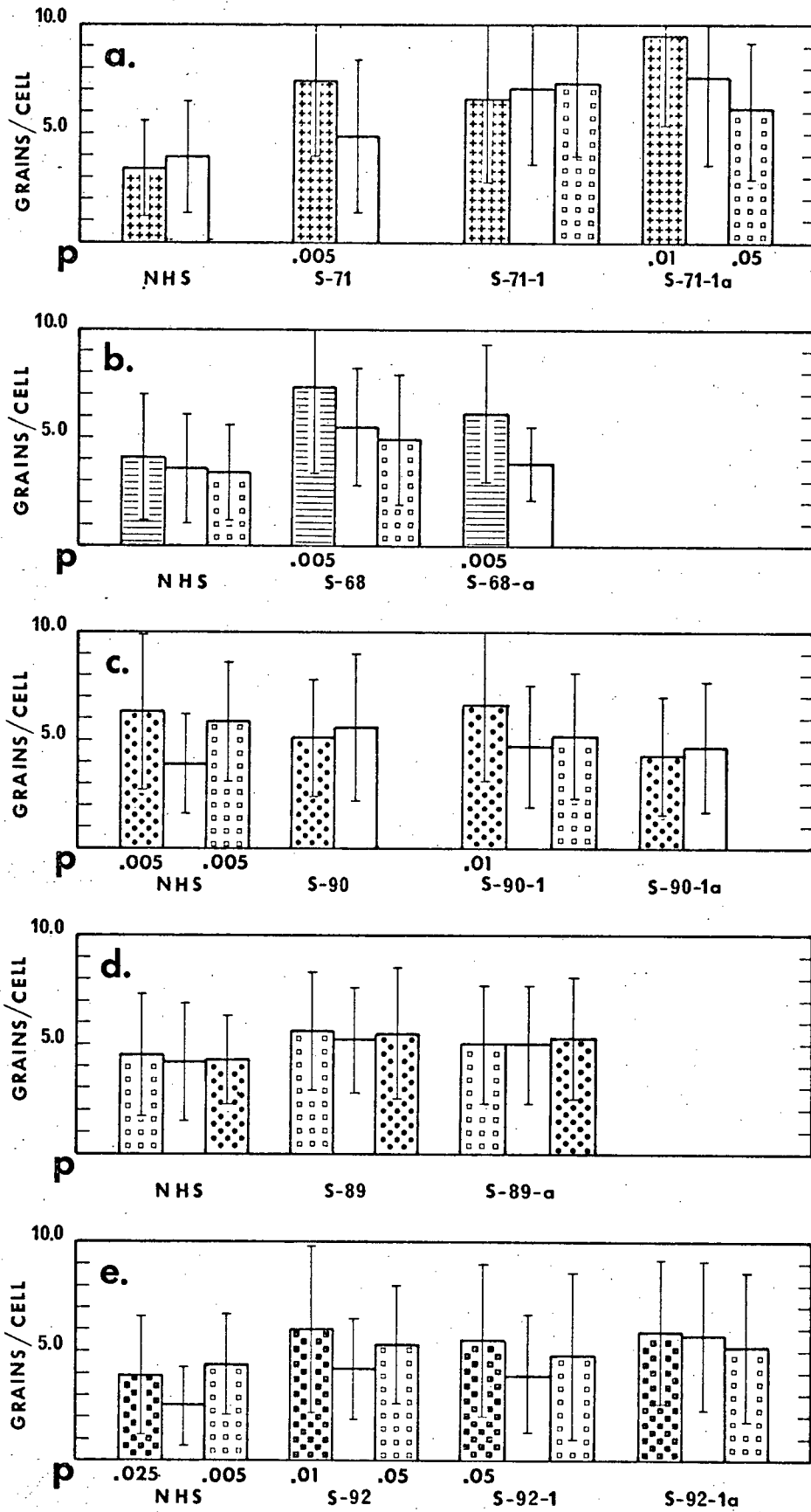
Fig. 38:

Fig. 38:   Indirect autoradiography assay for the binding of antibody
           from normal human serum (NHS) and cancer patient serum
           against homologous tumour reactions and heterologous normal
           lung and tumour tissue sections. Serum fractions were also
           tested.

    S -   : serum sample

  - 1   : $(NH_4)_2SO_4$ precipitate fraction

  - a   : high mw fraction eluted from Sephadex G-150 column
              in HOAc.

    SD is given for all grain counts and the probability (p) is
    given where the difference in grain counts between tumour
    and normal tissue sections was significant by the Student's
    t test.
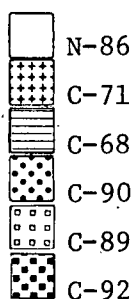
    ▢ N-86   a.   S-71   vs.   C-71
    ▦ C-71   b.   S-68   vs.   C-68
    ▤ C-68   c.   S-90   vs.   C-90
    ▨ C-90   d.   S-89   vs.   C-89
    ▥ C-89   e.   S-92   vs.   C-92
    ▦ C-92

5), or the presence of immune complexes not composed of tumour antigen-antibody. The latter possibility could be due to an autoimmune process, perhaps related to the neoplastic disease (40). S-71 (Fig.38a) was a post-mortem specimen and S-68 (Fig. 38b) was a 3 month post-operative specimen from a patient with multiple metastases already present (Appendix C). (Tumour specimen C-68 was an osteogenic sarcoma variant (see Appendix B) included because of the availability of materials and because post-operative serum samples were difficult to get). Immune complexes could be present in such sera. There was no specific binding to the tumour sections by normal serum for C-71 and C-68 (Fig. 38a,b).

Specific binding of label to the tumour sections was not detected with sera S-90 or S-89 (Fig. 38c,d). The results with S-92 (Fig. 38e) suggest that antibody might be present in this serum, but the low grain counts on the normal lung tissue sections by normal human serum makes this equivocal.

The unaccountable differences occasionally encountered severely limit the usefulness of this technique as an assay for biochemical studies of tumour antibody or antigens, but it may still be applicable for surveying different serum samples for tumour antibody. Because antibody could not be detected more readily with the serum fractions, only whole serum was used for subsequent experiments. The inability to detect more antibody with serum fractions could be because: tumour immune complexes were not present in the serum, the higher backgrounds could have interfered with quantitation, the antigen was large enough that it was not separated from the antibody, or some of the antibody activity could have been lost during the fractionation procedure.

A survey was set up to test 16 sera against 15 different tissue sections. The experiment was to test for the presence of antibody in the sera and for relationships between the antigens in the tissues. Statistical analysis for significance was by the Least Significant Difference method,

comparing the mean grains/cell in the presence of patient serum with the mean

of normal human serum. The tumours include osteogenic sarcomas (C-68 and

C-91) and a rhabdomyosarcoma (C-77) (Appendix B) which were added for extra

controls. For more information on tumour pathology and the sera, see

Appendices B,C. The results of Table V and Fig.39 suggest that the serum

of cancer patients contains antibodies reactive with tumour sections, and

that in some cases there is a high degree of cross-reactivity, even with

tumours of different pathology. Because of the latter cross-reactivity and

because there appeared to be clustering of positive results associated with

certain tumours, the results appeared somewhat suspect. However, all the

specimens were frozen and stored under the same conditions and only the time

of storage was different. The tumours which had the most cross-reactivity

(C-68 to C-77) were stored the longest, so the clustering of positive results

was not due to loss of antigenic activity. Also, these results (Table V)

are in agreement with those presented in Fig.38 regarding positive results

with S-68 and S-71 (and S-92?) and negative results with S-89, S-90 and the

normal control tissue sections, N-86. The results of Table V are also

comparable to those presented by Morton et al (59) and Moore et al (91),

using an analogous IF technique, in the demonstration of wide cross-reactivity

within malignant melanomas and sarcomas. The latter report (91) also found

some cross-reactivity between sarcomas and carcinomas.

