Failure of Cell-Mediated Effector Mechanisms in Lung Cancer[1,2]

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ABSTRACT—The status of cell-mediated effector mechanisms was studied in 28 patients with lung cancer (25/28 in stage III). Patients' precultured peripheral blood mononuclear leukocytes, isolated by Ficoll-Hypaque, were tested for lymphocyte proliferation responses to alloantigens in mixed lymphocyte culture (MLC). The influence of autologous patients' sera was studied further on MLC responses from patients and controls. Cell-mediated lympholysis (CML), with the use of allogenic blast cells as targets, and antibody-dependent cell-mediated cytotoxicity (ADCC) against human red blood cells also were tested. Major differences between the cancer patients and controls were not demonstrated by MLC. Inhibition or enhancement of MLC responses by the autologous serum was shown; bimodal influence was significant; 72% of the sera caused inhibition and 28% caused enhancement. CML was depressed in 54% of the patients, and ADCC was depressed in 50%. The decrease in both cytotoxic responses was significant ($P<.005$). Thirteen patients died after initiation of the investigative protocol; in 11 of 13, CML or ADCC was diminished. The altered cytotoxic capabilities were more prevalent among the epidermoid type, including the deceased patients. This study provides evidence that a severe impairment of cell-mediated effector mechanisms is frequent in advanced lung cancer and may be associated with poor clinical course and with the histologic type.—JNCI 1984; 73:1-6.

CMI has been postulated as one of the main antitumor defense mechanisms (*1*). Evidence that patients with lung cancer are capable of mounting CMI to their tumors has come from histologic studies documenting the presence of cellular reactions (*2, 3*), from in vitro studies on lymphocyte reactivity (*4-6*), and from delayed cutaneous hypersensitivity tests (*7*). A variety of antigens that appear to be associated with lung cancer has been described (*8-12*). In addition, antibodies reacting against epidermoid and adenocarcinoma tumor cells have been shown in lung cancer tissues and pleural effusions (*13*), although their demonstration is particularly difficult. Along these lines, we designed a protocol dealing with several aspects of the effector phase of in vitro CMI in advanced lung cancer.

MATERIALS AND METHODS

Patients.—Seven female and 21 male lung cancer patients, aged 42-68 years, were studied. The diagnosis was confirmed by histologic or cytologic examination. Sixteen patients had squamous cell (epidermoid) carcinoma, 7 had adenocarcinoma, 3 had large cell carcinoma, 1 had small cell carcinoma, and 1 had undifferentiated carcinoma. All the patients were carefully grouped by the TNM classification and by the stage grouping of the American Joint Committee for Cancer Staging and End Results Reporting as shown in table 1 (*14*); 25 of these patients had cancers belonging to stage III. All patients included in this protocol were studied before any form of treatment (surgery or chemotherapy) was given. The control group consisted of healthy adult blood bank donors.

Preparation of peripheral blood mononuclear cells.—Peripheral blood mononuclear cells from the heparinized blood of patients and controls were separated on Ficoll-Hypaque gradients; the method originally described by Boyum (*15*) with minor modifications was used. The lymphocyte-enriched population collected in the interphase was washed three times in RPMI-1640 medium and resuspended in 10×10^6 cells/ml in RPMI-1640 supplemented with 1 mmol L-glutamine/ml, 100 IU penicillin/ml, 100 μg streptomycin/ml, and 2% heat-inactivated normal human serum.

Preculture of the lymphocyte.—All the CMI assays were performed with lymphocytes precultured for 18 hours in RPMI-1640 medium (supplemented with 100 IU penicillin-streptomycin), 1 mmol glutamine/ml, and 10% normal human serum (heat inactivated) in an atmosphere of 95% air-57% CO_2.

Preparation of the patients' sera.—The sera were obtained from peripheral nonheparinized blood samples after clot retraction and centrifugation at 1,800 rpm for 20 minutes. The sera were aliquoted and stored at −70°C before use. The aliquots to be used as autologous sera were heat inactivated at 56°C for 30 minutes and stored at −20°C.

MLC.—The MLC reaction was performed following the microtest developed by Hartzman et al. (*16*). The

ABBREVIATIONS USED: ADCC=antibody-dependent cell-mediated cytotoxicity; CIC=circulating immune complex(es); CMI=cell-mediated immunity; CML=cell-mediated lympholysis; Clq-BA=Clq binding assay; MLC=mixed lymphocyte culture(s); NHS=normal human serum; RPI=relative proliferation index; TNM=tumor-node involvement-metastasis.

