SOLUBLE TUMOR ANTIGENS USED IN CLINICAL TRIALS OF IMMUNOTHERAPY

Ariel C. Hollinshead, Ph.D., Professor Emerita
Department of Medicine
The George Washington University
Washington D.C., USA

INTRODUCTION

In this chapter, we (I.)define **tumor-associated antigens** (TAA) used in laboratory evaluations as well as in their role as immunogens. (II.)summarize briefly **early studies of antigens in animal tumors and in human tumors.** (III.)summarize **methods of separation, identification, purification and analysis of antigens.** (IV.)focus upon those TAA **identified with lung cancers, and describe the unique nature of lung cancer,** the complexities presented by a **dynamic spectrum** of lung cancer types, as shown previously using histopathologic, electron microscopic, hormonal, cultural, and epidemiologic evidence and show the way in which TAA as biomarkers participate in this interrelationship as well as suggest genetic evidence. (V.)discuss the various **forms of immuno-therapy** and their usage alone and in combination and put into **perspective** the rationale for usage of a selected therapy, specific active immunotherapy, for lung cancer. (VI)describe the quality-controlled, tested preparations of **lung TAA immunogens in batches used in clinical trials.** Finally, (VII), we discuss what we do not know and what we have learned about the **mechanisms involved in the induction and maintenance of the control of lung cancer using specific active immunotherapy and consider how they might inter-relate with the possible role of dormancy,** the mechanism which is the subject of this workshop. Detailed reports of most of the work, here briefly reported, are available for study[1].

I. TUMOR ASSOCIATED ANTIGENS

TAA are polypeptides which are purified from proteins separated from the isolated, washed cell membranes of cancer cells, and which are identified and characterized for their ability to produce cell-mediated immune reactions specific for the TAA by the tumor-bearing host, both by in vitro and in vivo testing.

These TAA are designed for testing as specific active immunotherapeutic or immunoprophylactic biologic drugs both for safety and for efficacy in the treatment of human cancer and for further purification and use in producing simple and hybridoma cell line antibodies recognizing the TAA, for further preparation of TAA epitopes, for definitive protein analyses and gene identifications and other studies.

II. STUDIES OF ANIMAL AND HUMAN TUMORS

In 1962, we introduced the idea that tumor cell membranes contain unique proteins which might affect the growth of tumors[2]. The studies were performed in animal models, first using cancer cells in tissue culture and studying their growth in a privileged immunological site in Syrian hamster cheek pouches. My technicians Willis Wheeler and Heijia Lee helped to prepare HeLa cells grown in spinner culture, and to separate the cell constituents chemically or by ultracentrifugation methods after breakup of the cells by ultrasonic procedures. The DNA and RNA fractions were analyzed chemically and the purity of the microsomal fraction and membrane material were assessed using electron microscopy. Hamsters were given the DNA fraction suspended in Freund's adjuvant, whereas controls received Freund's adjuvant alone. Ten days after the first injection, HeLa cells were implanted in each cheek pouch. Animals were not conditioned by cortisone or by irradiation in order to avoid introduction of nonspecific factors into the response mechanism. Results of this initial experiment suggested that DNA was actually enhancing tumor size (Table 1). However, using saline injections as control and slightly increased cell inoculum, DNA was found to have no effect upon size or number of tumors. In fact, the Freund's adjuvant had reduced the average tumor size, although there was no effect upon the number of tumors (Table 1). Similarly (Table 2), RNA or microsomes from these cells had no effect, whereas the membrane components somewhat reduced the size and the number of tumors. This was the first report of the identification of membrane components separated from the rest of the cell and their effect in reducing the size and the number of tumors.

There were also a group of studies, first on randombred hamsters with transplantable adenovirus type 12-induced tumors in which the separated, identified, soluble tumor-specific transplantation antigens derived from the soluble materials of separated tumor cell membranes showed a strong, titration-dilution curve effect in blocking tumor growth[3-7]. In our experimental studies, we investigated a variety of materials including the hexons, fibers and core antigens of a series of wild and domestic types of adenovirus inoculated before, during the incubation period, and after challenge in random-bred hamsters with transplantable adenovirus 12-induced tumor. These studies generally led to disappointing observations. Only the hexons

appeared to protect the hamsters, and only to a moderate degree. Strikingly, one group of hamsters that had been injected with Sephadex fraction II of the solubilized adenovirus 12 tumor cell membranes looked as though challenge had been forgotten. In the light of our earlier cheek pouch studies, we undertook a further investigation which resulted in the identification of a soluble tumor-specific transplantation antigen (TSTA) from the membrane fraction of adenovirus tumor cells. The TSTA was highly effective in protecting randombred hamsters. These studies were repeated[8] in carefully inbred hamsters and an immunizing dose-response relationship as well as the specificity of the reaction were established (see Figure 1). At a 60 microgram protein dose, 50% of hamsters were protected and at 100 microgram TSTA protein, there was 100% protection. These animals were further studied as follows: Tumors were excised from a number of the control group and from those with tumor who received the 60 microgram dose. The animals from the control group had a recurrence of tumor growth but those in the 50% immunization group did not have a recurrence of tumor. Animals totally protected against tumor were given a different type of tumor, SV-40 cells, and the tumors which occurred were unique to SV-40. Soluble membrane antigens that affected tumor growth were also found in carcinogen-induced tumors[9]. In the virus-induced tumors, the sera of protected animals had antibodies reactive with the membranes of adenovirus 12 abortively-infected cells as demonstrated by indirect immunofluorescence, mixed hemad-sorption, and indirect paired radioiodine-labeled antibody tech-niques. Antibodies were not determined using the Chromium-51 release technique and, therefore, do not appear to be cytotoxic. In a separate study, noncytotoxic antibodies formed during the growth of carcinogen-induced tumors, giving evidence of an immune response by the host. Neutralization of C-3H anti 6C-3HED fluorescence by incubation of soluble antigen and 5X diluted antiserum was specific. These studies with virus-induced and carcinogen-induced tumors revealed in the sera of vaccinated animals the presence of noncyto-toxic antibodies specifically reactive with the antigens used for vaccination (Figure 1).

Additional experiments using Freund's complete adjuvant (FCA) were carried out[10]. Three groups of 24 hamsters were injected with 10^4, 10^5, and 10^6 tumor cells, respectively. Half of the animals in each group were given FCA intraperitoneally 17 days before subcutaneous adenovirus-induced tumor cell challenge; the other half were not pretreated. All animals injected with FCA developed tumors, whereas in the hamsters receiving no adjuvant, one-third of those inoculated with 10^4 cells, one-half of those inoculated with 10^5 cells, and all of those inoculated with 10^6 cells developed tumors. Thus, these findings illustrated that general stimulation of the lymphoreticular system by nonspecific materials might produce deleterious results. The effect of this stimulation greatly depends upon the type and site of tumor and the type of immune response

predominant in the host at the time of injection with nonspecific adjuvant. For example, BCG cell-wall skeleton oil-water, BCG or FCA might stimulate the existing host response we reported as present in early stage melanoma, whereas the use of these nonspecific adjuvants in the late stage of the disease might cause havoc since we find that the majority of these patients react to the wrong antigens[11]. In our studies, we reported[12] that the opposite occurs in many breast cancer patients, and the use of nonspecific active immunotherapy in early stage breast cancer seems inappropriate.

Finally, after study of cell surface components in developing human fetal organs, adult normal organs and benign disease adult organs, a study of cell surface membrane components in primary organ-related human cancer cells was initiated and resulted in the identification of human tumor-associated antigens (TAA). In brief, hundreds of fresh primary tumors and control tissues were collected and immediately processed, surgical and pathology reports and patient histories were collected. All cells, membranes, soluble proteins and separated fractions, regions and individual proteins were studied, measured for quantity and size, characterized and evaluated for ability to induce cell-mediated immune responses both in vivo and in vitro. Active proteins (antigens) were further analyzed, characterized, cross-tested and candidate tumor-associated antigens identified for usage in therapies. Properly prepared purified peptides which are highly concentrated and of uniform protein concentrations are an important key to the success of producing predictable CMI responses. It was an enormous undertaking and required development of meticulous procedure, development of important quality controls and safety measures, as well as intensive training of all personnel involved in these tasks. In 1968, at a Gordon Conference in New Hampshire, we presented the results of our studies of both fetal and normal, benign and cancerous adult colon cells, and identified both nonspecific and specific proteins related to the developmental changes as well as to the overt tumor, and showed the results of delayed hypersensitivity clinical testing of these materials and control materials in cross-tests of matched non-colon and colon cancer patients[13].