In the studies reported here, there would have been an advantage in

using normal control and tumour tissue from the same patient. Then the

possibility of interpreting histocompatibility differences (eg. A,B,O. blood

groups) as tumour specific would be eliminated. However, such an approach

would not have been suitable for comparing different sera and tumour sections.

Ideally, both homologous and heterologous normal tissue should have been

used, but the difficulty of quantitation precluded this for the present.

Sera	Tissue Sections														
	N-86	C-68	C-71	C-74	C-77	C-79	C-85	C-87	C-88	C-89	C-90	C-91	C-92	C-93	C-94
Normal															
MG	1.3	2.0	1.7	2.0	1.2	2.7	1.5	1.5	1.6	2.0	1.9	1.2	0.9	1.4	1.3
HM	1.3	1.9	1.7	2.3	1.7	2.1	2.1	1.7	1.3	1.3	1.6	1.3	1.0	1.6	1.3
Cancer Patient															
S-68	1.1	3.6	2.7	3.8	2.1	2.5	1.6	1.1	1.4	1.2	1.2	1.1	1.0	1.6	1.2
S-71	1.2	2.2	3.3	3.7	2.0	1.9	1.5	1.4	1.6	2.1	1.2	1.2	1.0	1.8	1.2
S-74	1.1	2.9	2.7	3.2	2.4	3.4	1.5	1.5	1.1	0.8	1.9	1.1	0.8	1.7	1.5
S-77	1.0	2.7	1.8	3.7	1.7	1.8	1.0	1.5	1.7	1.4	1.6	1.5	0.9	1.5	1.1
S-79	1.3	2.2	2.2	2.6	1.8	1.7	1.3	1.3	1.4	1.5	1.6	.9	1.1	1.4	1.8
S-85	1.1	2.3	2.3	1.8	1.3	2.0	1.2	1.2	1.4	2.1	2.1	1.8	1.8	2.1	1.6
S-87	1.4	5.1	3.1	4.4	3.0	2.3	1.9	1.6	1.9	2.2	1.7	1.7	1.3	2.0	1.6
S-88	1.1	2.3	2.4	1.8	1.7	2.6	1.6	1.7	1.2	1.6	1.6	1.3	1.1	2.1	2.1
S-89	1.3	1.8	1.9	1.9	1.1	2.7	2.3	2.1	1.1	1.0	1.8	1.5	1.1	1.9	1.4
S-90	1.1	1.7	2.0	2.7	1.1	2.8	1.9	1.7	2.1	1.9	1.3	1.7	1.3	1.8	1.9
S-91	1.2	1.8	2.0	2.7	1.0	2.0	1.4	1.7	1.8	1.1	1.7	1.2	1.4	1.5	1.2
S-92	1.6	2.9	3.0	3.4	1.4	2.2	2.0	1.5	2.3	1.7	1.1	1.9	1.4	2.0	2.0
S-93	1.3	1.8	2.1	1.9	1.7	2.0	1.5	1.9	3.0	1.6	1.7	1.4	1.5	1.8	2.0
S-94	1.6	2.6	3.2	2.7	1.8	2.7	1.9	1.3	1.7	1.6	1.4	1.4	1.1	1.5	1.5

Table V: Indirect autoradiography assay for the binding of antibody from normal human sera and cancer patient sera to various tumour and normal tissue sections. Tumour pathologies are presented in appendix B and information on the sera in appendix C. The results are in grains per cell. The significance of the grain counts with patient serum compared to normal serum for each tissue section was evaluated by the method of Least Significant Difference.

———— p <0.05

════ p <0.01
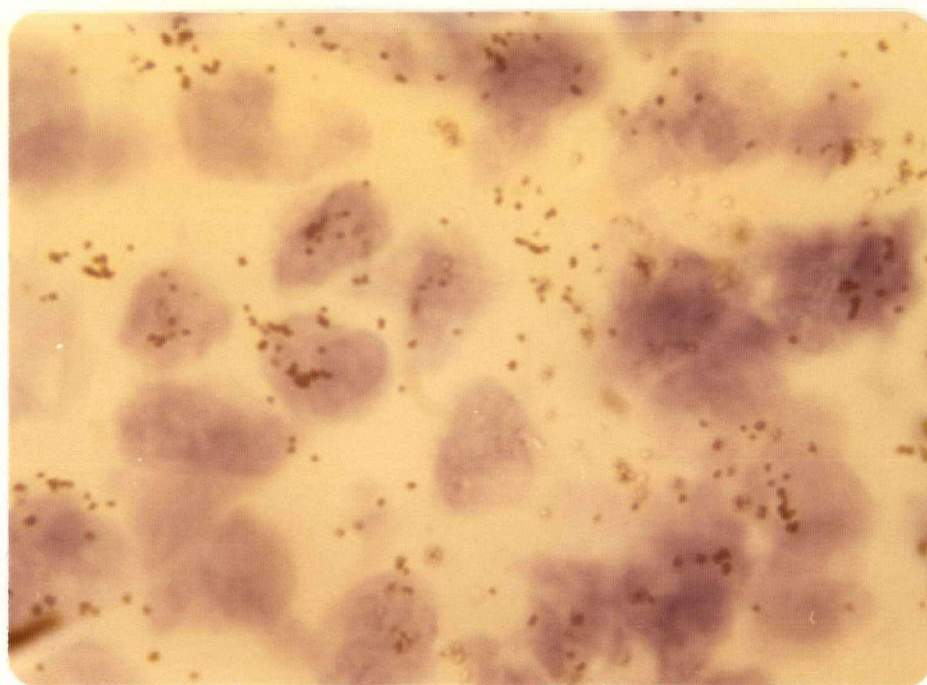
Fig. 39a
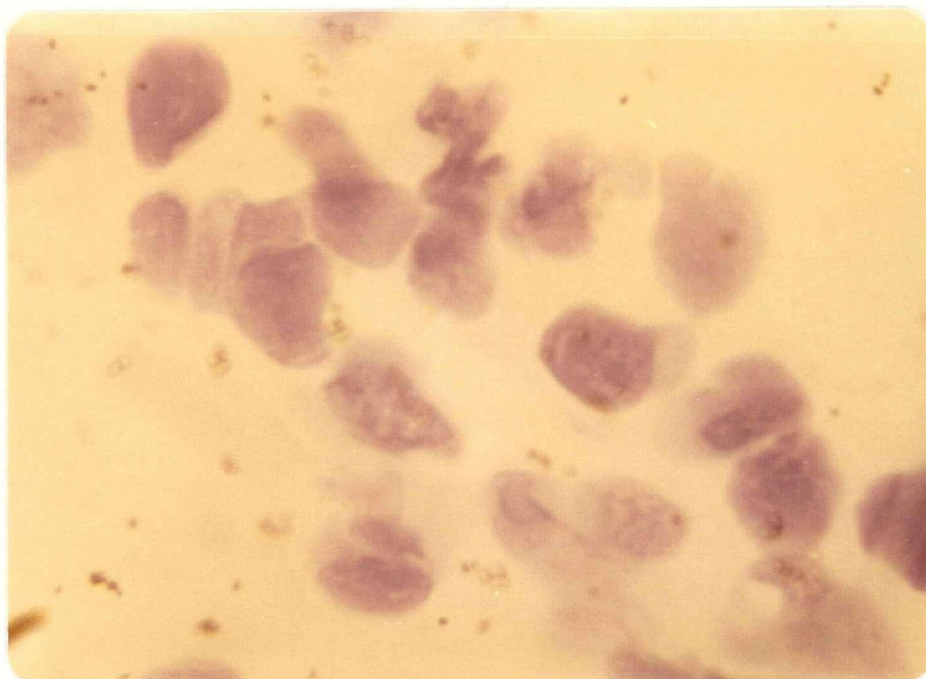
Fig. 39b

Fig. 39:   Photographs of indirect autoradiography results with
            serum 68 tested against: a. C-68 tumour tissue section
            and b. N-86 normal tissue section.