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TABLE 1.—*Lung cancer patients*

Case[a]	Histologic type	TNM
1	Epidermoid carcinoma	T1N0M0
2	Adenocarcinoma	T3N2M0
3	Adenocarcinoma	T3N2M0
4	Epidermoid carcinoma	T3N0M0
5	Epidermoid carcinoma	T3N0M0
6	Epidermoid carcinoma	T3N0M0
7	Large cell carcinoma	T3N0M0
8	Adenocarcinoma	T3N0M1
9	Adenocarcinoma	T3N2M1
10	Epidermoid carcinoma	T3N0M1
11	Epidermoid carcinoma	T3N2M0
12	Epidermoid carcinoma	T3N2MX
13	Adenocarcinoma	T3N2MX
14	Epidermoid carcinoma	T3N0MX
15	Adenocarcinoma	T3NXM1
16	Large cell carcinoma	T3N2M1
17	Epidermoid carcinoma	T2N2MX
18	Epidermoid carcinoma	T3NXM1
19	Epidermoid carcinoma	T3N2MX
20	Small cell carcinoma	T3NXMX
21	Adenocarcinoma	T3NXMX
22	Epidermoid carcinoma	T3NXMX
23	Epidermoid carcinoma	T3NXMX
24	Epidermoid carcinoma	T3NXM0
25	Large cell carcinoma	T3NXMX
26	Undifferentiated carcinoma	T2NXMX
27	Epidermoid carcinoma	T3NXMX
28	Epidermoid carcinoma	ND[b]

[a] n=28.
[b] Not determined.

responder cells were precultured for 18 hours and adjusted in RPMI-1640 (containing 10% heat-inactivated normal serum) at a concentration of 1×10^6 cells/ml; the cells (5×10^4 cells/0.05 ml) were added to wells of round-bottom Cooke microculture plates. The stimulator cells also were precultured for 18 hours and adjusted at 1×10^6 cells/ml in RPMI-1640, and then they were treated with mitomycin (25 μg/ml for 30 min at 37°C) and added at a concentration of 1×10^5 cells/well (1×10^5 cells/0.1 ml) to obtain a stimulator-to-responder ratio of 2:1. The MLC reaction was performed in the presence or absence of autologous serum. The plates were covered and incubated for 5 days at 37°C in an atmosphere of 95% air-5% CO_2. MLC were terminated on day 6; the plates were pulsed with [^{3}H]thymidine (1 μCi/well); after 18 hours the cells were collected on glass filters by means of a Mash II cell harvester (M.A. Bioproducts, Walkersville, Md.). The filters were dried at 150°C for 2 hours, placed in scintillation vials, and counted on a liquid scintillation spectrometer. The proliferative responses were expressed as RPI as described by Dean et al. (*17*). RPI is the ratio between the net counts per minute of the studied subject and the net counts per minute of 3 controls or more assayed simultaneously.[7] Cutoff values (established as the lower 10th percentile of normal RPI values) for alloantigens based on 80 normal controls was ≥0.66. This procedure allowed us to define depressed or enhanced proliferative responses with accuracy. All determinations were done in triplicate.

CML.—Effector T-cells were generated in vitro by one-way MLC following the procedure of Lightbody and Bach (*18*). The MLC was conducted in Falcon tissue culture tubes (17×100 mm) with 10^7 responder cells and 10^7 stimulator cells in a total volume of 10 ml (RPMI-1640 with 20% heat-inactivated normal human serum). The cultures were incubated for 5 days at 37°C in 5% CO_2. Eighteen hours prior to harvesting, 0.2 ml from each tube was labeled with 2 μCi [^{3}H]thymidine to determine the degree of proliferation. The cytotoxic effector cells were collected from the tubes on day 6. In a similar fashion, target cells were cultured; collected blast cells were centrifuged, resuspended in 0.3 ml saline, labeled with 250 μCi sodium chromate, and incubated for 1 hour. After incubation the cells were washed three times in saline in the cold, diluted to 10^5 cells/ml in RPMI-1640 (containing 10% NHS), and utilized as target cells in CML.