TABLE 1. *Effect of cell-specific DNA[a] with and without Freund's adjuvant on the growth of tumors in the hamster pouch*

Day after cheek pouch implant	Average tumor size mm²	No. pouches with tumors	Average tumor size mm²	No. pouches with tumors	Average tumor size mm²	No. pouches with tumors	Average tumor size mm²	No. pouches with tumors
	Group 1 DNA 1 mg × 1 (10 pouches) (Freund's adjuvant)		Group 1 controls (6 pouches) (Freund's adjuvant)		Group 2 DNA 1 mg × 3 (10 pouches) (Freund's adjuvant)		Group 2 controls (6 pouches) (Freund's adjuvant)	
7	19.5	9	4.2	6	21.3	10	0.5	6
10	14.6	9	3.7	4	20.9	10	4.1	6
14	9.8	8	1.2	1	13.1	8	1.3	3
17	5.6	7	2.2	1	6.0	6	0.2	2
22	1.8	6	1.7	1	6.0	4	0	0
24	0.6	3	0.5	1	2.6	2	0	0
	Group 3 DNA 1 mg × 1 (10 pouches)		Group 3 controls (10 pouches)		Group 4 DNA 1 mg × 5 (10 pouches)		Group 4 controls (10 pouches)	
5	24	10	19	10	20	10	18	10
8	11	10	13	10	13	10	9	10
12	4	4	4	5	6	6	2	5
15	2	2	1	4	3	5	0	4
19	1	2	0	0	2	2	0	0

[a]DNA (plus Freund's in groups 1 and 2) was injected i.p. once in groups 1 and 3 and as indicated, at weekly intervals in groups 2 and 4. Ten days after the initial DNA injection, a 1 × 10⁶ HeLa cell suspension (group 1 and 2) or 1.5 × 10⁶ cells (groups 3 through 7) from serum-free spinner cultures was implanted in each hamster cheek pouch. Experiments I and II were performed upon a different shipment of hamsters than experiments III, IV, V, VI, and VII (see Table 2 for last three groups).

TABLE 2. *Effect of cell-specific RNA, microsomes, and membranes on the growth of tumors in the hamster cheek pouch*

Day after cheek pouch implant	Average tumor size mm²	No. of pouches with tumors	Average tumor size mm²	No. of pouches with tumors
	Group 5 RNA 1 mg × 5 (10 pouches)		Controls (10 pouches)	
4	30	10	25	10
7	18	10	13	10
11	9	8	4	6
14	5	6	2	3
18	2	6	2	2
25	1	3	2	2
	Group 6 Microsomes[a] × 5 (10 pouches)		Controls (10 pouches)	
4	23	10	25	10
8	10	10	8	9
10	5	10	3	8
13	3	7	1	3
17	0	0	0	0
	Group 7 Membranes[b] × 5 (10 pouches)		Controls (10 pouches)	
4	31	10	26	10
9	2	2	10	10
14	1	1	2	4
19	1	1	0	0

[a]Microsomes from 8.3×10^8 cells per inoculum.
[b]Membranes from 9.6×10^8 cells per inoculum (approximately 100 γ membrane protein).

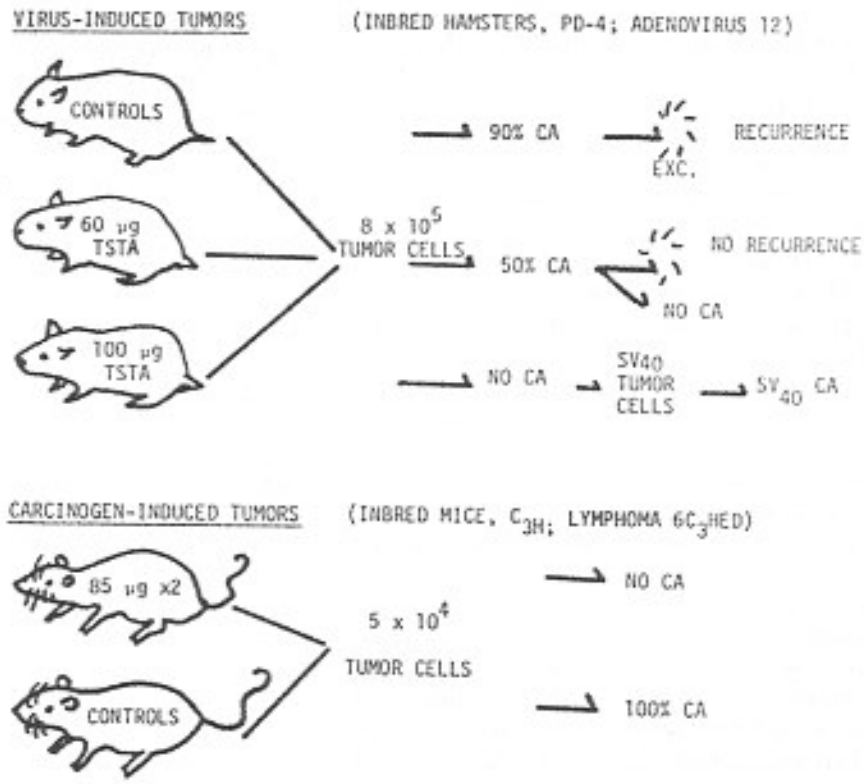

FIGURE 1 (This and preceding Tables 1 & 2 are reproduced, courtesy of Raven Press, from a section by A. Hollinshead et al. entitled 'Tumor-associated antigens: their usefulness as biological drugs', page 501-504 of *Lung Cancer: Progress in Therapeutic Research*, edited by F. Muggia and M. Rozencweig. Raven Press, New York 1979)

III. METHODS OF SEPARATION, IDENTIFICATION, PURIFICATION AND ANALYSIS OF ANTIGENS.

We reprint here, courtesy of J.B. Lippincott Co., a summary of methods which appears in Appendix I (here presented as Table 3) and Appendix 2 (here presented as Table 4) of a paper by Hollinshead et al. entitled 'Specific Active Lung Cancer Immunotherapy. Immune Correlates of Clinical Responses and an Update of Immunotherapy Trials Evaluations' in Cancer 62: 1662-1671, 1988.
In addition, Figures 2 through 7 are added, to illustrate some of the methods used.

TABLE 3

Pure TAA is biologically active and immunogenic. It is used for immunotherapy. To prepare pure TAA, separated tumor membranes (preferably solubilized by low frequency sonication) are placed into protein components while maintaining activity by certain of following methods, depending on TAA: (1) according to size by ultrafiltration chromatography; (2) according to charge by polyacrylamide gel electrophoresis; (3) according to charge by ion exchange chromatography; or (4) according to affinity for monospecific or monoclonal antibodies by affinity chromatography.

Ultrapure TAA is biologically active and immunogenic and is used in characterization, identification, hybridoma, and epitope preparation. Ultrapure TAA preparation includes the prior steps and appropriate additional methods to increase purity while maintaining its biologic activity and immunogenicity.

One method is isotachophoresis (a principle based on migration in an electric field of ion species of the same sign, all having a common counter-ion). The advantages to this are small sample requirements, short analysis time, ease of quantification, and accuracy. It is an electrophoretic separation.

Analytical separations (LKB 2127 Tachophor), in which separation takes place in a thermostat capillary tube with no stabilizing medium, are a type of isotachophoresis. Preparative separations, in which sample zones migrate with sharp boundaries between a leading and a terminating buffer stacked one behind the other in order of their electrophoretic mobility, are often used. The length of each zone is regulated by the amount of sample in the zone. Ampholine spaces (non-UV-absorbing) are chosen for mobilities between sample zones. All zones then migrate down column with the same velocity, ensuring that each zone comes off the bottom of the column at the same rate. This means that the peak heights of eluted zones are independent of the mobility of different components in the sample and last zones will have the same resolution and sharpness as first zones coming off column. Although more difficult than analytical separation, good results can be achieved using LKB 7900 Uniphor.

Another method is affinity chromatography in which purification is carried out by using affinity of monospecific or monoclonal antibodies for the antigen.

Identification and Characterization

TAA can be identified and characterized according to the following methods:

1. By polyacrylamide gel electrophoresis, including use of SDS-PAGE gel staining and comparison of densitometry profiles in gel separations and migration in relation to control proteins of known molecular weight determined by reactions with monospecific antisera in gel double diffusion.
2. By immuno-diffusion immuno-electrophoresis against a battery of hyperimmune and immune and control sera and prepared monospecific and monoclonal abs.
3. By specific lymphocyte stimulation assays.
4. By delayed hypersensitivity reaction skin testing in titration assays.
5. By enzyme immunoassays to characterize TAA using tumor-related and control sera.
6. By testing stability at various temperatures and times at said temperatures.
7. By isotope tagging of TAA abs to show localization in tumors.
8. By indirect immunofluorescence studies of cancer tissues, cells, or subsets or cell-sorted groups thereof to TAA antibodies.

Methods 1, 2, 4, 5, and 6 are used in combination, although all eight are often used where appropriate.

TABLE 4

Flow Diagram of Lung TAA Separation

Cancer or Control Tissues

Immediately washed and minced, pressed through 60-mesh stainless steel sieve, and further dispersed by aspiration with a wide-bore pipet.

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Dispersed Cells

Counted with white cell diluent, trypan blue, and neutral red. Sterility monitoring at this point and throughout further separation. Cells frozen and thawed and membranes extracted by modified Davies procedure with isotonic to hypotonic saline (by rapid stepwise techniques to minimize degradative enzymatic activity).