The IA technique has severe limitations when used with tissue sections. The expected sensitivity was not achieved because of the high backgrounds and the necessity to use dilutions of the test antiserum. The difficulty in quantitation precluded multiple testing and the inclusion of some controls. It was also difficult to define the experimental parameters which would allow specific reactions to be detected maximally and to keep backgrounds to a minimum. It was not possible to locate antigens within the tissue or cells.

It is possible that if viable single cell suspensions could be used from either fresh tumour or tissue cultured cells for these studies, many of these problems could be eliminated. Unfortunately, the use of normal lung cells as controls, as well as tumour preparations, would be exceedingly difficult at present.

VIII. Skin Tests of Patients with Extracts of Their Own Tumours

Perhaps the simplest, most direct assay for TAA is to skin test cancer patients with extracts of their own or heterologous tumours. Although skin testing is a less sensitive method of detecting cell-mediated immunity than some in vitro tests (72), it more closely parallels the clinical state of the patient (50,83). The latter result is possibly due to components such as blocking factor and immunosuppressive factors produced by tumours, which may operate in vivo but would be washed from the effector cells or diluted in vitro, or to a possible requirement for tissue structure. Therefore, besides assaying for TAA, this technique could serve as a diagnostic and prognostic aid as well as monitoring the effects of chemotherapy and/or immunotherapy.

For ethical reasons and because of the possibility of detecting histo-compatibility differences, we did not wish to inject patients with extracts

of tumour or normal tissue from other patients. Similarly, injection of

healthy persons with any of the extracts was out of the question. In order

to control the experiments, the patients were skin tested with extracts of

their own tumour and DEAE cellulose fractions which were concentrated to the

original volume of the extract. Autochthonous normal lung extracts were not

available when these experiments were done, but future work should include

them. The expected positive result would be a skin test reaction against the

whole extract and an equal or smaller reaction to one (or possibly more) of

the fractions.

The first patients skin tested with extracts at DEAE cellulose fractions

of their tumours were osteogenic sarcoma patients about to receive immuno-

therapy (BCG plus acetoacetylated tumour extract). Patient 68 was tested with

undiluted (Fig. 40a,b) and 1/10 diluted (Fig. 40c,d) materials to determine

the effects of protein concentration on cutaneous reactivity. Induration and

erythema was measured after different time intervals to aid in determining

the types of response. There appears to be little effect of protein concen-

tration on skin test reactivity over the range tested (Fig. 40), except for

the reaction to C-68-Ace (acetoacetylated extract) which was probably non-

specific due to the high protein concentration of 21.0 mg/ml. Other workers

(53) have reported non-specific reactivity to materials over 3.0 mg protein/ml,

but C-68 at 8.6 mg/ml (Fig. 40) induced no delayed skin reaction at all.

Since a classical delayed hypersensitivity reaction reaches a maximum

at 24 to 48 hours, the reaction observed against C-68 is probably a residual

immediate reaction instead (specific or non-specific), although it is possible

that part of the reaction at 24 hours was cell-mediated. It should be noticed

that the reactivity to some of the fractions was greater than to the whole

extract which contained much more protein. This finding was common to all

of the patients tested.

Fig. 40a,b,c:

142
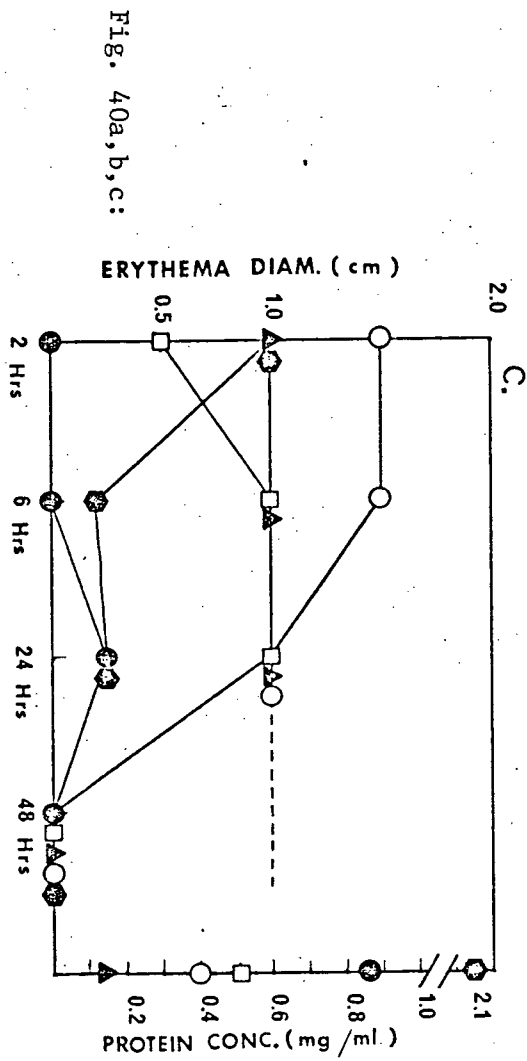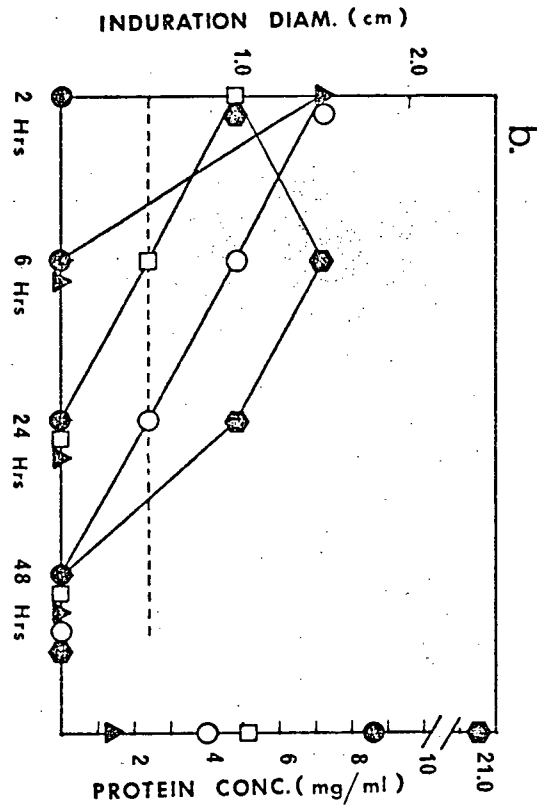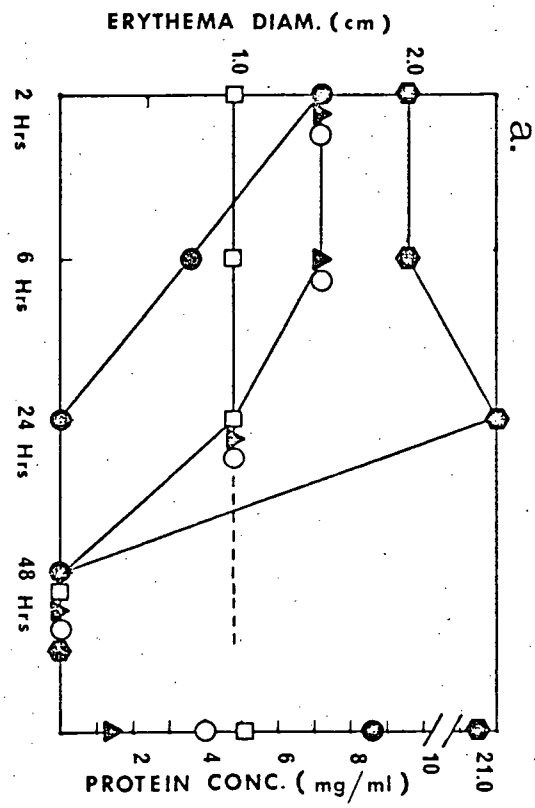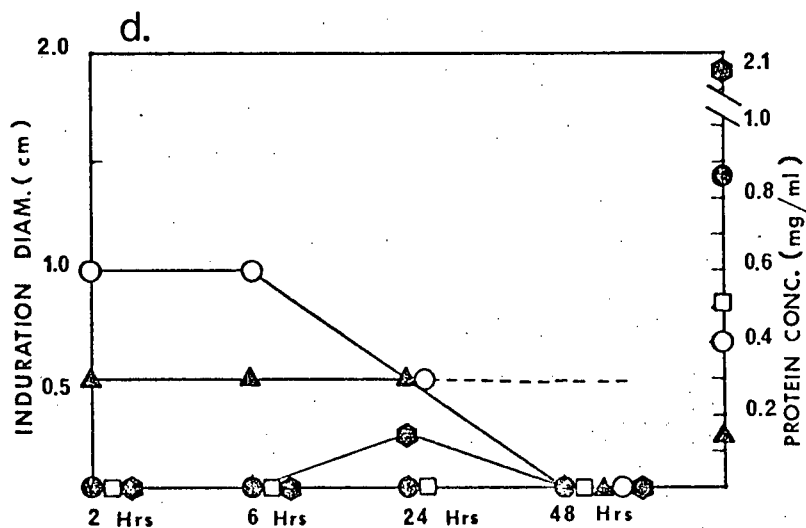
Fig. 40:   Skin tests of osteogenic sarcoma patient with whole extract
(C-68), DEAE cellulose fractions (I, II and III), and aceto-
acetylated (Ace) extract of autochthonous tumour.

