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Role of T-Lymphocytes in Production of a Cancer-associated Protein Factor in Serum from Lung Cancer Patients

HANS KR. KOTLAR*† and TORE SANNER‡

Laboratory for Environmental and Occupational Cancer, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

Abstract—*Oligoclonal T-cells have been generated by sensitization of peripheral blood mononuclear cells from lung cancer patients to a lung cancer tumor-associated antigen (TAA). A factor similar to the antigen-specific glycoprotein factor in the serum of these cancer patients was found in the supernatant of the oligoclonal T-cells.*

The factor from the T-cell supernatant had specificity for lung cancer TAA and induced stimulation of normal lymphocytes of the CD8 phenotype when mixed with lung cancer TAA. Furthermore, the factor blocked the ability of lymphocytes from lung cancer patients to recognize lung cancer TAA.

Both the factor from lung cancer serum and from the oligoclonal T-cells were absorbed on a lung cancer-associated antigen-coupled immunosorbent column. On FPLC-gel filtration the desorbed fractions from the immunosorbent column from both sources showed activity in the same molecular weight range, 70–90 kD. Heteroantisera raised against the factor from serum and against the factor from the oligoclonal T-cell supernatant bound about the same portion of lymphocytes from lung cancer patients as measured by immunofluorescence, while only a minor fraction of cells from patients with unrelated cancers and from healthy persons were labelled on incubation with the antisera.

These results support the hypothesis that an antigen-specific factor found in serum of cancer patients is produced by antigen-stimulated T-cells, possibly of the CD8 phenotype. This putative antigen-specific suppressor factor and the tumor antigen-reactive lymphocytes of the patient seem to share similar idiotopes.

INTRODUCTION

ALTHOUGH promising results for early detection of cancer have been obtained with tests assumed to measure cellular and humoral immunity [1, 2], such tests have gained little application. This may be due to several factors. The tests are relatively complicated to perform, and seem to be influenced by factors which are not yet under control. Thus, one laboratory may have difficulties in reproducing the results of another laboratory. Furthermore, the immunological reactions underlying the response of the tests are not understood and it is thus difficult to make intelligent approaches to develop simpler and more reproducible procedures.

The leukocyte adherence inhibition (LAI) test, developed by Halliday and Miller [3], is assumed to give a measure of cellular immunity [4]. Since the test requires lymphocytes from the person under study, the test will probably never gain general use due to logistic problems. Some years ago the humoral leukocyte adherence inhibition (H-LAI) test was developed in our laboratory [5, 6]. Serum for use in the H-LAI assay can be kept frozen for many years, and less than 1 μl is necessary for performing an assay. Trypsin-treated leukocytes from healthy persons are used as indicator cells in the test. The indicator cells may be cryo-preserved and used for repeated assays. Promising results have been obtained in the cases of patients with cancer of the lung, breast, ovaries and oral cavity [5–10]. The results obtained with this test have been confirmed by other groups [11–14]. The events

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*Fellow of the Norwegian Cancer Society.
†Present address: Bionar A/S, Strømsdalsjordet 4, N-3700 Skien, Norway.
‡To whom requests for reprints should be addressed at: Laboratory for Environmental and Occupational Cancer, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo 3, Norway.

involved in the specific triggering of the response are, however, not known and non-specific stimulatory factors may from time to time cause difficulties in performing the analysis.

The serum factor involved in the H-LAI response is specific for different types of cancer. The lung cancer factor has been found in serum even in the preneoplastic state of the disease [7]. The molecular properties of the factors seem to be the same for cancer at different sites. The factor has been isolated from serum of lung cancer patients and found to be a glycoprotein with molecular weight of 70–80 kD and pI = 4.5 [15].

Studies on the mechanisms involved in the H-LAI reaction have shown that the response is dependent on T8-cells, both as effector and responder cells [16]. In earlier studies on the cellular LAI (C-LAI) reaction evidence has also been obtained that T8-cells play an important role [17]. Moreover, in studies where we have compared the response obtained in the C-LAI and H-LAI test systems, the responses obtained have been found to be very similar. This suggests a relation between the C-LAI and H-LAI test systems.

The purpose of this investigation has been to study further the mechanisms of the LAI tests and especially the role of T-lymphocytes in the formation of the serum factor responsible for the H-LAI reaction.

MATERIALS AND METHODS

Collection of serum

Serum from patients with squamous cell carcinomas of the lung was obtained from blood samples drawn the day after admittance to the Norwegian Radium Hospital. The patients were untreated prior to hospitalization.

