Specific Active Lung Cancer Immunotherapy

Immune Correlates of Clinical Responses and an Update of Immunotherapy Trials Evaluations

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The mechanisms of action of the specific active immunotherapy of solid tumors have not been defined. In an attempt to characterize some of these mechanisms, we report controlled studies of humoral immune responses and cell-mediated immune (CMI) responses in lung cancer patients with Stage I and Stage II adenocarcinoma and squamous cell cancer receiving pure tumor-associated antigen (TAA) specific active immunotherapy or combination immunochemotherapy. At 5 to 6 months postimmunotherapy, the humoral immune response measurements are predictive of response to therapy/survival in early lung cancer patients, permitting decisions as to whether to continue therapy. Patients with adenocarcinoma respond to combination chemoimmunotherapy by showing stronger or earlier responses to tests of immunity. Cell-mediated immunity to TAA at 17 to 24 months was far greater in patients receiving immunotherapy or immunochemotherapy compared with control patients, and also correlated with early humoral immune response and with 5-year survival. Here we report a further subset analysis of Stage I and Stage II lung cancer patients in a successful Phase III US specific active immunotherapy trial as substantiating the experience with Stage I patients in a successful Phase II Canadian trial. We analyze failures and suggest additional therapies, especially a chemoimmunotherapy trial indicated by our analyses of humorocellular immune variables reported here.

Cancer **62:1662–1671, 1988.**

IN OUR PILOT STUDIES before the large clinical trials,[1] the use of specific active immunotherapy alone and with chemotherapy was assessed by both laboratory and clinical evaluations of immune variables.[2,3] One drug, methotrexate, which was studied as a single agent, induced a rebound overshoot phenomenon of *in vitro* lymphocyte performance measured for patients on the drug, and this peaked at approximately 7 days after commencement of drug administration.[4] In a Phase II trial, a cohort of lung cancer patients received either methotrexate alone or methotrexate followed by tumor-associated antigen (TAA) immunotherapy introduced at the peak of drug-induced rebound overshoot response. The number studied was too small for reliable data on survival differences. However, those on immunochemotherapy showed a striking increase in the level of cell-mediated immune (CMI) responses to TAA skin tests for delayed hypersensitivity.[5]

In an attempt to further establish whether the increase in the survival of patients on immunotherapy alone was indeed due to increases in antigen-specific immune functions as a result of immunotherapy, and also whether there were any differences for patients receiving immunochemotherapy, we studied 64 representative patients (selected from all patients studied thus far) for both humoral response to TAA and cell-mediated response to TAA.

As indicated by the data below, high serum antibody levels at 5 to 6 months after initiation of immunotherapy correlated with increased 5-year survival in both histologic types of lung cancer chosen for study, and in both Stage I and Stage II patients. The degree of delayed hypersensitivity at 17 to 24 months, a measure of CMI, also was correlated with increased 5-year survival and with humoral response levels for the same patients.

Three clinical trials have been previously reported individually, in detail, for lung cancer patients randomized to adjuvant specific active immunotherapy using purified TAA associated with primary lung tumors. Protein concentrations of 500 μg well-homogenized with adjuvant

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The authors thank O. Lee, C. DeAngelo, R. Hamilton, and V. Deas-Moore for expert technical assistance; and N. Petrovic for manuscript preparation.

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Accepted for publication August 1, 1988.

once per month for a total of three intradermal injections commencing 2 to 3 weeks after surgery were administered. Only then was there an overall pragmatic, detailed analysis (March 1986) of 234 patients studied in these three trials, as reported previously with appropriate individual trial review and references.[1] Here we report a further analysis (January 1988) of the US trial. This analysis includes an important subset analysis that indicates that the significant increase in the 5-year survival rates of patients with Stage I and Stage II lung cancer undergoing specific active immunotherapy is a repeat of the results of an earlier trial[1,5] in Stage I lung cancer patients.

Patients

Sixty-four patients with squamous cell lung cancer or adenocarcinoma of the lung, 34 of whom had received immunotherapy or immunochemotherapy and 30 of whom had received no treatment postsurgery, were selected for study. Five serial sera taken from each patient after the first, second, and third months of immunotherapy, at 5 to 6 and 9 to 12 months after the first course of immunotherapy, and at comparable times from patients in the control group (chosen from those postsurgical Stage I and Stage II patients, randomized to control groups, who lived at least 18 months) were available for each patient except one. This patient died before the last time period. This yielded a total of 319 serums for study.