Cytotoxicity assay.—CML was performed following the technique of Brunner et al. (*19*). The cytotoxic effector cells were centrifuged for 10 minutes at 1,000 rpm, resuspended in RPMI-1640 (containing 10% NHS) at a concentration of 10^7 viable cells/ml, and added to round-bottom microculture plates (10^6 cells/0.1 ml) already containing the labeled target cells (10^4 cells/0.1 ml). The mixture was then incubated for 18 hours 37°C in an atmosphere of 95% air and 5% CO_2. Prior to harvesting, the plates were centrifuged for 10 minutes at 1,000 rpm; 0.1 ml of the supernatant was taken from each well and counted to determine the amount of ^{51}Cr released. The spontaneous release was obtained by incubation of 10^4 target cells alone in 0.2 ml RPMI-1640 medium, and the maximal release was achieved by the lysis (freeze-thawing) of 10^4 labeled target cells in an equal volume of RPMI-1640 medium. The percentage of lysis was calculated by the following equation: Percent lysis=$\bar{X}$ experimental release − $\bar{X}$ spontaneous release/$\bar{X}$ maximal release − $\bar{X}$ spontaneous release × 100. In our laboratory, values under 10% are considered to be an index of low cytotoxic activity, since 50 normal individuals showed values over 10%.

ADCC.—The ADCC of peripheral blood mononuclear cells was determined with the use of ORh (D^+) human erythrocytes as target cells. The target cells were labeled with 100 μCi sodium chromate in 0.1 ml saline, incubated for 90 mintues at 37°C, washed twice in saline, and diluted to 12×10^5 cells/ml in RPMI-1640 with 2% heat-inactivated fetal calf serum. The labeled erythrocytes were added to round-bottom Cooke microculture plates (6×10^4 cells/0.05 ml), and then 50 μl of a 1:25 dilution of specific IgG anti-D serum (previously titered) was added to each experimental well, and the plates were incubated for 30 minutes at 37°C in an atmosphere of 95% air-5% CO_2. After incubation 0.1 ml of effector cells was added to the microplate wells

[7] It was calculated by this formula: RPI=net cpm of test individual/mean net cpm of 3 controls assayed simultaneously.

(containing the sensitized and labeled erythrocytes) at different dilutions to obtain different effector-to-target cell ratios; after centrifugation at 1,000 rpm for 5 minutes, the plates were incubated for 18 hours at 37°C in an atmosphere of 95% air-5% CO_2. Finally, the plates were centrifuged at 1,000 rpm for 10 minutes, and 0.1 ml supernatant was counted to determine the amount of ^{51}Cr released. The spontaneous release was measured by incubation of target cells alone in an equal volume. The maximum release was determined by lysis of 6×10^4 labeled erythrocytes with zapo-globin II (Coulter Electronics Inc., Hialeah, Fla.). Another experimental control included 6×10^4 labeled nonsensitized erythrocytes incubated with effector cells under the same conditions. The percentage of ^{51}Cr released from each effector-to-target cell ratio was calculated by the following equation: Percent ^{51}Cr release=$\overline{X}$ experimental release − $\overline{X}$ spontaneous release/$\overline{X}$ maximal release − $\overline{X}$ spontaneous release × 100. Results were expressed as the number of mononuclear cells capable of lysing 50% of the sensitized erythrocytes (K); K-values were calculated by the Van Krogh equation as modified by Trinchieri et al. (*20*): $X=K\ [y/(A-y)]^{1/n}$, where X=number of lymphoid cells per well, y=the percentage of lysis obtained at each cell concentration, A=the maximum percentage of cytotoxicity obtained in the presence of mononuclear lymphoid cells and anti-D serum, K=the number of mononuclear cells needed to mediate 0.5 of A, and $1/n$=the slope. Plotting the log X versus $y/(A-y)$, we obtained a straight line by linear regression, permitting the estimation of K-value, since at the point where $y=0.5\ A$ log X=log K. K-values were obtained from 30 blood donors. The normal range calculated as the 80th percentile of these 30 controls was 9.1×10^4 to 11×10^5 mononuclear cells. Statistical analysis was performed according to the Kolmogorov-Smirnov test and chi-square test.

CIC.—CIC were measured by C1q-BA (*21*) and Raji cell (*22*) assays. CIC levels in 100 healthy controls were previously established in our laboratory.

Statistical analysis.—All the statistical calculations were done by chi-square contingency analysis and paired t-test, and the significance was expressed by P-value.

RESULTS

Allogeneic response (MLC).—The MLC reactivity from patients and controls showed normal proliferative responses in all the experiments (mean RPI value: 1.15±0.43 vs. 1.05±0.31).

Influence of autologous serum on patient MLC and normal individual MLC.—Inhibitory action of autologous serum was found in 72.2% of the cases (table 2). In addition, 27.7% of the sera tested were enhancers. The bimodal influence (either inhibition or enhancement) was significant ($P<.005$) when compared with values obtained with NHS. These effects also were noted when homologous normal lymphocytes were incubated with the patient's serum ($P<.005$).