● C-68, whole

□ C-68-I

▲ C-68-II

○ C-68-III

⬢ C-68-Ace

The dashed line represents a borderline reaction. The protein
concentrations of the skin test materials are given on the
right.

a. Diameter of erythema with undiluted materials

b. Diameter of induration with undiluted materials

c. Diameter of erythema with 1/10 diluted materials

d. Diameter of induration with 1/10 diluted materials

Similar results were obtained with another osteogenic sarcoma patient, 96.

Patient 73 gave a maximal reaction to two of the fractions at 24 hrs. (Fig.41). While the complete lack of a reaction at 48 hrs is not typical of the time course of delayed cutaneous hypersensitivity, the increased induration at 24 hrs (Fig.41b) shows that a residual immediate reaction was not responsible and suggests that the reaction was delayed hypersensitivity. Note that the protein concentration of C-73-III was only 1/5 that of C-73-Ace. (It may be of interest that this patient is clinically free of disease over a year since surgery).

The results of Figs.40 and 41 show a good correlation between erythema and induration, and appear in agreement with reports that a negative reaction can be defined as less than 1.0 cm diameter of erythema (if induration is also present) (123) or less than 0.5 cm diameter of induration (94,137).

Three bronchogenic carcinoma patients were skin tested with dilutions of extract and fractions of their tumour. Although the results were not quantitated, they can be summarized as follows:

No reactions were observed at 48 hrs and no reactions were observed against the 1/10 dilution after a few hrs.

There were no reactions to any dilution of or undiluted C-67 materials after a few hrs.

There were no reactions to the 1/5 dilution of C-57 materials but there was a positive reaction to the undiluted C-57-I and III at 24 hrs.

There were positive reactions to 1/5 diluted and undiluted C-53-I and II at 24 hrs.

These results clearly paralleled those of the osteogenic sarcoma patients (Figs. 40 and 41) and suggested that more work should be done to
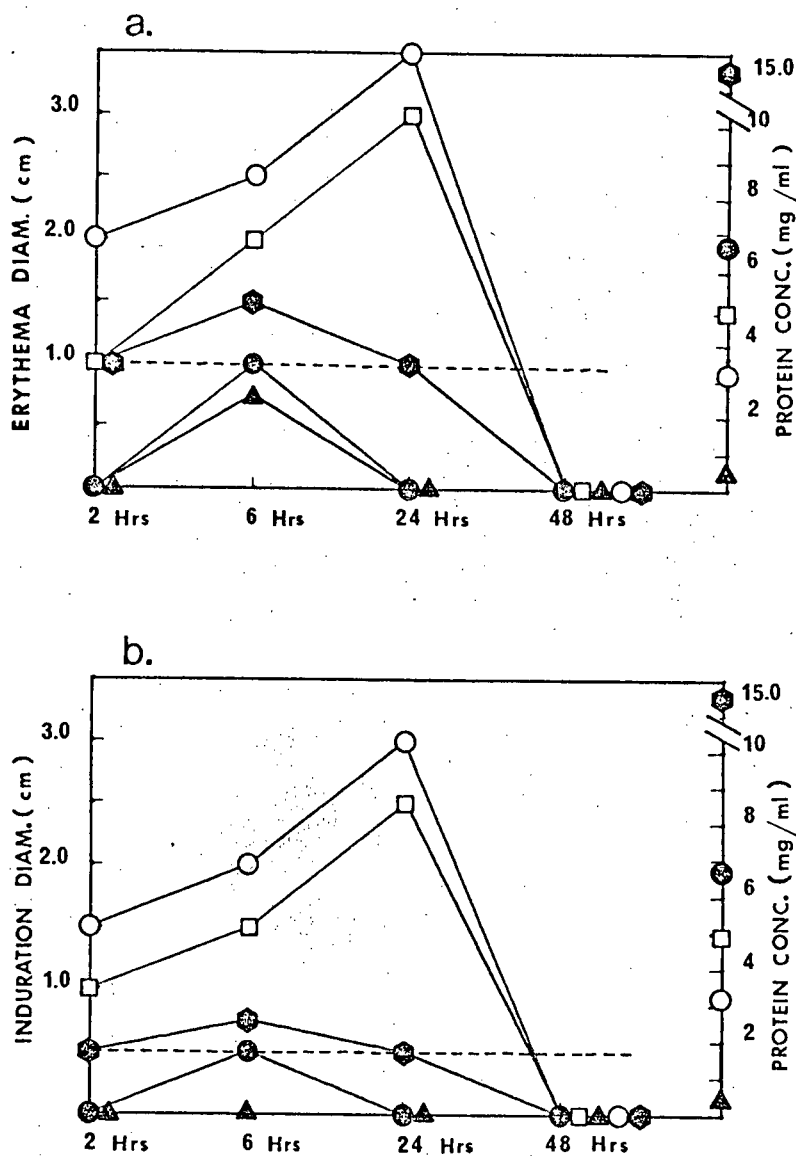
Fig. 41: Skin tests of osteogenic sarcoma patient with whole extract
(C-73), DEAE cellulose fractions (I, II and III) and aceto-
acetylated (Ace) extract of autochthonous tumour.

● C-73, whole

□ C-73-I

▲ C-73-II

○ C-73-III

⬣ C-73-Ace

The dashed line represents a borderline reaction. The protein
concentrations of the skin test materials are given on the
right.

a. Diameter of erythema   b. Diameter of induration

determine the cause of the delayed skin reactions to some of the fractions but not the whole extracts.

To test the fractions for the presence of non-specific skin reactive factors, 4 extracts and their fractions were tested undiluted in non-immune guinea pigs before testing in the patients. The results of 3 of the animals were similar to Fig.43, and the fourth gave reactions to C-76 and fractions, which were all near 1.0 cm diameter. This is not a good test for non-specific factors because all of the materials stimulated a reaction although some of them were negative in the patients (Fig.43). However, in 3 of 4 guinea pigs the reaction was somewhat greater to fraction III than the whole extract, although the whole extracts contained more protein (Fig.42). On this basis, there could be non-specific skin reactive factors in fraction III of the extracts, but the time course of the reactions (Fig.42) does not suggest a delayed hypersensitivity reaction.