Cancer antigens

Potassium chloride (3.5 M) extracts from the cell line Calu-1 and MCF-7 were used as lung and breast cancer-associated antigen, respectively. The Calu-1 cell line was established from a squamous cell carcinoma of the lung by Dr J. Fogh, Sloan-Kettering Institute, New York, NY. The MCF-7 cell line was derived from a pleural effusion of a patient with breast cancer and was provided by Dr M. Rich, Michigan Cancer Foundation, Detroit, MI.

H-LAI assay

Indicator cells. Blood from normal healthy persons was purchased from Røde Kors Blodsenter (Oslo, Norway). The mononuclear cells were separated according to the method of Bøyum [18]. The cells were washed and treated with 0.025% trypsin as previously described [5, 6]. The trypsin-treated indicator cells were cryopreserved and thawed before use in the H-LAI assay [9].

H-LAI. Details of the procedure have been published elsewhere [5, 6]. In brief, serum (0.5 μl) or supernatant from T-cell cultures (50 μl, diluted 1:5 with Eagle's minimum essential medium (EMEM) (Gibco, Paisley, U.K.) and antigen (5 μg protein) were incubated in a total volume of 150 μl EMEM at 4°C for 1 h. Indicator cells (10^6 cells) were added to the above mixture and incubated for 30 min at 37°C. Aliquots of the cell suspension were subsequently transferred to hemocytometers and incubated for another hour at 37°C. At the end of the incubation, the cells were counted in nine predetermined squares on each side of the hemocytometer. The coverglass was removed, the surface gently rinsed to remove non-adherent cells and the same squares were recounted. Each test was performed in duplicate. The response of the test is expressed by the LAI index:

$$\frac{A_a - A_p}{A_a} \times 100$$

where A_a and A_p represent the percentage of adherent cells in the absence and presence of antigen, respectively. Altogether between 4000 and 5000 cells were counted by the use of an image analyzer. On the basis of previous experience, a LAI index greater than 10 was considered a positive test [5–10].

C-LAI assay

The procedure used in the C-LAI assay is similar to that of the H-LAI assay except that the cancer patients' own mononuclear cells are stimulated directly against the tumor-associated antigen. The procedure has been published elsewhere [5]. In brief, the cancer patients' lymphocytes (0.05 ml: 10^6 cells), EMEM (0.10 ml) and an antigen extract (0.05 ml: 10 μg non-hemoglobulin protein) were mixed and incubated at 37°C for 30 min in an atmosphere of 5% CO_2 in air. From this point the procedure is identical to that of the H-LAI assay.

C-LAI blocking assay

The procedure was performed as above for the C-LAI assay, except that serum from a cancer patient (0.05 ml, diluted 1:100 in EMEM) or supernatant from the oligoclonal T-cells (0.05 ml, diluted 1:5 in EMEM) was incubated in a total volume of 200 μl mixture of antigen and mononuclear cells.

T-Cell supernatants

Supernatants were collected from oligoclonal T-cells cultured according to a modification of the method of Mustafa *et al.* [19]. Peripheral blood

mononuclear cells (PBMC) were prepared from patients with verified lung cancer. 20×10^6 PBMC (2×10^6 cells/ml) in complete medium (RPMI 1640 + 20% FCS + 1% penicillin/streptomycin) were cultured with lung cancer associated antigen (10 μg/10^6 cells) or irradiated (50 Gy). Calu-1 cells (10^5 Calu-1 cells/10^6 PBMC) in 25 cm^2 flasks (Nunc, Roskilde, Denmark). The flasks were incubated at 37°C in an atmosphere of 5% CO_2 in air. On day 6, the cells were washed and recultured with cryopreserved autologous or allogenic irradiated (25 Gy) feeder cells + antigen. On day 9, viable cells were recovered on a Lymphoprep (Nycomed, Oslo, Norway) gradient and recultured with feeder cells + antigen + 25% interleukin 2 (IL-2, Biotest, Frankfurt, F.R.G.). 25% IL-2 alone was added on day 4 after reculturing, and the cycle of adding feeder cells + antigen + IL-2 on day 1 and IL-2 alone on day 4, was repeated every seventh day. Collection of supernatants started after the first cycle.

Supernatants from two different T4-cell clones stimulated against BCG as well as supernatant from a clone of the CD8 phenotype of cells stimulated against PAA were obtained from Dr Mustafa (Department of Pathology, Institute for Cancer Research, Oslo, Norway).