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Crude Membrane Extract

Protein determination by Lowry procedures. Sequential low frequency sonication at 9 to 10 kc (more rigorous sonication tends to produce detrimental thermal effects, solubilization of unwanted materials, increased risks of protein degradation, and loss of antigenic activity).

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Soluble Membrane Sonicate

↓

Protein Determination

↓

Gel filtration on Sephadex G-200 → Gel electrophoresis

Sephadex G-200 soluble antigen fractions: crude molecular weight determinations.

Run on gradient polyacrylamide gel, bands measured, and a separation of proteins by slicing, eluting, and concentrating by ultrafiltration for selected protein concentration. Molecular weight determinations.

Pure TAA: Separated Soluble Antigens or Gel Regions

1. Sterility and pyrogen tests; protein, carbohydrate, and lipid analyses; graphing, photography, and densitometry.
2. Storage at −70°C is crucial in maintaining activity of an antigen preparation. Proteolytic activity and hydrolysis of proteins is greatly increased at 4°C and temporary storage at this temperature is not recommended. Serum, not TAA, is stored at −70°C and −18°C.
3. *In vivo* assays of pure and ultrapure TAA.
4. *In vitro* assays of pure and ultrapure TAA.

↓

Ultrapure TAA

5. Separation by isotachophoresis and/or monoclonal antibody affinity chromatography.
6. Monoclonal antibodies for ELISA, for isotope tagging for use in testing localization in tumors, as well as affinity chromatography separation of candidate epitopes.
7. N-terminal amino acid sequencing.

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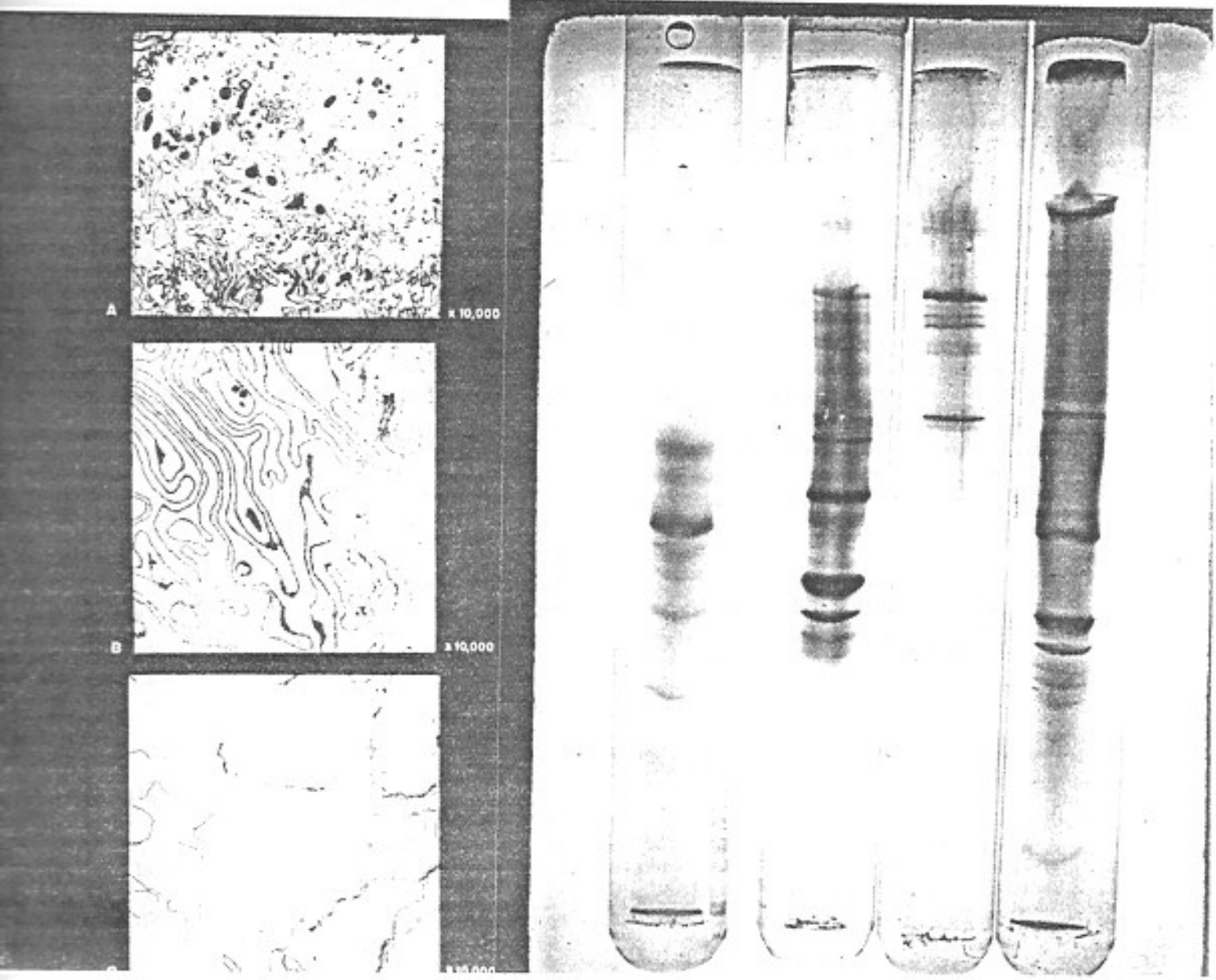
TAA Epitopes

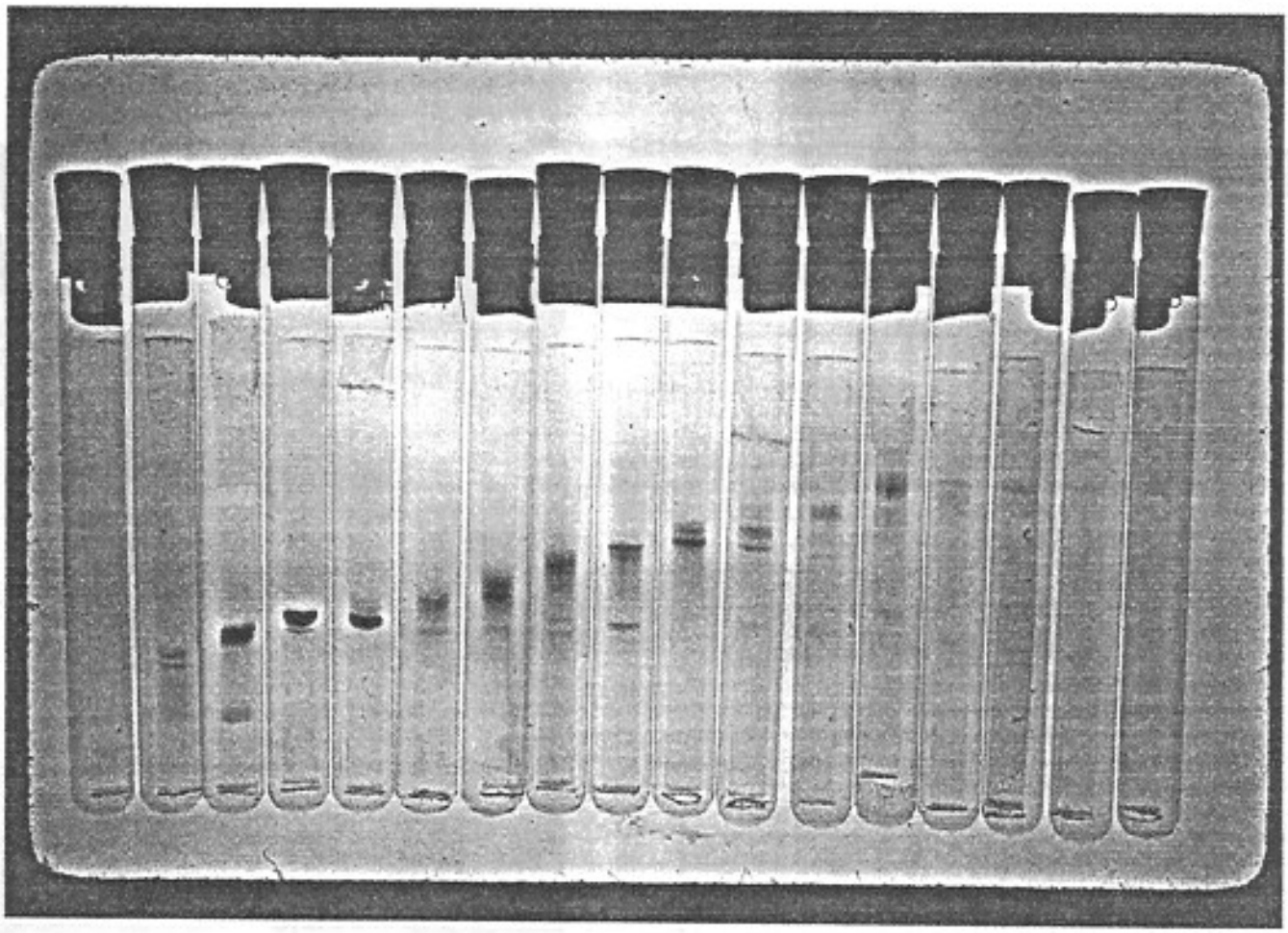
1. Measurement of specific and nonspecific antibody responses.
2. Identification and characterization of epitopes from primary and metastatic TAA (for presence of epitopes and for identification of changes, modulations, and alterations as well as similarities).
3. Testing of selected epitopes with a battery of sera from patients undergoing therapy.
4. Use of TAA monoclonal antibodies and monoclonal antibody-derived epitopes in preparation of a cascade of idiotype, anti-idiotype, anti-anti, and anti-anti-anti.
5. Studies of epitope and combinations with one or more epitopes for effectiveness in inducing CMI responses *in vitro* and *in vivo*.
6. Amino acid sequencing and studies of responsible oncogenes.