Therapy

Procedures used in the preparation and identification of both pure and ultrapure TAA from various cancer types are listed in Appendix 1. As described elsewhere,[6] ultrapure lung TAA preparations were cross-tested in patients with and without lung cancer. These preparations were subjected to 952 tests in 56 lung cancer patients (52 of whom submitted to 17 tests each) to establish the activities of individual antigens. The average amount of individual TAA per cell is approximately 0.3 pg or approximately 2% of soluble cell membrane protein. Thus, 1×10^9 tumor cells yield approximately 300 μg of TAA.

In addition to being tested individually, each ultrapure TAA also was tested in 42 patients in combination with other TAA to determine the best synergistic selection for the strongest induction of CMI per a given amount of protein. Specific lymphocyte stimulation titration assays were performed for individual patients where possible and correlated well with skin tests for delayed hypersensitivity. For reasons of efficiency and economy (considering the expense of preparing large amounts of ultrapure antigens), we tested the use of selected soluble antigen fractions within an inclusive range of molecular weights, separated by column chromatography with solute behavior measured independently of bed dimensions and packing of gels. Recovery was three times better, with only 14% to 16% impurity. Appendix 2 summarizes the steps in preparation of and some of the studies on lung TAA, pure TAA, ultrapure TAA, and TAA epitopes derived from lung TAA. Pure TAA used in clinical trials was essentially free of nucleic acids, major tissue antigens, pyrogens, bacteria, or viruses.

TAA essential for induction of a required level of CMI included three in the squamous cell lung vaccine at approximate molecular weights of 37 kilodaltons (KD), 49 KD, and 100 KD as determined by sodium dodecyl sulfate (SDS) gel electrophoresis, and two in the adenocarcinoma lung vaccine at 51 KD and 77 KD. There was good reproducibility of content and activity between batches.

Immunotherapy consisted of 500 μg of TAA homogenized in complete Freund's adjuvant and delivered slowly intradermally to patients once a month for 3 months with no further immunization. The homogenate was given intradermally into the deltoid region of the arm, the thigh, and the arm again. Ulcers developed slowly at the site of injection with erythema and thinning of the skin. Ulceration lasted 7 to 10 days and was followed by gradual healing that was complete within the year (usually by 7 months). The ulcers looked unsightly but caused little discomfort. Occlusive dressing with sterile vaseline gauze was useful for the first 2 months. In the immunochemotherapy group, 300 mg of methotrexate was given by rapid intravenous infusion and then 700 mg was infused over a period of 6 hours. A normal creatinine clearance was mandatory and urine was alkalinized by giving patients sodium bicarbonate every 6 hours (1.2 g) *per os,* starting 24 hours before the infusion and continuing during the folinic acid rescue period of 60 hours (12 mg intramuscularly every 6 hours). The urine pH was monitored every 6 hours to ensure that the pH remained above 6. Patients then received immunotherapy, as described above, starting 7 to 9 days after administration of chemotherapy. Data on age, sex, performance status, type of surgery, stage at the time of initiation of therapy, and 5-year survival status of the patients we studied are given in Table 1 for reference as pertinent results are given throughout this report.

Methods

Humoral Immune Correlates

Humoral immune responses to the D36h6 epitope of squamous cell TAA (37 KD) and to the G10r8 epitope of adenocarcinoma TAA (77 KD) were measured by a reverse enzyme-linked immunosorbent titration assay using increased concentrations of epitope (range, 10 to 60 ng of epitope).[1,7-9] These enzyme immunoassays of early antibody responses based on TAA-derived epitopes were

TABLE 1. Description of Patients Evaluated for Humorocellular Immune Response Correlates