TABLE 2.—*Influence of autologous serum on MLC response*[a]

Cases	NHS	AS[b]	Inhibition, %	Enhancement, %[c]
A	1.24	0.36	70.97	—
B	0.93	<0.01	100.00	—
C	0.85	<0.01	100.00	—
D	0.87	0.35	59.78	—
E	1.16	1.38	—	15.95
F	1.70	0.54	68.24	—
G	3.90	0.60	84.62	—
H	1.88	2.64	—	28.79
I	0.83	0.07	91.57	—
J	0.80	0.13	83.75	—
K	0.91	0.09	90.20	—
L	0.75	0.20	73.34	—
M	1.16	0.57	50.87	—
N	0.89	1.05	—	17.97
O	1.17	1.07	8.55	—
P	0.82	0.14	17.35	—
Q	0.79	0.93	—	18.53
R	0.76	0.95	—	25.00

[a] RPI (normal values ≥0.66).
[b] AS=autologous serum.
[c] $P<.005$ (significance expressed as P-value from paired t-test).

Expression of CML.—T-cell cytotoxic capacity was investigated in both the lung cancer and the control groups. In the lung cancer group, CML was depressed in 14 of 26 of the cases (54%). The difference between the lung cancer group and the controls was highly significant (table 3).

ADCC.—The ADCC capability to IgG-sensitized ORh (D^+) red blood cells was examined in 20 patients (table 4). Depressed responses ($K>11\times10^5$) were found in 50%, whereas only 2 showed enhanced capability (less number of cytotoxic cells to lyse 50% of the red blood cells).

Correlation between ADCC and histologic type.—In regard to ADCC, it can be noted (table 5) that the epidermoid type showed the greatest association with depressed capacity when it was compared with non-epidermoid types ($P<.005$). A similar trend was observed in relation to CML, where 10 of 14 epidermoid types were associated with depressed CML (data not shown).

Effector responses of patients who died during the period of investigation.—We analyzed the results of cytotoxic mechanisms in 13 patients who died within

TABLE 3.—*T-cell effector capacity (CML)*

Group	No. of subjects	Lysis, %[a]
Control	25	$\overline{X}$=17.95±5.73
Lung cancer, normal capacity	13	$\overline{X}$=19.92±8.92
Lung cancer,[b] depressed capacity	14	$\overline{X}$=3.71±2.81

[a] Values are means ± SD. Values <10% of lysis are considered as an index of low response.
[b] $P<.005$ (significance expressed as P-value from chi-square contingency analysis).

TABLE 4.—*ADCC*

Capacity	No. of patients, n=20	K-value[a]
Depressed	10	$K>11\times10^5$
Enhanced	2	$K<9.1\times10^4$
Normal[b]	8	9.1×10^4–11×10^5

[a] K=No. of cells required to mediate 50% of lysis.
[b] Determined in 50 controls.

13 months after the onset of the study; 11 of 13 (table 6) showed either depressed or absent cell-mediated effector mechanisms (CML or ADCC); the failure in the cytotoxic capacity was particularly prevalent in the epidermoid type.

CIC.—CIC were measured in 21 of 28 patients; only 4 (Clq-BA) and 2 (Raji cell) sera, respectively, showed elevated levels.

DISCUSSION

In lung cancer investigations only a few reports (*23–38*) have focused on the evaluation of CMI reactions, measuring isolated in vitro tests, suggesting depression of CMI in these patients; furthermore, very little is known about cell-mediated effector mechanisms (CMI) and their possible correlation with staging and clinical course. We have performed a research protocol in 28 untreated patients with lung cancer and have examined simultaneously their capacity to recognize alloantigens, to generate killer T-cells, and to express CML activity against the sensitizing blasts. In addition, we have explored their capacity to mediate cell cytotoxicity against an antibody-sensitized target and have investigated the possible influence of autologous serum factors on the proliferative response of patients and controls. The results were correlated further with the histologic type and stage of the disease.

Proliferative responses to alloantigens have been found to be diminished in several types of cancer (*39–41*). However, our results, with the use of precultured cells, showed an intact response to alloantigens. This step may allow the shedding of probable serum factors that could interfere by blocking membrane receptors with the in vitro response pattern of

TABLE 5.—*ADCC function in lung cancer patients*

Group	No. of patients, n=20	ADCC capacity[a]			
		Normal, 9.1×10^4–11×10^5	Depressed, $K>11\times10^5$	Enhanced, $K<9.1\times10^4$	
Nonepidermoid	10	5	3	2	$P<.005$[b]
Epidermoid	10	3	7	0	

[a] K-value.
[b] Significance expressed as P-value from chi-square contingency analysis.