The results of the patient skin tests with these materials are presented in Fig.43. Patients 87 and 85 who failed to give a positive reaction (at 24 hrs) to their tumour materials (Fig.43a,b), also did not respond to any of the recall antigens, thus demonstrating impaired immune responsiveness. Similar results have been reported by others (94,137).

The time course of the reaction of patient 72 to the fractions (Fig. 43d) suggest that it is a residual immediate reaction, possibly with no delayed hypersensitivity at all. Again, however, there is some reaction against the fractions and not the whole extract.

The time course of the reaction of patient 76 to fraction II and III (Fig. 43c) is suggestive of delayed hypersensitivity, while the reaction to C-76 is smaller and possibly not delayed hypersensitivity.

These results are different from studies reported on two transplantable methylcholanthrene-induced guinea pig sarcomas (55). The animals gave a
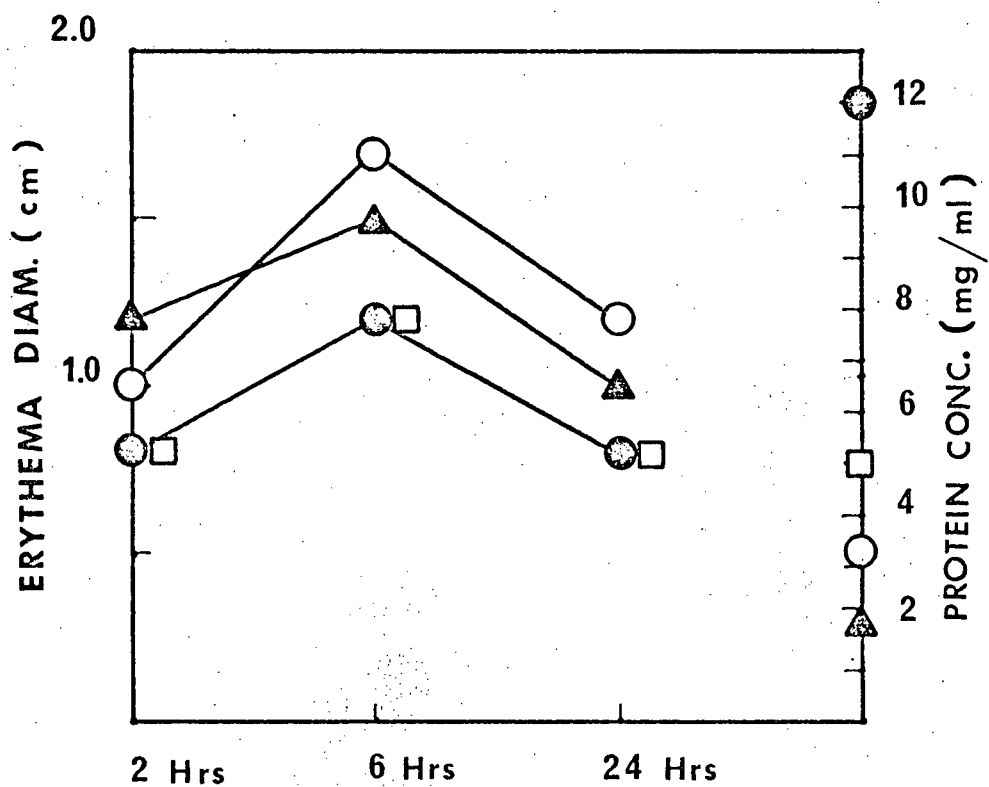
Fig. 42:   Skin tests of a non-immune guinea pig with C-87 extract
and DEAE cellulose fractions. The protein concentrations
of the skin test materials are given on the right

● C-87, whole
□ C-87-I
△ C-87-II
○ C-87-III
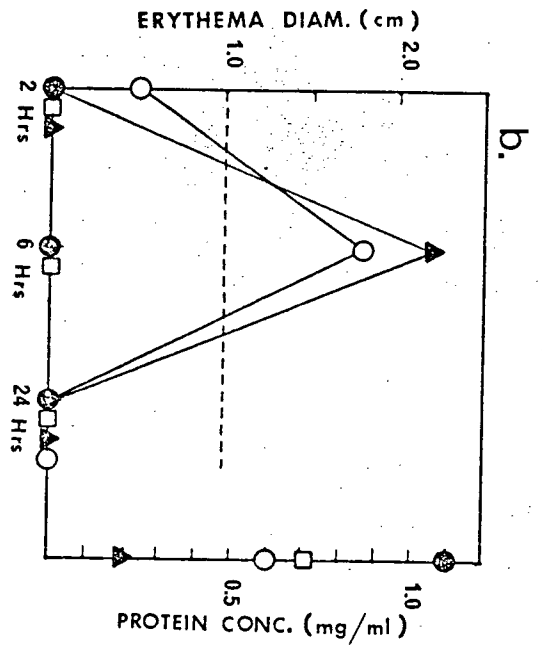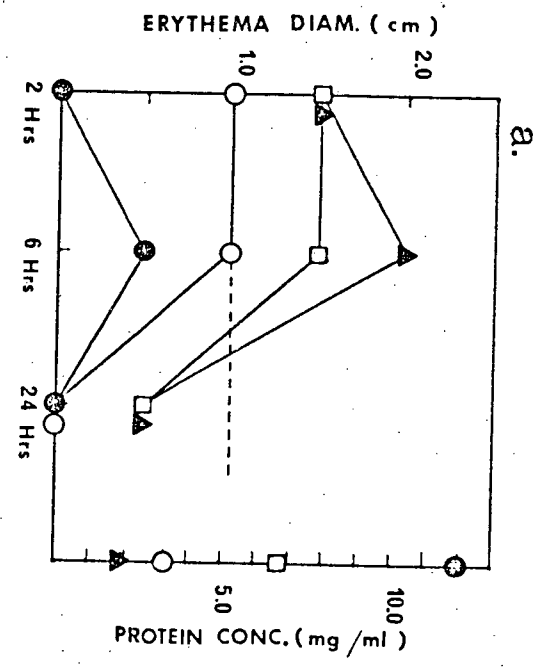
Fig. 43a,b,c:

Fig. 43: Skin tests of bronchogenic carcinoma patients with extracts and DEAE cellulose fractions (I, II and III) of their own tumours.

● whole extract
□ fraction I
▲ fraction II
○ fraction III

a. patient 87
b. patient 85
c. patient 76
d. patient 72

delayed hypersensitivity reaction to whole extracts and one of the fractions upon skin testing with tumour extract prepared by sonication of tumour cells and 5 DEAE cellulose fractions. The reactions were specific for the individual tumours and although the positive fractions were eluted under different conditions, both would be eluted from the DEAE cellulose under the conditions for the preparation of fraction III of Figs.40 to 44 .

Other workers (83,132) using whole 3.0M KCl extracts of human tumours, were able to detect positive delayed cutaneous hypersensitivity in approximately 50% of the immunocompetent patients tested. Vanky et al (132) also tested patients with 3.0M KCl extracts of normal tissue and found a few weak reactions, suggesting the possibility that the reactions were not tumour specific.

In a study by Wells et al (137), 7 of 14 immunocompetent lung cancer patients demonstrated delayed cutaneous hypersensitivity to autologous tumour cell membranes. However 2 of these 7 patients also reacted to autologous leukocyte membranes and 1 reacted to normal lung cell membranes.