Patient no.	Age (yr)/Sex	PS	Surgery	Stage	5-year survival status
Squamous cell: immunotherapy					
1	62/M	1	lu lobectomy	T1N1	NED
2	66/M	1	ru lobectomy	T2N0	NED
3	62/M	2	l pneumonectomy	T2N1	NED
4	44/M	1	r pneumonectomy	T2N2	NED
5	76/M	1	lu lobectomy	T2N0	NED
6	62/M	0	rm & l lobectomy	T1N0	EXP 32m
Squamous cell: immunochemotherapy					
7	59/F	1	rl lobectomy	T1N0	NED
8	40/M	1	lu lobectomy	T2N0	NED
9	57/M	1	l pneumonectomy	T1N0	NED
10	64/M	0	ll lobectomy	T2N0	NED
11	62/M	1	r pneumonectomy	T1N1	NED
12	48/M	2	lu lobectomy	T2N1	EXP 41m
Adenocarcinoma: immunotherapy					
13	61/F	1	ru lobectomy	T1N0	NED
14	52/F	1	lu lobectomy	T1N1	NED
15	44/F	0	ru lobectomy	T2N0	NED
16	57/F	1	lu lobectomy	T2N1	NED
17	53/M	0	ll lobectomy	T2N0	NED:1fu 50m
18	56/M	1	rl lobectomy	T1N0	NED:1fu 49m
19	58/M	0	ru lobectomy	T1N0	NED:1fu 43m
20	66/M	1	ru & rm lobectomy	T1N1	EXP 18m
21	66/M	1	ru lobectomy	T1N1	EXP 5m
22	40/F	1	rm lobectomy	T1N0	NED
23	71/M	0	lu lobectomy	T1N0	NED
Adenocarcinoma: immunochemotherapy					
24	60/M	0	ll lobectomy	T1N0	EXP 39m
25	66/M	0	lu lobectomy	T1N0	NED
26	56/F	1	ru lobectomy	T2N0	NED
27	45/M	1	r pneumonectomy	T2N0	NED
28	66/F	1	ru lobectomy	T2N0	EXP 51m
29	45/M	1	l pneumonectomy	T2N0	NED
30	60/F	1	rm & ll lobectomy	T2N1	NED
31	48/F	1	r pneumonectomy	T2N1	NED
32	57/M	1	ru lobectomy	T2N0	NED
33	54/M	1	ru lobectomy	T1N0	NED
34	45/F	1	lu lobectomy	T2N1	NED
Control group 35–64 (matched for age, stage, and type of cancer)					

PS: performance status; lu: left upper; ru: right upper; l: left; r: right; rm: right middle; rl: right lower; ll: left lower; NED: no evidence of disease; EXP: expired.

performed on the 319 sera drawn after the times of, or in the case of patient controls at comparable times of, first, second, and third immunizations and at 5 to 6 and 9 to 12 months thereafter. The two sets of assays (squamous cell and adenocarcinoma) were performed using the double-blind method (in triplicate) with a graded scale of seven titrated epitope concentrations per assay, with the assay-well designated '0' actually containing 70 ng (the non-specific cutoff point within the limitations of the balanced *in vitro* assay variables) for a total of 6699 enzyme immunoassays. The principle of the assay was to measure the smallest concentration of epitope giving a positive reaction with antibodies in the serum tested.

Cell-Mediated Immune Correlates

Delayed hypersensitivity responses of 63 patients were measured 17 to 24 months posttherapy by intradermal injection of 100 μg of TAA containing the appropriate epitope of squamous cell lung cancer or adenocarcinoma of the lung. The area of induration was measured in millimeters by Sokal's method as described in detail elsewhere.[6]

Results

Table 1 shows the patients with squamous cell lung cancer who received immunotherapy or immunochemo-

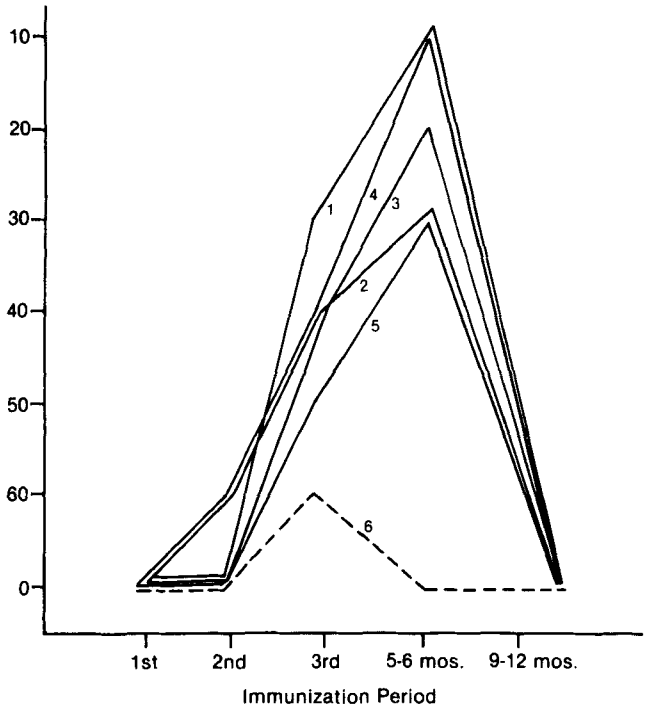


FIG. 1. Monitoring for early response to immunotherapy for squamous cell lung cancer (enzyme immunoassay of serial sera *versus* epitope D36h6 from TAA (37 KD); dilutions titered 10 to 60 ng). Numbers on graph indicate patients (see Table 1). Dotted line shows Patient 6, a nonsurvivor.