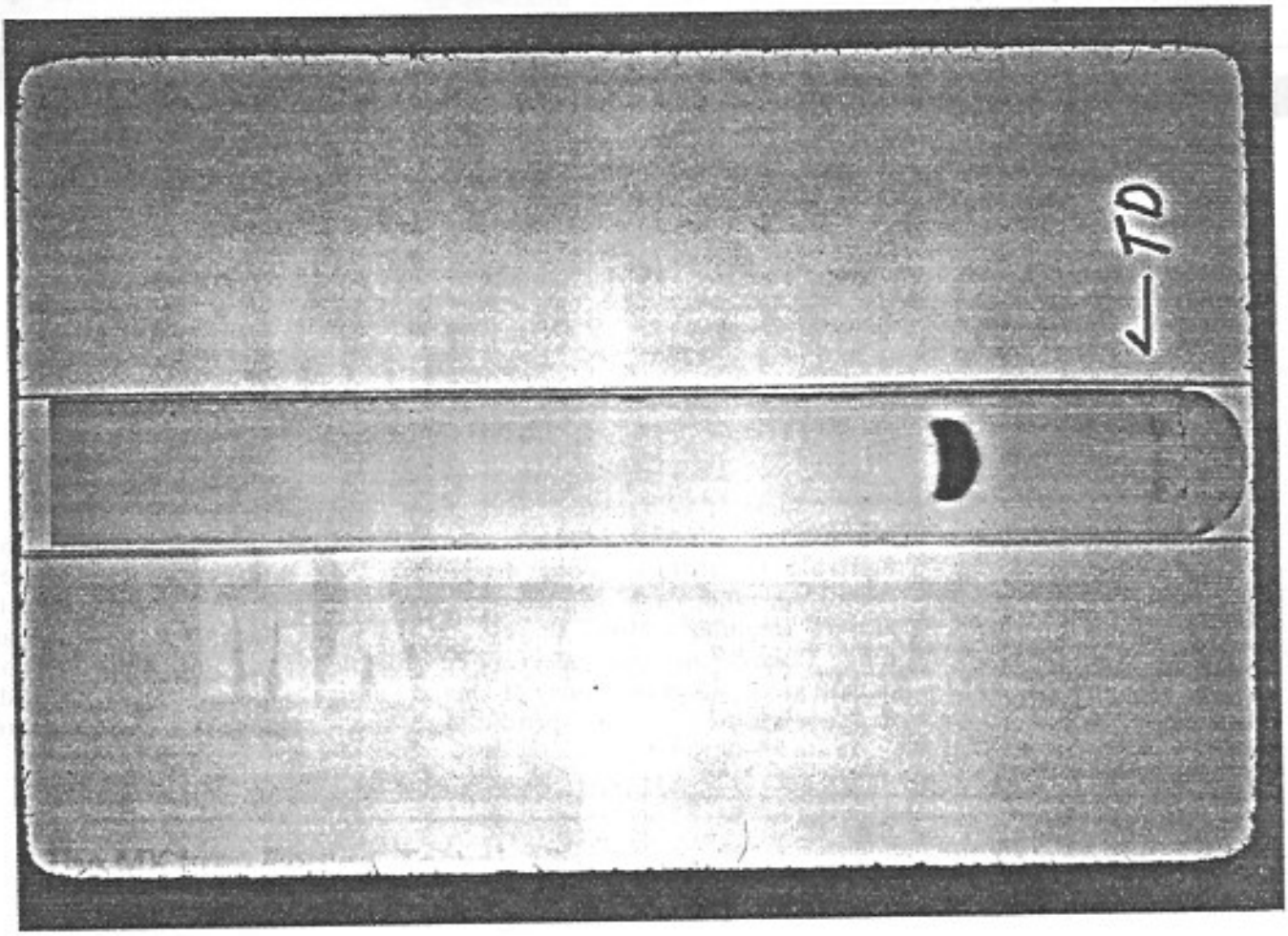
FIGURES 2 through 7

Figure 2 (on left) shows cell membrane preparations before washing, and after the first and third washing, to prepare them before sonication. Figure 3 (on right) shows a bladder cancer cell membrane soluble pool (right) before separation, and Fractions I (2nd from right), II (2nd from left) and III (left) after Sephadex chromatography. Both cells, membranes, soluble pool and Fractions are tested for ability to produce CMI. Figure 4 (top) shows further separation of an identified bladder cancer Fraction into Regions for further testing of each of the Regions. Figure 5 (bottom) illustrates the further purification of larger amounts of an identified melanoma band in an active Region for further study and testing. Figure 6 (left) illustrates the use of preparative electrophoresis.On lower right, a purified melanoma PAGE region R-5. On lower left, a Sephadex fraction IA for contrast. The type of further purification depends upon the chemistry and immunoreactivity of an identified band. In some instances isotachophoresis was preferable and in others affinity chromatography using TAA monoclonal antibody.

Figure 7 (right) illustrates a type of DHR-skin testing performed, e.g. of purified bands from melanoma regions, using the Sokal ballpoint pen technique of defining areas of induration.



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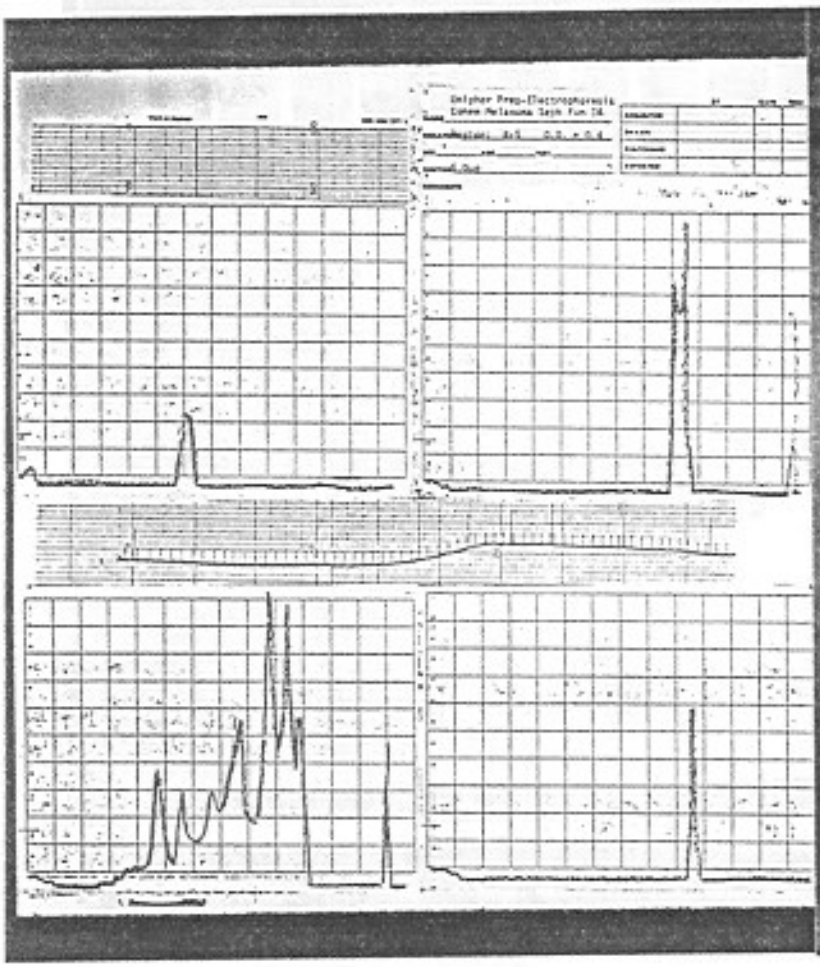
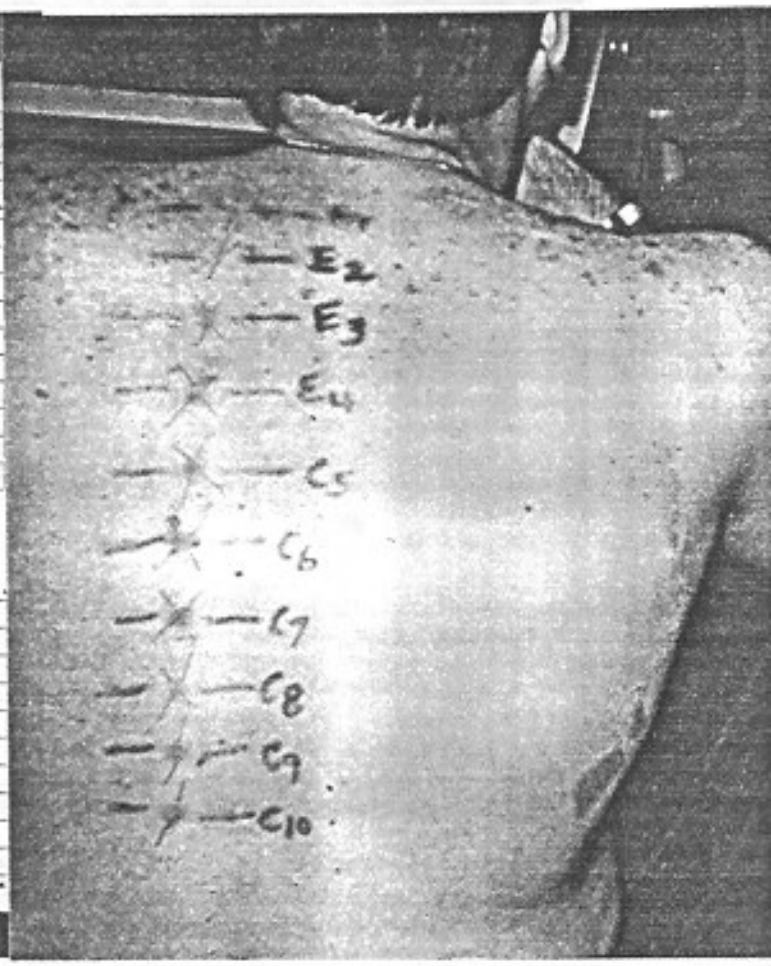