Hollinshead et al (53) detected no delayed hypersensitivity to immunocompetent lung cancer patients skin tested with tumour cell membranes. However, 50% of these patients demonstrated delayed cutaneous hypersensitivity (confirmed by histology) to Sephadex G-200 fractions or gradient polyacrylamide gel fractions of sonicated membrane extracts. (There was no indication (53) that any patients were tested with the sonicated membrane extract before it was fractionated). Their results correlate very well with those presented here in Figs. 40, 41, 43, 44 although the extraction and fractionation methods were different. They also compare their lung cancer results (53) with the results of their studies on different types of tumours under the same conditions. Membranes for intestinal cancer and leukaemia cells induced delayed hypersensitivity in the respective patients, while the reactions to membranes from breast cancer and malignant melanoma cells were weaker and less frequent.

There is no explanation given for the difference in reactivities of different types of tumours, but it is suggested that the poor reactivity to the membranes is due to blocking factors which were removed from the antigens in the fractions. (Blocking factors were probably not responsible for the lack of reactivity of the 3.0M KCl whole extracts of Figs. 40,41,43,44) because the DEAE cellulose fractionation procedure would not be expected to dissociate antigen-antibody complexes). Some reactions were also observed when patients were tested with autologous and heterologous normal lung extract fractions (53), although it was possible to differentiate between lung specific and tumour specific antigens.

Averkieva and Trakhtenberg (3) also report frequent delayed cutaneous hypersensitivity of lung cancer patients to protein-containing tumour extract fractions and less frequent reactivity to non-malignant tissue extract fractions. They detected reactions to tumour extract fractions by 2 of the 130 normal subjects tested.

In a recent study on breast carcinoma antigens, Hollinshead et al (51) report poor delayed hypersensitivity reactions to tumour cell membranes, and sonicated membrane extracts, and frequent strong reactions to some fractions of the extracts. Four of 8 patients reacting to tumour extract fractions also reacted to similar fractions of normal tissue extract. Further fractionation by gradient polyacrylamide electrophoresis could differentiate between a normal tissue antigen and a tumour-associated antigen (51). Reactivity to normal extract was also detected in the human peripheral blood MIF studies (II).

Because some of the recent studies on delayed cutaneous hypersensitivity to tumour extracts (51,53,132) clearly show that some of the reactivity was against normal antigens, and because the patient access required to clearly establish whether the specificity of the reactions are due to organ, virus,

or tumour antigens was not available here at the time, this approach was deemed unsuitable as an assay for biochemical and immunochemical studies of tumour antigens. Nevertheless, I have tried to demonstrate if it is even possible that the reactivity seen against some of the extract fractions (Fig. 41,43c) is against tumour antigens. It is possible that greater reactivity to some of the fractions than the whole extract was due to:

Contamination of the fractions (ie. chemical or bacterial).
Modification of existing materials (eg. by exposing an antigen or a non-specific skin reactive factor).
An immune suppressor (specific or non-specific) present in the extract, which is capable of inhibiting skin reactions to tumour and/ or normal antigens. Such a suppressor would be separated from anti-gen or lost by the fractionation procedure.

To test these possibilities, patients were skin tested with 1:2 dilutions (in 0.15 M NaCl) of the fractions and extract of their tumour and a 1:1:1 dilution of the 3 DEAE cellulose fractions. All 5 patients tested were shown to be immunocompetent by reacting to at least one recall antigen. Erythema and induration were measured at 2 and 24 hrs, but only the diameter of induration is presented in Fig.44 because the primary concern is the delayed hypersensitivity reaction. The results of Fig.44a,b show positive reactions only against the mixture of the fractions. This is not readily explainable, but does show that the fractionation procedure produced a change in these extracts which could not be reversed by reconstitution. The results of Fig. 44c,d,e are much more interesting. (It should be emphasized that the mixtures contained as much of each fraction as the dilution of the fractions used for skin testing). From Fig.44c it can be seen that C-87-I and C-87-II
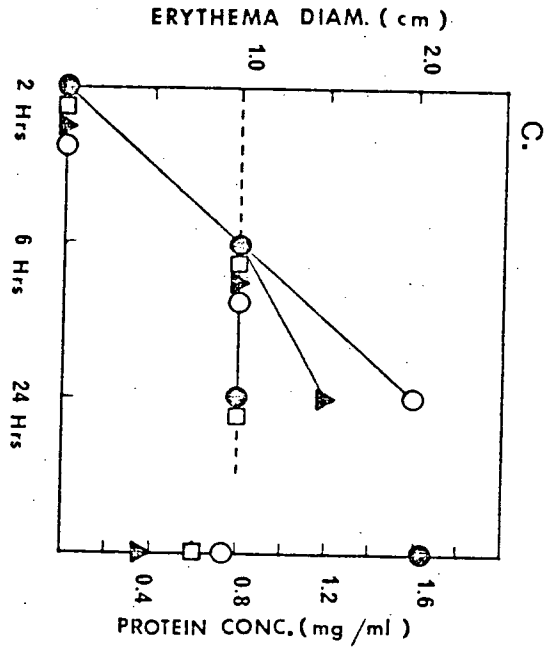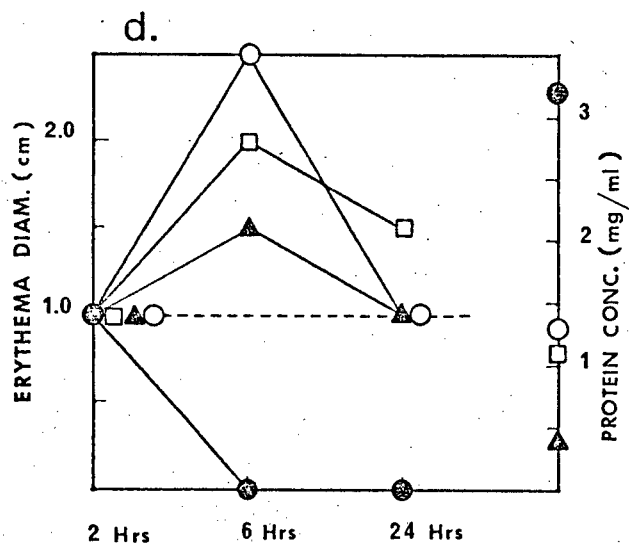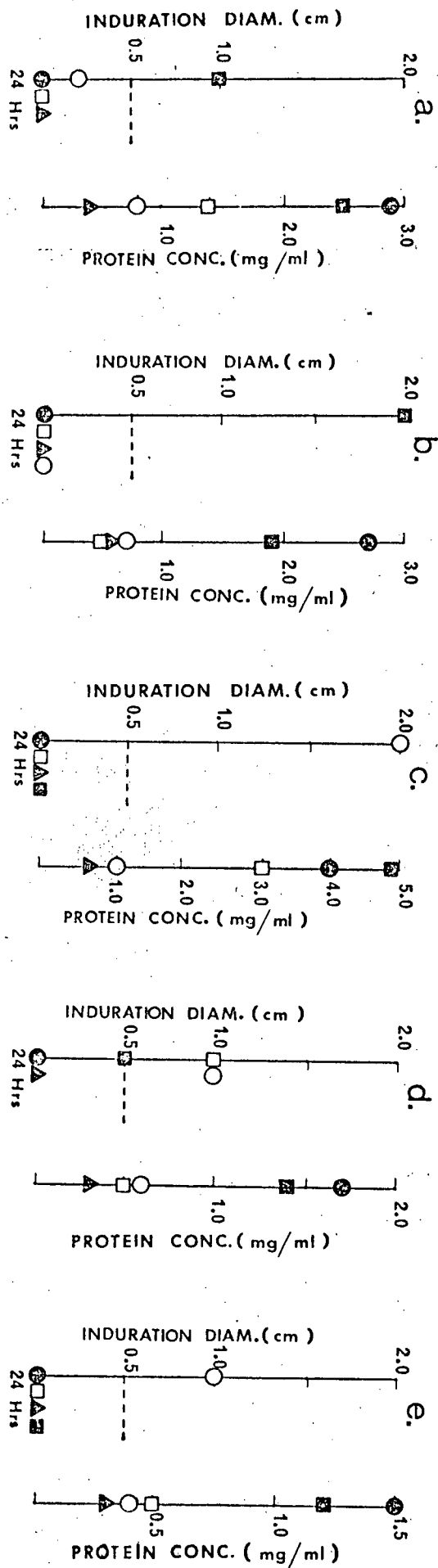
Fig. 44: Skin tests of bronchogenic carcinoma patients with extracts, DEAE cellulose fractions (I, II, and III), and these fractions mixed 1:1:1. Only the diameter of induration at 24 hr is presented here for clarity. The whole extracts and the fractions were diluted 1:2 with saline.