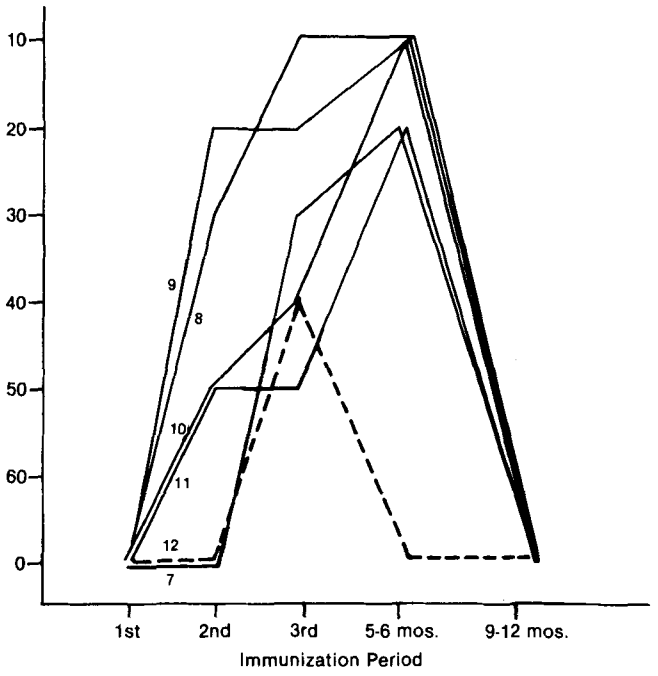


FIG. 2. Monitoring for early response to immunochemotherapy for squamous cell lung cancer (enzyme immunoassay of serial sera *versus* epitope D36h6 from TAA (37 KD); dilutions titered 10 to 60 ng). Numbers on graph indicate patients (see Table 1). Dotted line shows Patient 12, a nonsurvivor.

therapy and the patients with adenocarcinoma lung cancer who received immunotherapy or immunochemotherapy, respectively. Age, sex, surgical procedure, stage, and 5-year survival status are listed. Data are not shown for the control group (see the Patients section). Titers of antibody response are plotted for each patient at the times of first immunization, second immunization, third immunization, and at 5 to 6 and 9 to 12 months after initiation of therapy. Figures 1 through 4 show these measurements for the patients listed in Table 1. Antibody was measured (Figs. 1 and 2) to epitope D36h6 from squamous cell TAA (37 KD) in epitope dilutions of 10 to 60 ng (weakest to strongest) with a 70-ng concentration per well on assay plates designated 0, the nonspecific cutoff point as described in the Methods Section. Similarly, antibody was measured to epitope G10r8 from adenocarcinoma TAA (77 KD) (Figs. 3 and 4).

As shown in Figure 1, one patient (Patient 6) in the squamous cell immunotherapy group (Table 1) showed a transient rise in titer at the third immunization. However, this rise subsided to low anti-TAA antibody levels at 5 to 6 months. The 0 level was consonant with the nonspecific reactivity observed in sera from all 30 matched patients with squamous cell lung cancer and adenocarcinoma who had received no treatment postsurgery and was, therefore, considered negative. Levels were consid-