IV. LUNG CANCER TAA. THE DYNAMIC SPECTRUM OF LUNG CANCERS

Lung TAA studies have been reported elsewhere (14-90). Major lung TAA produce strong cell-mediated immunity per equivalent protein concentrations. All antigens are titered, compared, and each of the antigens identified for use in specific active immunotherapy shares in 75-97% (mean: 93%) primary lung tumors of precise related histology, as selected by our pathologists. Combinations of major antigens were tested in order to determine those producing the strongest cell-mediated

immunity per given protein concentration in synergistic testing. Table 5 contains the dynamic spectrum of major lung TAAs used in therapy.

TABLE 5. LUNG CANCER IMMUNOGENS

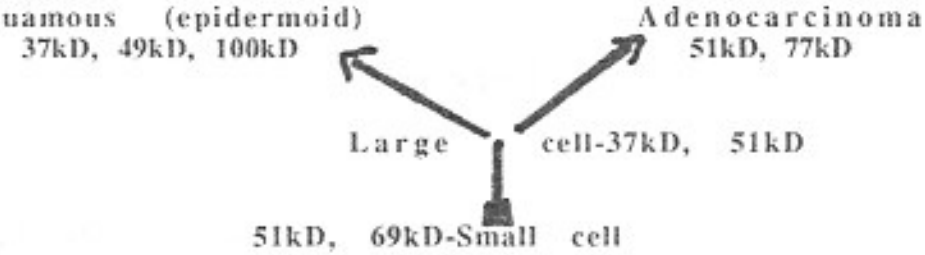
TAA Immunogens	Molec. Weight	Description
Oat cell	51kDa	unique* protein
	69kDa	unique lipoprotein
Large cell	37kDa	unique protein
	51kDa	unique protein
Squamous cell(epidermoid)	37kDa	unique protein
	49kDa	fetal protein
	100kDa	fetal lipoprotein
Adenocarcinoma	51kDa	unique protein
	77kDa	unique protein
Bronchioalveolar	77kDa	unique protein
	100kDa	fetal lipoprotein

*(not found in this form in normal lung)

These TAA also act as Biomarkers which participate in the interrelationship and dynamic spectrum of lung cancer[80,89]. Dr. Raymond Yesner has defined the dynamic spectrum indicating a common origin in endoderm (Y-construct) and has supported this theory by histopathologic, electron microscopic, hormonal, cultural and epidemiologic evidence. This theory is now further supported by biomarkers, as illustrated in Figure 8. As shown, large cell carcinomas (LCC) at the Y-center exhibit markers 37kD and 51kD. Squamous cell carcinomas (SCC) at one of the Y-arms have markers 49kD and 100kD and share 37kD with LCC. Adenocarcinomas at the other Y-arm have 77kD and share 51kD with LCC. Small cell carcinomas at the Y-foot have 69kD and share 51kD with LCC. Bronchoalveolar carcinomas (not shown) share 77kD with adenocarcinomas, marking them as an adenocarcinoma subclass, and 100kD with SCC, which relates to

the keratin expression of pneumonocytes. The vast clinical evidence of shared and mixed lung cancer types, of changes from primary type to different types seen at autopsy, alterations in type as a result of chemotherapies, and a whole host of data serve to confirm the dynamic spectrum (Y-construct) amongst the lung cancer types.

FIGURE 8. MAJOR SYNERGISTIC TAAs:Biomarkers show lung cancers inter-relationship and dynamic spectrum



Another set of evidence for this dynamic spectrum comes from numerous reports in the literature on genetic studies of lung cancer. In general cytoplasmic oncogenes such as Ha-ras, Ki-ras, erbB-1 and 2, RB are associated with the induction of malignant processes, whilst nuclear oncogenes such as c-myc, N-myc and c-myb may activate during tumor progression and possibly might serve as second genes required for independent neoplastic change or growth. Approximately 18 protooncogenes (c-erbB-1, c-erbB-2, N-myc, c-myc, L-myc, c-myb, c-ras-Ha, N-ras, c-ras-Ki, c-fes, c-fos, c-fos-2, c-fms, c-mos, c-abl, P53, PDGFA chain, c-sis) have been detected as altered or with point mutations, abnormal, deleted, amplified or over-expressed in human lung tumor cells. The changes may be directly or indirectly related or merely associated with cancer development and are shared amongst lung cancer types. So far, these studies seem to support the conclusion that there exists a dynamic lung cancer spectrum.

Since TAA are purified gene products, the usage of TAAs in important forms of therapy is not only justified, but further studies using tailored immunogens containing selected or more polyvalent TAA combinations may be made to cover the dynamic spectrum of possible cancer forms during therapy.

V. CONSIDERATIONS OF THE USE OF TAA IN SPECIFIC ACTIVE IMMUNOTHERAPY AS COMPARED WITH OTHER IMMUNOLOGICAL APPROACHES FOR TREATMENT OF LUNG CANCER.

We have developed a form of specific active immunotherapy using well defined tumor cell membrane-derived

purified protein immunogens with clear evidence of inducing a specific immune response directed against the tumor cells. The specificity has been proven both in vivo and in vitro as a direct induction of a long-lasting cell-mediated immune response, accompanied by an early-acting humoral immune response induced mainly by IgM subclass antibodies. We have produced monoclonal antibodies by fusion of human spleen cells from immunized patients with human cell lines and shown their ability to induce specific lymphoblast stimulation with resultant lysis of target tumor cells. We have produced and tested monoclonal antibody-derived epitopes and developed highly relevant, specific monitoring tests and have learned much about changes induced by specific active immunotherapy in humoral immunity as well as in cell-mediated immunity in our patients, and have considered the interaction between these forces. These effects have been measured in many patients in-cluding those with colon cancer, lung cancer and other malignancies[1].

A critical evaluation of the status of current research, how other types of immunotherapy compare with specific active immunotherapy, and their appropriateness for the treatment of lung cancer.may be in order. So far, these approaches[69,91] have been used but have not been very effective against the solid lung tumors Little or no effect was noted using immune stimulation with BCG, C.parvum, lentinen, OK432 and other nonspecific agents. A group of synthetic substances have been shown to have partial, non-specific stimulatory effects on the immune system and includes such substances as levamisole, isoprinosine, and azimexon. In the hematologic diseases (lymphomas, leukemias), cyclophosphamide appears to increase delayed hypersensitivity responses, but when used for lung cancer, in the long run the drug is immunodepressive. Natural and recombinant nonspecific biologic response modifiers such as interleukin-2 and -4, IL-2 stimulated lymphokine-activated killer (LAK) cells, as well as nonspecific cytostatic or cytotoxic cytokines like interferon and tumor necrosis factor have been tried. Interferon has some effects on hematologic cancers like hairy cell leukemia, cutaneous T-cell lymphoma, CML and Hodgkin's lymphoma. IL-2 is highly toxic and the recombinant IL-2 is even more toxic; they cause an increase in lymphocytes but also produce eosinophilia. Workers at NCI and others have shown[69, 91, 92]that IL-2 induces a subset of normal lymphoid cells to lyse tumor cells (LAK); however, only one-tenth of LAK-treated patients, mainly with hypernephromas and melanomas, experience complete remission and only one-half of this one-tenth of patients have long remissions. Usage of tumor-infiltrating lymphocytes for these cancers proved 100X more effective than LAK cells. However, the methods of in vitro production and the constant needs for re-injection of patients with this passive, adoptive form of immunotherapy were fraught with complications and this led the groups of workers at NCI and other centers to seek new forms of therapy. At present the work is directed at finding cancer genes which produce TAA. As a study model, the group has managed to insert a gene for tumor necrosis factor into tumor-infiltrating