● whole extract 1:2   a. patient 57
□ fraction I; 1:2   b. patient 93
▲ fraction II; 1:2   c. patient 87
○ fraction III; 1:2   d. patient 88
▨ I, II, III; 1:1:1   e. patient 60

could eliminate the 2.0 cm reaction to C-87-III and that the mixture contains 3 times the protein concentration of C-87-III. C-88-II could reduce a 1.0 cm reaction against C-88-I and C-88-III to 0.5 cm (Fig. 44d). A 1.0 cm reaction against C-60-III was eliminated by mixing with C-60-I and II (Fig.44e). Thus, a factor(s) in fraction I and/or II (II in the case of C-88) was shown to be capable of eliminating or diminishing a delayed skin reaction to autologous tumour extract antigens.

The results presented here (Fig.43c,44d) and the results of others (53) have shown delayed skin reactivity to more than one lung tumour extract fraction. This suggests that either more than one antigen was present or that the antigen was present in different forms, such as aggregated or in association with other materials. Although it is possible that normal tissue antigens were present, the detection of reactivity to more than one fraction (53) when no reactivity was detected against normal antigens suggests that in some lung tumours more than one tumour antigen may be present. This would support the results from the studies by immunodiffusion with absorbed anti (tumour extract) serum (IV.1) and indirect autoradiography with patient serum (VII.2.).

Whether the antigens are tumour or normal tissue associated, the detection of a factor which can diminish a cell-mediated immune response in vivo could have tremendous implications for the immunotherapy of patients with malignant diseases. Further studies on the nature of this factor(s) are presently under investigation. These studies are beyond the scope of this thesis and will not be included.

GENERAL DISCUSSION

The original goal of this project was to definitively study the biochemical and immunochemical properties of antigens associated with human bronchogenic carcinoma. The absence or the presence of common or unique antigens could tell whether immunotherapy might be feasible and it is possible that purified tumour antigens could be used for specific immunotherapy. Ultimately, immunization methods might be developed which would protect against malignant diseases. If the TAA are common or cross-reactive (but not necessarily antigenic in the patient), the detection and quantitation of antigen in the serum could have diagnostic and prognostic value and possibly allow the screening of large numbers of high risk individuals for cancer. This last factor would be of particular value for lung cancer because high risk patients are known and lung cancer diagnosis is often late in the course of the disease. Such tests would require purified antigen and highly specific antibody.

Studies on the biochemical and immunochemical relationships of TAA could supply information about the possible causative agents for malignant diseases, such as the studies on RNA virus antigens and human leukaemia (36,116) and information about the nature of oncogenesis, such as the relationship between histocompatibility antigens and TSTA.

One of the chief problems in this study arose from misleading reports in the scientific literature. The method of inducing neonatal tolerance in rabbits was shown to be ineffective in this study and by other workers (Dr. J. Berczi, Department of Immunology, University of Manitoba, personal communications). The significance of certain immunofluorescence techniques might also be questioned because they measure the percentage of fluorescent labelled cells in a population rather than the amount of fluorescence on

each cell. There are many reports of tumour antigen detection using cancer patients' immune responses, such as the MIF test and delayed cutaneous hypersensitivity. However, the MIF test results reported in this study and the skin test results of others (51) where normal control results were reported, demonstrated that the reactivity to tumour materials was not necessarily tumour specific (although careful analysis could resolve tumour specific reactivity (51)). Such non-tumour-specific reactions to tumour materials could be autoimmune phenomena, perhaps related to malignant diseases as suggested by Hall (40).

The detection of immune responses of cancer patients to tumour antigens is a prerequisite for studies on TSTA. The skin test results reported here suggest that tumour antigens may be present (although tumour specificity was not ascertained) in some of the tumour extracts, but were not detectable until the extracts were fractionated on DEAE cellulose because of an immunological inhibitor which could be separated from the antigen.

The results of the indirect autoradiography studies (Table V) where tissue sections were reacted with patients' sera, do not appear to be due to autoimmunity because the normal lung tissue sections and the two normal sera were not associated with significant binding of radioactivity. However, many more normal controls would need to be studied before the possibility of autoimmunity could be eliminated. An interesting observation from Table V is the negative results with some homologous sera where heterologous sera gave a positive reaction. It is certainly possible that false positive and negative results can occur or that antibody might induce a change in phenotypic expression of immunogenicity (57), but the non-reactivity with homologous serum could also suggest that multiple antigen systems are present. This would be in agreement with the results of the immunodiffusion studies using absorbed antisera (section IV.1.) Non reactivity in the homologous system

when reactions were detected in a heterologous system was also reported by McCoy et al (75), using an MIF technique to study extracts of human breast cancer.

The large quantity of normal antigen and the low concentration of TAA in the tumour extracts were the main problems in the detection of TAA by xeno-antisera. Although methods of inducing tolerance to normal antigens were unsuccessful, some good results were obtained by using solid phase immunoadsorbents to remove antibodies to normal antigens from the serum of rabbits hyperimmunized with tumour extract and testing these absorbed anti-sera by immunodiffusion against normal lung and tumour extracts. The system reported here probably demanded too much from the techniques because anti-bodies to normal antigens were often still present after multiple absorptions, but it does indicate a direction for further tumour antigen studies. The results of this system should be greatly improved by minimizing the antibody response to the normal antigens during hyperimmunization. (Immunoadsorbents would still be required.) This could be done a few different ways:

Non-human primates instead of rabbits could be immunized with tumour extracts (85,86).

Rabbits could be treated with normal tissue extracts neonatally or with cyclophosphamide before hyperimmunization with tumour extracts.

The tumour extracts could be pre-absorbed with an immunoadsorbent of antibodies against normal antigens. This technique could also augment other studies on tumour antigens in extracts.

If monospecific antibody were prepared, more sensitive antigen detection methods such as radioimmunoassay could be used.

We can be confident that the antigens detected by the immunodiffusion

technique are tumour-associated since they were not detected in any of the 9

normal lung extracts or the 3 normal serum samples employed at higher protein

concentrations than the tumour extracts. (These antigens were also not

detected in any of the cancer patients' sera). However, it is possible that

the antigens detected are from infection by bacteria or opportunistic viruses.

It is unlikely that the antigens would be bacterial, since more would be

expected in the normal lung extracts than the tumour extracts and the con-

ditions of extraction and storage did not encourage bacterial growth. Studies

with preparations of different viruses would be required to demonstrate a

viral origin of these antigens. If further studies with normal controls

still showed tumour specificity of such viral antigens, these antigens could

be of value both clinically or scientifically.

The results of immunodiffusion studies presented here also suggest

that more than one antigen may be present in the tumour extracts and that

some of these antigens may be common to tumours of different pathology. A

similar common antigen was detected by several of the antisera in a precipitin

band which formed near the central (antiserum) well on the immunodiffusion

plates. One of the antisera demonstrated a partial identity reaction with

this antigen and an antigen in the foetal extracts. This antigen was shown

to be neither carcinoembryonic antigen nor α_1-foetoprotein.

Other TAA (in C-77 and C-94) were also detected, but not as often as

the TAA in the inner precipitin band. At least one of these antigens could

be detected in larger quantities than others as evidenced by a heavy pre-

cipitin band, and it was shown to be inactivated by repeated freeze-thawing.