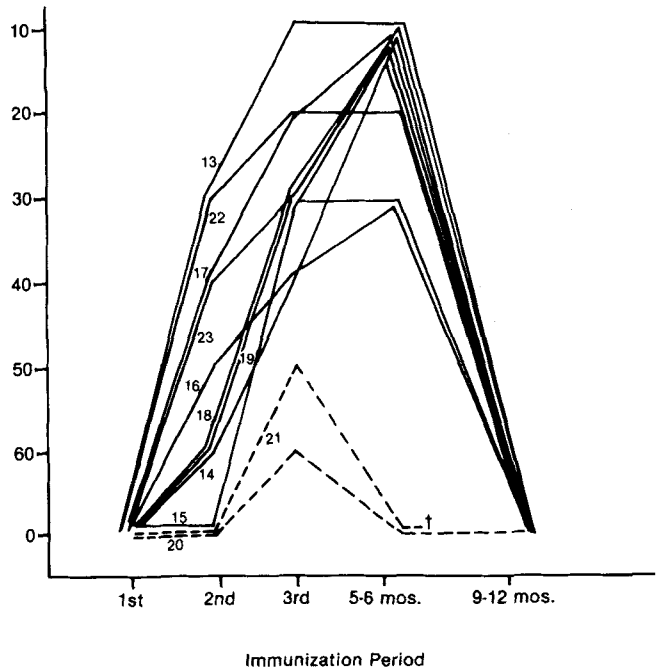


FIG. 3. Monitoring for early response to immunotherapy for adenocarcinoma of the lung (enzyme immunoassay of serial sera *versus* epitope G10r8 from TAA (77 KD); dilutions titered 10 to 60 ng). Numbers on graph indicate patients (see Table 1). Dotted lines indicate Patients 20 and 21, both nonsurvivors.

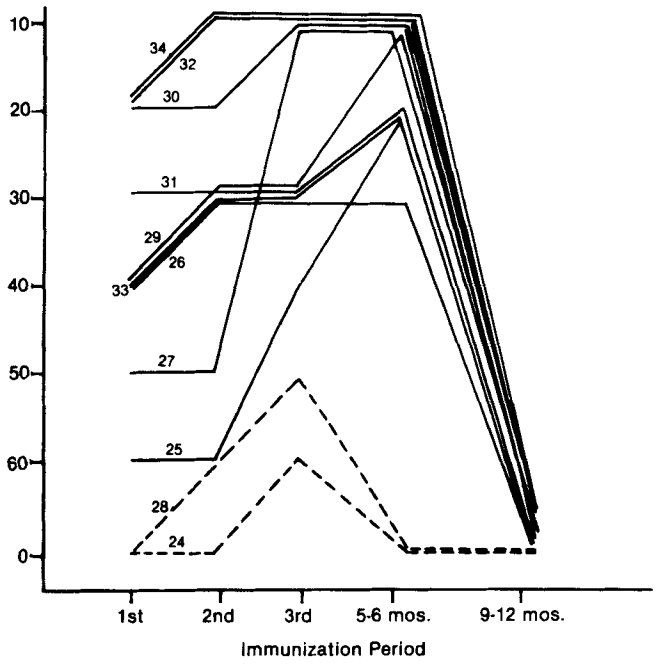


FIG. 4. Monitoring for early response to immunochemotherapy for adenocarcinoma of the lung (enzyme immunoassay of serial sera *versus* epitope G10r8 from TAA (77 KD); dilutions titered 10 to 60 ng). Numbers on graph indicate patients (see Table 1). Dotted lines indicate Patients 24 and 28, both nonsurvivors.

erably higher in the 5-year survival group. Notice that by the second immunization there were two responders, with five responders at 30 ng or below by 5 to 6 months and a disappearance of humoral response by 9 to 12 months as reactivities subsided to the tissues.