lymphocytes (TIL) and has demonstrated TNF tumor localization in patients. This has encouraged work on a mouse melanoma gene discovered in Israel[92]. It is probable that the number of gene insertions into the weak TIL cells is limited, and that programing only one form of T cells may not effect the total strategy of body defenses, including macrophages and humoral components. Thus, it is interesting to identify genes for various TAAs, but to out-program nature may be self-defeating. It may be better to effectively stimulate or to program the immune system using gene products like TAA. Even if successful initially, the inserted gene may cease to produce a protein product in a higher concentration. Products from histones may cause gene shut-off, or prevent proper chromosome unfolding. We have already shown that TAA take various physical forms and chemical compositions but contain various common epitopes. It is better to identify these epitopes and to use them for monitoring the natural, varied but selective in vivo production of TAA-induced defenses. Other workers are studying monoclonal antibodies (Mab) as therapeutic agents. Mabs and fragments of Mabs alone, because of size and other problems, have failed in clinical trials. However, many groups and drug companies are attempting to link whole Mab or Fc fragments to toxins or drugs to be used in passive immunotherapy; this approach will require constant administration, and side effects may be numerous, along with fear of reversals of desired effects as time goes on. So far, there is no evidence that these approaches will be useful in the treatment of lung cancer. In addition, except for a few small cell lung cancer patients, there is little evidence that current chemotherapies, unless used in combination with new therapies, will produce long term survival in lung cancer patients.

In view of the dynamic spectrum of lung cancer types and the complexity of this disease, the approach of using specific active immunotherapy with well-defined gene products as antigens(TAA) to induce long-lasting cell-mediated immunity makes sense. There is still a need for many additional studies and improvements in this approach, but further work is certainly justified on the basis of reported and current observations.

VI. LUNG TAA IMMUNOGEN BATCHES USED IN CLINICAL TRIALS

All Quality Control procedures will not be included here but are documented and reported regularly, including those required by FDA regulations. These procedures were carried out routinely, including methods of procuring and processing specimens, air, reagent and temperature monitoring throughout the process, proper equipment and supplies maintenance and care, and all animal and laboratory evaluations.

In preparing lung TAA immunogen ('vaccine') lots for use in clinical trials in Canada and the USA, we always reviewed donor histories, excluded those who pretested positive for hepatitis (now, in addition, for HIV), and skin tested two 10-fold dilutions of each lot to assure predictable strength in inducing delayed hypersensitivity responses within a given range of

protein concentration. We improved as we progressed, and developed criteria (shown below) for standardized, controlled assurance of lot to lot consistency. We added the use of specific lymphocyte stimulation tests to back up the CMI measurement. And, we developed a sensitive measure of humoral immune responses for retrospective analyses and future prospective analyses[58,61,66,70,71,72,73] (discussed in Section VII of this paper). Only lots 1, 4 and 6 do not have the required complete balance of component, synergistic TAAs for the particular lung cancer type. Lot 11 had one questionable test for hepatitis and was used only for research purposes. In Table 6, parts A to E., we delineate the features of each of the TAA lots. For example, a review of Table A. showing squamous cell TAA lots records that lot number 15 had the required three major, synergistic soluble tumor antigens, produced predictable CMI and that the lot has the proper protein concentration range needed for therapy. Since we had described and reported every possible soluble cell membrane antigen which induces CMI, we also listed other, weaker but active antigens which might be in the preparation (for example, lot 15 contains fetal antigen 3). In this particular lot 15, the skin tests prepared for monitoring patients receiving immunotherapy would be prepared at 107 micrograms per 0.1 ml and the immunogens at 537 mcg per 0.2 ml. We review 22 lots of lung TAA immunogen (Table 6).

TABLE 6

LUNG TAA VACCINE LOTS FOR USA/CANADA TRIALS

(Pretested, selected antigens present (x) in vaccine per 100 mcg TAA protein separation on discontinuous stacked PAGE with densitometry quantification for verification of lot to lot consistency)

A. Epidermoid(squamous cell) TAA Immunogen:

Lot no.:	1	4	7	12	15	19	23
49kD	x	x	x	x	x	x	x
100kD		x	x	x	x	x	x
37k	x		x	x	x	x	x
Others:							
fetal ag 3	x				x	...x	
fetal ag 4					x		
Donor neg.hepat.test (at clinical center):	6-	7-	4-	7-	4-	7-	8-
DHR-ST:2 dilutions,10-fold, test-nonanergic pt. >5mm induration(+):							
mcg prot.	60/6	47/5	62/6	48/5	107/11	100/10	103/10
	++	++	++	++	++	++	++
Conc.Immunogen:							
mcg/ml:	475/.25;	472/.4;	620/.5;	487/.4;	537/.2;	498/.22;	500/.2

B. Adenocarcinoma TAA Immunogen:

Lot no.:	3	6	10	11	17	18	22
51kD	x	x	x	x	x	x	x
77kD	x		x	x	x	x	x
Others:							
Fetal ag 4		x					x
Donor neg.hepat.test (at clinical center):	3-	3-	1-	5-(1+)*	3-	7-	6-
DHR-ST:2dilutions,10-fold,test-nonanergicpt.>5mm.induration(+):							
mcg prot.	50/5	51/5	52/5	100/10	51/5	99/9	100/10
	++	++	++	++	++	++	++

Conc. Immunogen: mcg/ml.
496/.37;513/.4;522/.25;632/.25;508/.22;516/.23;500/.2
*(repeat test neg.; nevertheless this lot used for research only)

C. Large cell TAA Immunogen:

Lot no.:	2	8	16	21
37kD	x	x	x	x
51k	x	x	x	x
Others:				
Sq.fetal ag 1		x	x	
Sq.fetal ag 2	x		x	
Adeno/oat ag	x		x	
Oat TAA 3		x		
Donor neg.hepat.test (at clinical center):	2-	2-	4-	6-
DHR-ST:2dilutions,10-fold,test-nonanergic pt. >5mm induration(+):				
mcg prot.	50/5	54/5	99/9	100/10
	++	++	++	++
Conc. Immunogen:				
mcg/ml	504/.35	540/.5	496/.25	504/.2

D. Broncho-alveolar Immunogen:

Lot no.:	5	13	20
100kD	x	x	x
77kD	x	x	x
Squam.HSVTAA	x	x	
Squam.Fetal ag 1	x	x	
Adeno. TAA 1		x	
Oat TAA 3		x	
Donor neg.hepat.test (at clinical center):	4-	3-	3-
DHR-ST:2dilutions,10-fold,test-nonanergic pt. >5mm induration(+):			
mcg. prot.	54/5	112/11	100/10
	++	++	++
Conc. Immunogen:			
mcg/ml	536/.40	560/.5	500/.2

E. Oat cell Immunogen:

Lot no:	14
51kD	x
69kD	x
Donor neg.hepat.test (at clinical center):	4-
DHR-ST:2 dilutions,10-fold, test-nonanergic pt.	>5mm induration(+):
mcg prot.	107/11
	++
Conc. Immunogen:mcg/ml:	547/.17

The largest undertaking in our laboratory was the preparation of a sufficient quantity of lung TAA immunogen for Phase III trials conducted by Dr. Hiroshi Takita, Roswell Park Memorial Institute, Buffalo, New York for the USA, and by Dr. Thomas H.M. Stewart, Ottawa University Medical School for his next Canadian trial. This enormous undertaking would not have been possible without the loyal support of my staff, including Oh Bong Lee, Keith Tanner, Cindy Bryck, Marianne Dannbeck, Bob Hamilton and others. A summary of the four major lung TAA immunogen/vaccine lots prepared for uniform use in the two separate phase III trials is given in Table 7.

TABLE 7
LUNG TAA VACCINE LOTS FOR PHASE III USA/CANADA TRIALS

Type:	Adenocarcinoma	Squamous cell	Bronchoalveolar	Largecell
#skintests:	150	254	30	160
mcg/ml conc.:	99.73/.1	100.5/.1	100/.1	100/.1
#immunogens:	150	282	30	140
mcg/ml conc.:	516.1/0.23	497.6/0.22	500/0.2	504/0.2

TOTAL 602 vaccines and 594 skin tests = 1,196

The results of these two trials are published elsewhere[67,73,76] and are updated recently by Dr. Takita[90] and by Dr. Raman in another chapter of this book.