Another TAA (C-26) was detected in only a few tumour extracts.

Although the common TAA were frequently detected in extracts of

tumours of different pathologies, when the overall results for each antiserum

and tumour extract were compared according to tumour pathology, correlations

were observed. Squamous cell, anaplastic and oat cell carcinomas all showed a similar level of cross-reactivity with each other and lower cross-reactivity with adenocarcinomas (and possibly alveolar cell carcinomas). Adenocarcinomas and alveolar cell carcinomas cross-reactions were similar with each other and lower with squamous cell, anaplastic and oat cell carcinomas. The differentiation between these two groups of lung cancer pathology is of particular interest because most tumours in the latter group are carcinogen induced while the adenocarcinomas and alveolar cell carcinomas are not (66). There is some evidence of viral association with the latter two types of tumours (87,135). In order to ascertain whether the antigens are also present in normal tissue preparations and to answer other important questions such as quantitation, the presence in serum, location and the significance of the tumour antigens to the clinical situation, purified antigen and high titre monospecific antibody will be required.

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APPENDIX A

Protein concentration of extracts

Extract	mg protein/ml	Extract	mg protein/ml
N-pool	29	C-62	6.7
N-25	40	C-63	8.1
N-50	20	C-64	4.0
Norm	30	C-65	6.5
N-83	33	C-66	5.7
F-48	14.5	C-67	6.9
		C-68	8.6
C-1	6.9	C-69	11.6
C-2	17.6	C-70	5.7
C-3	6.3	C-71	4.4
C-4	4.7	C-72	3.2
C-5	13.8	C-73	6.7
C-6	15.4	C-74	11.1
C-24	9.5	C-75	8-2
C-26	18.0	C-76	8.2
C-30	27	C-77	7.9
C-36	1.9	C-78	6.6
C-40	19	C-79	6.5
C-41	20.1	C-81	10.4
C-45	13.7	C-82	7.9
C-46	18.5	C-85	5.5
C-53	11.0	C-87	12.1
C-54	7.5	C-88	5.0
C-55	7.2	C-90	7.9
C-56	5.1	C-91	6.0
C-57	8.6	C-92	7.0
C-58	4.9	C-93	8.2
C-60	4.6	C-94	6.3
C-61	6.0	C-96	18.3

APPENDIX B

All this information is from pathology or autopsy reports

Reference Number	Patient	Age/Sex	Autopsy or Surgical A	Autopsy or Surgical S	Cell Type	Size	Extent of Metastases
C-1	VER	76 M	✓		Squamous cell	'large'	none
C-3	LAN	61 M		✓	Adenocarcinoma		mediastinal lymph nodes
C-4	HOL	33 M		✓	Carcinoid		peribronchial nodes
C-5	PHI	M		✓	Adenocarcinoma		hilar nodes
C-6	NYS	63 M		✓	Squamous cell	7 x 6 cm	hilar nodes
C-24	DIC	62 M	✓		Adenocarcinoma		brain, liver
C-26	ROB	62 M	✓		Adenocarcinoma	10 x 6 cm	hilar nodes and widespread*
C-30	CON	49 M	✓		Alveolar cell carcinoma	'huge'	hilar nodes and brain
C-36	STE	72 M	✓		Adenocarcinoma	'huge'	local extension and liver
C-38	MIK	68 M	✓		Anaplastic	9 cm diam	widespread
C-40	CHA	44 M		✓	Oat cell	2.5 cm	local nodes and liver, bones
C-41	MOO	54 F	✓		Anaplastic (some squamous elements)	8 cm diam	widespread
C-45	LEH	54 M	✓		Adenocarcinoma	medium	widespread
C-46	DRY	60 F	✓		Anaplastic	'large'	widespread
C-53	JOH	67 M		✓	Squamous (recurrence of primary resected 3 yrs ago)		bilateral hilar nodes
C-54	MCW	50 F		✓	Anaplastic (viewed by some as a mesothelioma)		extensive chest wall invasion
C-56	ALL	71 M		✓	Anaplastic (some sarco-matous elements)	4 cm diam	residual disease
C-57	HOP	62 M		✓	Squamous (biopsy of metastatic lesion, lung primary)		distant site (floor of mouth)
C-58	FIS	M		✓	Adenocarcinoma (biopsy from metastatic nodule in abdomen from lung primary)		distant site (abdominal mass)
C-60	AND	72 M		✓	Squamous cell	3 cm diam	
C-61	TUC	M	✓		Squamous cell	123 gm	hilar nodes
C-62	CHAL	M		✓	Anaplastic	96 gm	
C-63	BUR	M	✓		Squamous cell	133 gm	mediastinium and brain
C-64	LeJ	M	✓		Oat cell	35 gm	widespread
C-65	AND	48 M		✓	Oat cell		
C-66	TRE	M	✓		Squamous cell	105 gm	widespread
C-67	DAV	F		✓	Anaplastic (some sarco-matous elements)	52 gm	lung and local lymph nodes
C-68	McP	54 M		✓	†Atypical osteogenic sarcoma	11 cm	none
C-69	CLI	F		✓	Squamous cell	>14 gm	pleural extension
C-70	LeB	F		✓	Oat cell	>15 gm	hilar nodes and residual disease
C-71	BAR	83 M	✓		Squamous	5x4.8 cm	lymph nodes
C-72	CAR	F		✓	Oat cell	>12 gm	local nodes
C-73	FLI	9 M		✓	Osteogenic sarcoma		none

* Widespread: numerous organs involved with metastatic disease
† Tumour tissue origin - bone

APPENDIX B (Continued)

Reference Number	Patient	Age/Sex	Autopsy or Surgical A	Autopsy or Surgical S	Cell Type	Size	Extent of Metastases
C-74	MAL	M		✓	Squamous	>10 gm	none
C-75	SHU	M		✓	Squamous	35 gm	hilar
C-76	BAL	M		✓	Squamous	56 gm	
C-77	McC	M		✓	Rhabdomyosarcoma§	46 gm	sub pleural extension
C-78	HAR	F		✓	Oat cell	>13 gm	pulmonary lymph node
C-79	FOL	73 M	✓		Squamous cell	'large'	local extension and distant sites
C-81	MON	65 M		✓	Alveolar cell (2 primaries)	both 'small'	none
C-82	MEL	63 M	✓		Mixed (squamous and adenocarcinoma elements)	5 cm diam	local nodes
C-85	WAL	60 F		✓	Adenocarcinoma	5 x 3 cm	local nodes and satellite nodules
C-87	POB	68 M		✓	Squamous	'large'	local nodes
C-88	GOR	69 F		✓	Adenocarcinoma	3 cm diam	none
C-89	FOS	53 F		✓			
C-90	CHA	62 F		✓	Squamous	5 x 3 cm	extensive local
C-91	DIX	12 F		✓	Osteogenic sarcoma		
C-92	BAL	60 M		✓	Anaplastic	6 x 6 cm	local nodes
C-93	SMI	58 M		✓	Squamous		
C-94	MOR	64 M		✓	Squamous	6 x 5 cm	local nodes
C-96	LAN	15 M		✓	Osteogenic sarcoma		

§ Tissue of origin - (Chest wall) striated muscle

APPENDIX C

Serum sample	Condition of donor when serum taken	Metastases detected within 2 months of serum collection
S-53	5 mo. post-op.	−
S-66	autopsy	
S-68	3 mo. post-op.	+
S-71	autopsy	
S-74	pre-op.	−
S-77	3 wk. post-op.	+
S-79	autopsy	
S-85	pre-op.	+
S-87	pre-op.	+
S-88	pre-op.	−
S-89	pre-op.	+
S-90	pre-op.	−
S-91	pre-op.	+
S-92	pre-op.	+
S-93	pre-op.	−
S-94	pre-op.	+