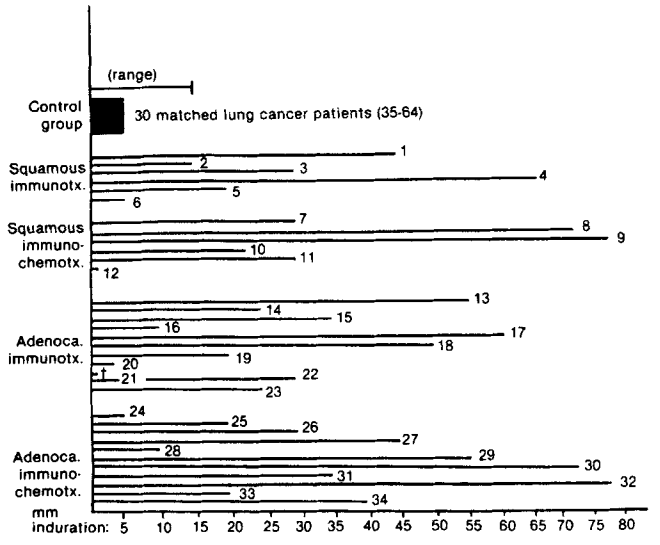


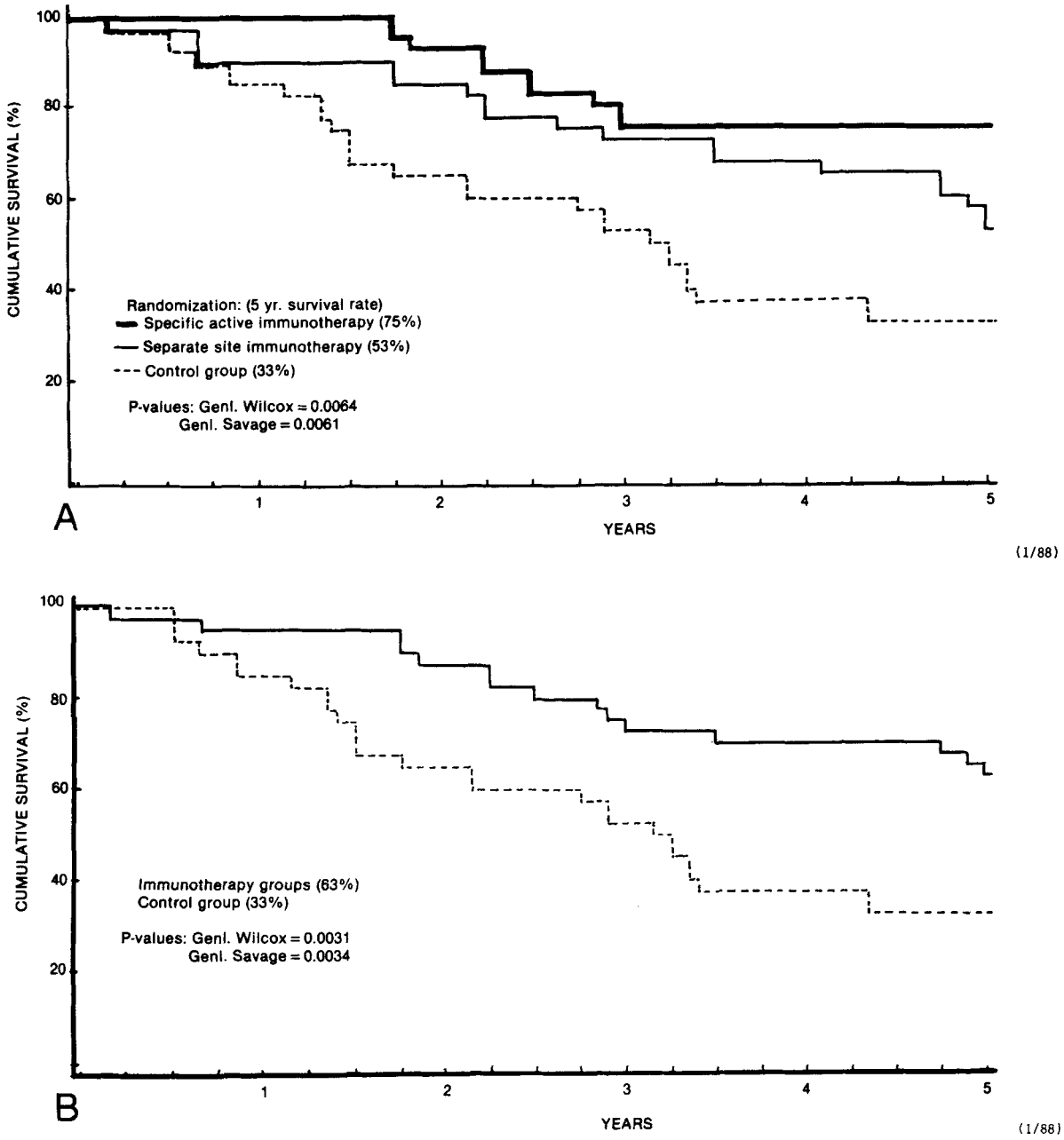
FIG. 5. Monitoring for continued cell-mediated immunity induced by immunotherapy (long-lasting delayed hypersensitivity monitored between 17 to 24 months posttherapy using 100 μg of TAA of matched histologic type). Numbers on graph indicate patients (see Table 1). Patient who failed therapy were Patients 6, 12, 21 (not measured), 24, and 28.

Figure 2 shows similar findings for patients with squamous cell lung cancer treated with immunochemotherapy. The patient (Patient 12) with Stage T2N1 lung cancer who died at 41 months (Table 1) showed a low titer at 5 to 6 months and did not survive to 5 years. By the second immunization there were four responders, with five responders at 20 ng or below by 5 to 6 months.

Figure 3 shows antibody titers to the adenocarcinoma epitope in the serums of 11 representative patients with adenocarcinoma who received immunotherapy. Notice that the response pattern in adenocarcinoma patients was similar to that of the squamous cell cancer patients. Notice especially that two patients (Patients 20 and 21; Table 1) of this group had a reduction in titer at 5 to 6 months, whereas the other nine patients' titers had increased progressively. These two patients (both Stage T1N1) died at 18 and 5 months, respectively. Eight patients had responded by the second immunization, with nine responders at 30 ng or below by 5 to 6 months.