TABLE 6.—*Effector responses of patients who died during the period of investigation*[a]

No. of patients, n=13	Altered mechanism[b]
4/13	↓ CML
4/13	↓ ADCC
3/13	↓ CML–ADCC
11/13[c]	↓ CML or ADCC

[a] Histologic type (9 epidermoid carcinomas and 4 adenocarcinomas).
[b] ↓=depressed cytotoxic response.
[c] Total No. of deceased patients with alteration of cytotoxic mechanisms (CML or ADCC).

fresh cells. Our observations in systemic lupus erythematosus (*42*), gastric cancer (*43*), and in paracoccidioidomycosis (*44*) tend to confirm such a possibility. The allogeneic response found among the patient group may represent a normal ability of the cells of advanced lung cancer patients (25/28 patients were in stage III) to recognize and proliferate when confronted with new antigenic specificities.

When T-cell-mediated lympholysis (CML) against the sensitizing lymphoblasts was investigated, we found a significant decrease in the patient group (54%) when compared with that in the controls. Even though we do not know the nature of this abnormality, the fact that some of the patients did show intact CML capability may suggest that in advanced lung cancer a defect of effector T-cells is prevalent, probably unrelated to either the recognition or the proliferating phases of T-cell-mediated immune response to alloantigens. Perhaps the defect may reside at the level of the actual cytotoxic process and/or one or more suppressor mechanisms may block the direct effect of the effector cell.

In relation to the ADCC assay utilized in the present study, an appropriate phase of standardization was accomplished in 50 normal donors (*43*). This in turn allowed us to calculate K-values in a normal range and to define whether a given individual needed more (low capacity) or fewer (high capacity) effector cells to lyse 50% of the erythrocyte suspension. Our results showed a prevalence of altered ADCC response in lung cancer; 10 of 20 patients had a decrease in ADCC response (low capacity), and 2 patients had an enhanced ADCC (high capacity). The finding of a bimodal expression of altered cytotoxic capability agrees with the previous report by Blanca et al. (*43*) on gastric cancer. The role of ADCC in the response of cancer patients to tumor cells is not understood completely; monocytic infiltration of solid tumors has long been recognized (*45*); furthermore, the probable nature of the effector cell against IgG-sensitized ORh (D^+) red blood cells seems to be the monocyte (*46, 47*).

The influence of patients' autologous serum on proliferative responses of patient or control lymphocytes against alloantigens was remarkable. All se tested showed either an inhibitory or an enhancing

effect on both groups (bimodal effect). Furthermore, the inhibitory sera depressed a normal MLC response to normal levels (RPI=<0.66) in 12 of 13 patients and in 7 of 10 controls, whereas the enhancing sera facilitated the response within the normal range. The possible consequences of this bimodal influence are unknown; blocking serum activity has been observed in mitogen-induced transformation (*48*), mixed leukocyte culture (*49*), cell-mediated cytotoxicity (*50*), the leukocyte migration technique (*51*), and antigen-stimulated lymphocyte blastogenesis (*52*). The nature of this inhibitory or blocking activity also is unknown; however, free antigen or antibody or else the presence of CIC could be participating (*39*). In our study, only 4 (C1q-BA) and 2 cases (Raji cells) showed elevated CIC levels; since the majority of the patients were in stage III of their disease, the low incidence of elevated CIC levels may be associated with tumor-bound complexes. The enhancing effect on MLC reactivity induced by the cancer sera on patient or control lymphocytes was reported previously from our laboratory (*43*) in gastric cancer. Its possible nature and significance remain to be determined.

Previous investigations on the possible relationship between CMI status and type of lung cancer have been contradictory (*53*). In our study the correlation of the in vitro cell-mediated cytotoxic mechanisms with the histologic type showed a marked tendency for altered T-cell and ADCC effector functions in the epidermoid type. Furthermore, 13 of 28 patients died during the first 13 months of observation; among them 11 had either depressed CML or ADCC responses. All the epidermoid carcinoma patients (9 cases) showed alteration in these mechanisms.

These findings may offer new and concrete evidence that a severe failure of the cell cytotoxic capabilities is prevalent in advanced lung cancer; this in turn seems to be associated with a severe clinical course, poor prognosis, and the histologic type (epidermoid carcinoma). Further investigations should allow us to delineate the possible mechanisms of the failure of CML and ADCC, as well as to be able to evaluate the clinical usefulness of this integrated immunologic workup in early stages and follow-up of lung cancer patients.

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