Throughout these trials, we thought seriously about the possibilities of providing more comprehensive protection, of possible usage in future of booster shots to assure long-lasting protection, and of many other considerations in improving the therapy. We also thought of how to expand this therapy to treatment of patients with somewhat later stages of lung cancer who were candidates for cytoreductive surgery, and how to use our observations during parts of our Phase I and II patient studies on the role of chemotherapy in combination with immunotherapy. We also had considered the importance of immunoprophylaxis, and had actually prepared a protocol, found a proper site in California's shipyards and obtained permission

from the local authorities and hospital for the conduct of a trial in workers doubly exposed to asbestos and tobacco and at high risk. In preparing for this prospective study and before submitting a grant proposal for the immunoprophylaxis trial, we tested various combinations of the five major lung cancer type immunogens and came up with a balance of synergistic antigens which we felt might be efficacious in prevention therapy. However, the FDA decided that the immunoprophylactic trial must await further results of clinical trials in cancer patients. In view of the dynamic spectrum of lung cancer, as discussed above in Section IV, we undertook the task of preparing a more comprehensive lung TAA immunogen, a polyvalent vaccine to cover mixed or changed cancer types, or to prevent metastases which might take different pathways. Shown below, as Table 8 is our current evaluation of what might constitute an ideal composite vaccine, as well as an actual version, a variation of this designed for a study of patients with non-small cell lung cancers.

TABLE 8
LUNG TAA POLYVALENT VACCINE

Per 100 mcg protein need proportionate appearance of :
37kD-squamous cell &large cell ca.; 49kD-squamous cell.ca.; 100kD-squamous cell.&bronchoalveolar cell ca..; 51kD-adenocarcinoma, large cell & oat cell ca.; 77kD-adenocarcinoma &bronchoalveolar ca..; 69kD-oat cell ca.

If doing all types of lung ca in protocol:
squamous cell.ca. 33.33%; large cell ca. 13.8%; broncheoalveolar ca. 16.66%; adenocarcinoma 13.8%; oat cell 22.16%

For our current studies: we made a lot with a variation since we had contemplated prophylactic trials or early stage entry. However, we did use a lesser amount of oat cell immunogen for possible variance in 51kD configuration. Bronchoalveolar is a subclass of adeno-carcinoma but we had no plans to include bronchoalveolar per se;therefore, we used a little for variance. So, the polyvalent vaccine we prepared for these purposes was as follows: 48% squamous cell., 13.6% large cell, 3.8% bronchoalveolar, 23.7% adenocarcinoma, 10.9% oat cell. Considerations were: major pathologies; cell differentiation; possible types of metastases.; synergies; clinical preferences.
So far, this immunogen lot has been used by Drs. Maroun and Stewart in a IL-2-TAA combination immunochemotherapy protocol. Specific abrogation of delayed hypersensitivity reactions to tumor antigen, with growth of measureable tumor resulted in discontinuation of the protocol.

VII MECHANISMS. STUDIES OF SOLUBLE TUMOR ANTIGENS : SPECIFICITY OF PATIENT RESPONSES, THE WAY THEY RELATE TO IMMUNOLOGICAL EFFECTS BY CELL AND SERUM COMPONENTS, BY DRUGS, BY INHIBITORY AGENTS, BY IMMUNE COMPLEXES, AND THE CHANGES INDUCED AFTER SPECIFIC ACTIVE IMMUNOTHERAPY. HOW MUCH DO WE KNOW AND HOW WOULD THESE ACTIVITIES RELATE TO INDUCTION OR CESSATION OF CANCER DORMANCY AS ONE OF THE MECHANISMS ?

The following list of TAAs have all been identified with the same care, precision, controls, standards, cross-tests and procedures described for lung cancer TAA identification. All are essentially free of nucleic acids, major tissue antigens, pyrogens, bacteria and viruses. All produce strong cell-mediated immune responses in nonanergic patients with that type of cancer, and are capable of inducing a response in most anergic patients after a series of two or three immunizations:

List of cancers for which all soluble cell membrane antigens producing CMI are identified:

Colon Cancer
Lung Cancer
Ovarian Cancer
Melanoma
Bladder Cancer
Breast Cancer
Prostate Cancer
Glioblastoma
Astrocytoma
Gastric Cancer
Ewing's Sarcoma
Laryngeal Cancer
Cervical Cancer
Meningioma
Hypernephroma
Leukemia (ALL, CML)

We must summarize here, and refer to detailed reports, some mechanisms discovered whilst conducting research in all of these systems. During the studies of normal embryonic, different stages of fetal development, adult, benign and cancerous cells of the same organ, we discovered some characteristics of the cancerous process which were held in common and others which were diversified.
Among the common characteristics were A. the existence of interfering or inhibitory substances on the cell membranes,

which could interfere with TAA expression if not separated from TAA[50]; B serum factors and cancer serum indices which could be measured quantitativly for helpful evaluations/predictions of anergy and of tumor present[93]. Diversification was found in A. the methods needed for separation of certain TAA[94], B. in patient responses to TAA at different stages in cancer progression[37], C. in TAA present on primary tumors and in some cases altered, missing or new TAA on metastatic tumors[95] D. the existence, mainly in advanced stages of cancer, of immune complexes: some nonspecific and some which specifically deterred the efficacy of immunotherapy[85]; E. in numbers and characteristics of TAA for a given cancer type (some cancer types produce only one cell membrane TAA, whilst others are less simple)[1]; F. immunologic responses to drugs and changes in B and T cells during combination therapies[84] and G. in TAA-specific humoral and cellular immunological changes during the course of therapy; we can measure humoral immunity using TAA monoclonal antibody-derived epitopes to serial sera from patients before, during and after immunotherapy to show the initial rise in antibody response which reaches its peak one-half year after therapy and then subsides within the year; meanwhile, in the tissues cell-mediated immunity, which also reaches its peak one-half year after therapy, takes over, plateaus at the one-half year mark and lasts for years in responding patients[73].

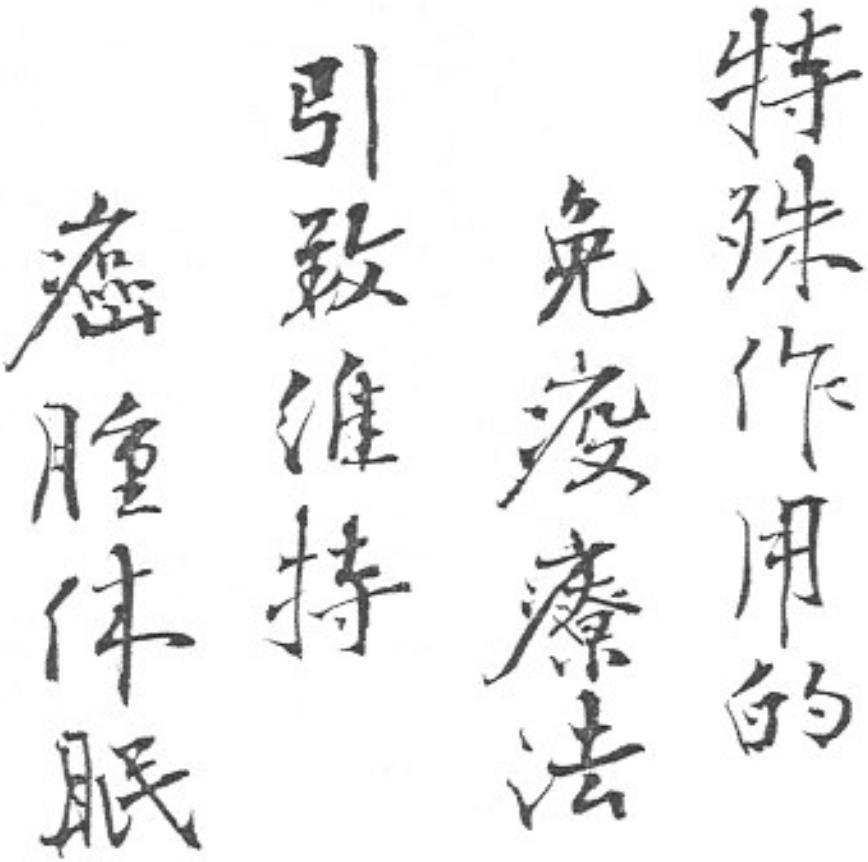
The studies of the humorocellular immune changes in patients with solid tumors are continuing, and in a few years we should be able to integrate and to make sense of much of what we are now measuring. We have discussed elsewhere[69] the changes in subsets of white blood cells and tissue cells and presented a list of studies which may lead to further understanding of immune phenomena. Our studies using combination chemoimmunotherapy in patients with lung cancer have indicated the usefulness of methotrexate[37,73] which causes an early white blood cell rebound overshoot, at which point TAA immunization is introduced to target these cells. The drug promotes adenosine release from endothelial cells and fibroblasts, thereby serving as a feedback inhibitor of neutrophil-mediated inflammation[96]. Our studies of other cancer types indicate that in some patients there exists a reasonable level of TAA immune response prior to immunotherapy, e.g. about 5% of colon cancer patients may have such a level and, therefore, the possibility of spontaneous remissions in such patients and of colon cancer dormancy in normal populations[95]; such levels have not been observed in lung cancer patients[73]. Other work also suggests that we have selected a therapy which draws the fine line between natural complexity and chemical simplicity. It has been reported that problems may occur with synthesized peptide and recombinant subunit vaccines , where, among other problems, there is a risk of vaccination with an incomplete repertoire of T-cell epitopes[97] It has been reported that a major obstacle in usage of monoclonal antibodies and/or to

immunotoxins attached to them are developments of antibodies to one or both[98] There is still a great deal to learn.