Figure 4 shows the responses in patients with adenocarcinoma who had received combination immunochemotherapy. Again, titers increased in most patients and were back to baseline by 9 to 12 months after immunization. A patient (Patient 24) with Stage T1N0 who died at 39 months and a patient (Patient 28) with Stage T2N0 who died at 51 months had negative responses at the 5 to 6 months. There was a difference, however, in this group. Notice that all patients in the 5-year survival group responded after the first vaccination, an exceedingly early time frame, with only one responder at 30 ng, three at 20 ng, and five at 10 ng or below by 5 to 6 months. This indicated some difference in the rate of response between adenocarcinoma patients on immunotherapy and those on immunochemotherapy.

Cell-mediated immunity of long duration induced by immunotherapy was tested 17 to 24 months posttherapy by intradermal injection of 100 μg of TAA of appropriate histologic type. As is evident from Figure 5, CMI response to TAA at 17 to 24 months posttherapy (as measured in millimeters induration) was far greater in patients receiving immunotherapy or immunochemotherapy than in 30 matched lung cancer patients of the same type who received no further treatment after surgery. Although the smaller number of patients precludes statistical analysis in this particular study, previous reports[4,5,10] have established this observation. A previous observation[10] that positive responders reacted more vigorously to skin tests for delayed hypersensitivity in the immunochemotherapy group is true for the long survivors of both types of cancer. In this study (Fig. 5), patients gave an average response of approximately 44 mm induration in the immunochemotherapy groups. This is 28% higher than in the immunotherapy groups, which have an average response of approximately 32 mm induration. Notice the great vari-



FIGS. 6A AND 6B. Survival: US adjuvant specific active TAA immunotherapy Stages I and II squamous cell lung cancer trial. (A) January 1988 update of 5-year survival curves for the three-arm trial testing immunotherapy given once a month for 3 months as homogenized with adjuvant for single site therapy *versus* adjuvant given in a separate site with smaller doses of TAA given as skin tests for an average total of 450 μg of TAA *versus* control group. (B) Results when both immunotherapy groups were combined for analysis. These results are essentially similar to those available for our previous report[1] using the March 1986 analysis. The breakout of the two separate therapy group survival experiences (January 1988) was not available at that time.

ability between patients. That those patients with high antibody levels at 5 to 6 months in the humoral immunity studies (Figs. 1 to 4) also had high cell-mediated immunity by this criteria can been seen by comparisons for each group (Fig. 5). In general, those patients with the greatest degree of cell-mediated immunity had a longer mean survival than those with a lower degree.

Immunotherapy alone has promise. We have discussed studies with 234 patients.[1] A more detailed analysis of one of these trials, the US randomized adjuvant trial of Stage I and Stage II squamous cell lung cancer patients[1] on specific active TAA immunotherapy, is of interest. In January 1988, we updated our analysis of the 5-year survival of all 81 patients. As shown in Figure 6A, 75% of

patients receiving specific active immunotherapy survived 5 years, with a plateau of 75% reached by the third year. Fifty-three percent of patients receiving adjuvant plus multiple skin tests, separate site therapy, and 33% of the control group survived 5 years. When the two therapy groups were combined (Figure 6B), there was still a 30% survival difference compared with the control group. Thus, immunotherapy alone can produce a 30% to 42% difference in survival. These data repeat the experience[1,5] of an earlier trial with 52 Stage I patients, where survival at 5 years was 78% for patients receiving specific active immunotherapy *versus* 46% for the control group.

Discussion

The data presented here indicate that in patients with squamous cell cancer or adenocarcinoma of the lung, Stage I and Stage II, immunotherapy with TAA in Freund's complete adjuvant produces a significant increase in humoral antibody to the relevant epitope of TAA by 3 months in most patients. The patients in whom this titer is maintained or in whom it rises further by 5 to 6 months are in the group of 5-year survivors, whereas those with low values at 5 to 6 months have shorter survival. A similar association was observed in both patient groups receiving combination immunochemotherapy. The measurements at 5 to 6 months show a differential of serum antibody levels that appears to be prognostic.

We have no explanation for the earlier humoral responses observed in adenocarcinoma patients on immunochemotherapy. A plausible interpretation may be that after preferentially affecting a regulatory class of lymphocytes, in the absence of which a rebound overshoot is seen, the surge of lymphocytes must contain a substantial component directed at TAA. This is so because we are dealing with a sensitized host, not a naive host. Stimulation within 24 to 48 hours may be due mainly to a sensitized host response.

After the third immunization detectable antibody levels were high in most 5-year survival patients, but declined to baseline by 9 to 12 months. This finding is important because it implies that during the 12 months to 5 years after therapy other mechanisms prevent tumor growth. Indeed, the degree of the CMI response after therapy, as measured by skin tests for delayed hypersensitivity at 17 to 24 months post-therapy, has a high correlation with 5-year survival. The reaction is antigen-specific, with sensitivity to TAA derived from both autologous and allogeneic tumors of the same histologic type.[6] In those patients with low levels of detectable serum antibody at the 6-month mark, other therapeutic interventions are indicated.

We realized early in our studies of individual TAA of the related histologic type of cancer that the synergism (combinations of selected TAA in a given protein concentration) was crucial in effecting a strong CMI patient response. As reviewed elsewhere,[6] after the separation of ultrapure TAA, testing of individual TAA from each tumor (both in the autologous host and in the controlled allogeneic cross-testing of patients) permitted identification of the entire spectrum of primary lung tumor cell membrane soluble antigens, each of which produced cell-mediated immunity in greater than 75% of nonanergic, postsurgical patients of the related primary lung cancer as our initial, major criteria. There were five candidate ultrapure antigens for squamous cell, four for adenocarcinoma, three for oat cell, and a mixed representation with two antigens most consistent for bronchioalveolar and two for large cell lung cancer, respectively. The mixed histology of many lung cancers and the history of recurrent tumors of different histology from the primary indicate the importance and usefulness of our early studies of ultrapure TAA related to well-defined primary tumor pathologies. These individual antigens were then combined in all possible ways for testing in patients of each category. Combinations of ultrapure TAA producing maximum cell-mediated immunity were selected for clinical usage, with three antigens (37 KD, 49 KD, and 100 KD) in combination necessary for maximum cell-mediated immunity production in patients with squamous cell cancer, two antigens for adenocarcinoma (51 KD and 77 KD), two for oat cell (51 KD and 69 KD), two for bronchioalveolar (77 KD and 100 KD), and two for large cell (37 KD and 51 KD mw) lung cancer, respectively. Notice that there is a sharing of certain antigens among these carefully evaluated pathologically well-defined lung cancer types. As there is a loss of antigen during preparation of ultrapure TAA, we selected pure TAA prepared from several tumors per category for clinical studies. There was batch to batch consistency for the quantity of each selected antigen, and titrated, even batch to batch cell-mediated immunoreactivities, with full characterization, quantitation, and identification of ultrapure TAA as part of the quality control procedures. In the future, especially for Stage II and beyond, we plan to study the use of additional TAA representing more than one category for even greater breadth of antigenicity. For this purpose we are studying currently a larger selection, from both primary and metastatic TAA, of epitopes derived from monoclonal antibody-affinity column separations. We hope to understand the alteration and modulation as the tumors metastasize.

In our design for the treatment of early stage lung cancers using TAA specific active immunotherapy, the polyvalency and synergism between appropriately selected antigen combinations assured a comprehensive approach to adjuvant therapy for this group.

Due to the antigenicity and purity of TAA, only three immunizations are necessary for most patients with early

stage lung cancers. Since we introduce simple antigens free from the major histocompatibility antigens, this permits the body to respond and to possibly complement the efficacy of the antigens through its own tissue antigen complex and endocrine and immune network in the initiation of interference with and prevention of tumor progression or metastases. The result is that many of our patients have experienced 12 years or longer of cancer-free existence.

In summary, this clinical experience using adjuvant immunotherapy (coupled with a better understanding of mechanisms of action) will now permit us to develop strategies of combination therapy for patients who fail to respond, incorporating a strategy of proven worth.

Additional therapy in the form of booster immunizations should be considered. Therapy with a broader spectrum of TAA in combination may be applicable. Other forms of therapy should be tested in combination with specific active TAA immunotherapy. We have begun a study to see whether or not the addition of interleukin-2 might be useful.[11] The study reported here indicates that another combination therapy study we have begun may prove important. Namely, the use of combination chemoimmunotherapy may prove effective in certain groups of patients.[12]

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APPENDIX 1

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.

APPENDIX 2

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

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Protein Determination

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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.

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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.