However, most principles are ancient, and it is important to relate new findings to the reports, the hard-won factual data of past researchers. To drive home this point, we present in Figures 9 and 10 the basic premises for this workshop; they are written in ancient Chinese calligraphy, with English at the bottom. The principles of dormancy have been with us for thousands of years. The methods of dormancy induction have been partly proven by animal experimentation. The existence of dormancy in patients with certain types of cancer has been well-documented. Why then, has lung cancer not followed this natural pattern? What exists in lung cancer initiation and development which has prevented any chance of a natural, dormant state taking place in certain patients with lung cancer?

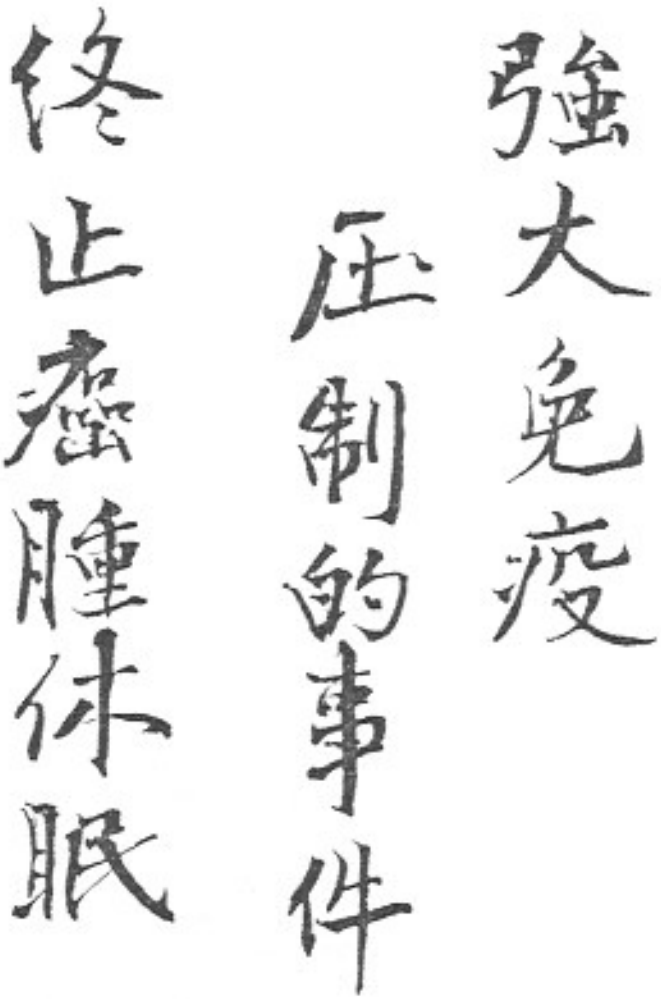
We believe that the above-summarized research of other mechanisms and the research described briefly in this chapter provide useful clues and partial answers to the question of why most patients with lung cancer escape dormancy, why specific active immunotherapy induces dormancy, and, ultimately, why certain patients fail to maintain dormancy induced by three immunizations 10 to 14 years earlier. Namely, 1. the discovery of an unusually large number of CMI-inducing antigens present in primary lung tumors, 2. the diversification of lung cancer types and the natural distribution of similar and unique TAAs amongst these types, 3. the ease with which we can measure changes in CMI responses and the way these responses are influenced by intrinsic and extrinsic factors, 4. the dynamic spectrum and the intermixing or changing characteristics of lung cancer types in nature, 5. the existence of specific immune complexes, which appear in late stage patients as well as in early stage patients who fail immunotherapy, and actually envelop TAA, preventing the action of TAA as immunogen--such complexes may well appear after immunologic insult in long-term survivors years after immunotherapy; 6. the lack of strong enough TAA immune responses in untreated lung cancer patients, a disease with little or no dormancy observed, as compared with other cancer types in which dormancy has been reported and in which pre-existing adequate TAA immune responses may be measured in a small percentage of patients.

FIGURE 9



TUMOR DORMANCY MAY BE INDUCED AND MAINTAINED BY SPECIFIC ACTIVE IMMUNOTHERAPY

FIGURE 10



TUMOR DORMANCY MAY BE TERMINATED BY STRONG IMMUNODEPRESSIVE EVENTS

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A Workshop

Cellular Immune Mechanisms and Tumor Dormancy

Ottawa General Hospital / October 1 – 2, 1991

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PROGRAMME

September 30, 1991

	Arrival at the Chateau Laurier Hotel
18:30	Ice breaker: an informal meeting with Workshop participants

October 1, 1991

08:00	Bus leaves the hotel for the General Hospital
08:25	Welcome - Jacques Labelle, President
08:30 - 08:55	E. Frederick Wheelock – An Overview of mechanisms responsible for tumor dormancy
09:05 - 09:40	Raymond Yesner – A pathologist's view of tumor dormancy
09:40 - 10:05	Suzanne A. Eccles – Animal models of tumor dormancy
10:05 - 10:30	DISCUSSION
10:30	COFFEE BREAK
10:50 - 11:25	E.Frederick Wheelock – Immune regulation of a murine T-cell lymphoma dormant state
11:25	Freda K. Stevenson – Idiotypic vaccination against B-cell lymphoma leads to dormant tumor
12:00	Jonathan W. Uhr – Tumor dormancy in a murine lymphoma
12:25 - 13:00	DISCUSSION
13:00 - 14:00	LUNCH
14:00	Shimon Slavin – Amplification of natural host defence mechanisms against cancer by recombinant IL-2 with induction of tumor dormancy
14:35	Robert L. Truitt – Graft versus leukemia effect of allogeneic bone marrow transplantation. Late relapse and tumor dormancy
15:00	DISCUSSION
15:30	COFFEE BREAK
15:50	Girish C. Moudgil – Effects of anaesthesia on the immune response and tumor metastasis

16:25	Joel Lundy – The effects of anaesthesia and surgery on the immune response and tumor metastasis
16:50	Eva Lotzová – Immunosuppressive effects of surgery in animals and man
17:15 - 17:45	DISCUSSION
18:00	Arrive back at the Chateau Laurier Hotel
19:30	Dinner at an Indian restaurant in the Byward Market – 2nd floor HAVELI

October 2, 1991

08:00	Bus leaves the hotel for the General Hospital
08:30	Darham P. Singal – Animal studies of the effect of blood transfusion on tumor growth
09:05	Paul Tartter – The effect of perioperative whole blood transfusion on the outcome of cancer surgery
09:40 - 10:05	Thomas H.M. Stewart – Negative effects of adjuvant therapy for Stage I and II non small cell lung cancer
10:05	DISCUSSION
10:30	COFFEE BREAK
10:50	Israel Penn – The effect of renal transplantation in patients with a remote history of curative cancer therapy
11:25	Jules E. Harris – Local tumor immunity and abnormal immunoregulations
12:00 - 12:30	DISCUSSION
12:30 - 13:30	LUNCH
13:30	Ariel C. Hollinshead – Soluble tumor antigens used in clinical trials of immunotherapy
14:05	Thomas H.M. Stewart – Evidence for the presence of dormant metastases inpatients treated by adjuvant specific active immunotherapy
14:40	Sankaranarayanan Raman – Statistical analyses of clinical trials of specific active immunotheray for non small cell lung cancer
15:15	COFFEE AND DISCUSSION
17:00	Bus back to the hotel
19:30	Bus to Le Cercle Universitaire d'Ottawa
22:30	Bus back to the hotel

PARTICIPANTS

Suzanne A. Eccles, Ph.D.
Sutton Surrey U.K.

Jules E. Harris, M.D.
Chicago, U.S.A.

Ariel C.Hollinshead, Ph.D.
Washington D.C., U.S.A.

Eva Lotzová, Ph.D.
Houston, Texas, U.S.A.

Joel Lundy, M.D.
New York, U.S.A.

Girish C. Moudgil,
M.B., B.S., M.Sc., FRCP(C)
Hamilton, Ontario

Israel Penn, M.D.
Cincinnati, Ohio, U.S.A.

Jonathan W. Uhr, M.D.
Dallas, Texas, U.S.A.

E. Frederick Wheelock, M.D., Ph.D.
Philadelphia, PA U.S.A.

Raymond Yesner, M.D.
New Haven, Connecticut, U.S.A.

Sankaranarayanan Raman, Ph.D.
University of Ottawa, Ontario

Dharam P. Singal, Ph.D.
Hamilton, Ontario

Shimon Slavin, M.D.
Jerusalem, Israel

Freda K. Stevenson, D.Phil., MRCPath.
Southampton U.K.

Thomas H.M. Stewart,
M.B., Ch.B., FRCP(C)
Ottawa General Hospital, Ontario

Paul I. Tartter, M.D.
New York, U.S.A.

Robert L. Truitt, Ph.D.
Wisconsin, U.S.A.