Concanavaline A (Con A) affinity chromatography

A sodium acetate buffer (0.03 M NaAC, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, pH 6.0) was used for equilibration of a Con A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.6 × 30 cm). Lung cancer sera or oligoclonal T-cell supernatants were diluted with buffer (2 vol), and applied to the column. The flow rate was 30 ml/h and 10 ml fractions were collected. The same buffer containing α-methyl-D-mannoside (0.3 M) was used for desorption.

Antigen immunosorbent column

A pooled antigen preparation from Calu-1 cells was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the instructions given by the manufacturer. The column (1.6 × 12 cm) was eluted with a bicarbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0). Lung cancer sera (1 part serum + 3 parts buffer) or supernatant from the T-cell clones (1 part supernatant + 1 part buffer) were administered to the column. A flow rate of 20 ml/h was used. The same buffer with 3 M guanidine–HCl was used for desorption.

FPLC-gel filtration

The Pharmacia FPLC System was used with a Superose 12 M column. Elution was carried out with a 10 mM phosphate buffer containing 0.5 M NaCl (pH 7.0). Dialyzed and concentrated desorbed fractions from the Con A as well as from the immunosorbent column were administered for FPLC. The molecular weight distribution was determined by running the Pharmacia Low Molecular Weight standard under the same conditions.

Immunofluorescence with heteroantisera and monoclonal antibodies

Heteroantisera against the active fraction of serum from lung cancer patients as well as against the active fraction separated from the supernatant of oligoclonal T-cells were raised in rabbits. The antiserum against the supernatant active fraction was designated 179 and the antiserum against the serum active fraction was given number 181.

The monoclonal antibodies OKT3, OKT4 and OKT8 were obtained from Ortho Pharmaceutical Corp. (Raritan, NJ). Sheep antirabbit-IgG–FITC-conjugated antiserum was purchased from Dakopatt (Copenhagen, Denmark). Goat-antimouse-IgG–FITC was obtained from Nordic Immunology (Tilburg, The Netherlands). The leukocytes or oligoclonal T-cells ($0.5–1.0 \times 10^6$) were centrifuged through 3 ml Hank's balanced salt solution (HBSS) (Gibco, Paisley, U.K.) containing 0.2% BSA and 15 mM NaN_3 (HBSS–BSA) and incubated for 30 min at 4°C with monoclonal antibodies or heteroantisera (5 μl OKT3, OKT4 or OKT8; 50 μl 179 or 181 diluted 1:200), all in a total volume of 100 μl HBSS–BSA. The oligoclonal T-cells were centrifuged on a Lymphoprep gradient in order to obtain viable cells prior to analysis. After incubation, the cells were washed twice with HBSS–BSA and subsequently incubated for another 30 min at 4°C in the dark with sheep antirabbit or goat antimouse FITC-conjugated antibodies. The cells were washed twice in HBSS–BSA before counting in a fluorescence microscope.

The manual fluorescence counting was also compared with flow cytophotometric counting on an EPICS cell sorter (Coulter Electronic, Hialeah, FL).

RESULTS

In order to ascertain whether the factors found in serum from cancer patients are synthesized by T-cells, a number of different experiments were carried out. Oligoclonal T-cells were obtained by stimulation of peripheral blood mononuclear cells (PBMC) with tumor-associated antigen extract or irradiated Calu-1 cells, propagated by IL-2 and feeder cells. The true T-cell origin of the cells was verified by immunofluorescence counting giving 87–93% positive response for CD3. The oligoclonal nature was shown by an average of 36% for the CD8 phenotype and 61% for the CD4 phenotype.

Table 1 shows results where supernatants from oligoclonal T-cells from different patients with lung

Table 1. H-LAI reaction with supernatant from oligoclonal T-cells

T-Cell supernatant	H-LAI (index)
Ca pulm patients	
IH	16
SS	34
MH	17
MBH	30
UAS	28
Controls	
Medium	5
$T4_{BCG1}$	2
$T4_{BCG2}$	3
$T8_{PHA}$	4
$PBLs_{ConA}$	2

The H-LAI index was determined with supernatants of oligoclonal T-cells from lung cancer patients stimulated with Calu-1 antigen. The controls represent the medium used as well as supernatants from different T-lymphocyte clones stimulated with BCG, PHA- and ConA-stimulated PBLs.

cancer were tested against a lung cancer tumor-associated antigen in the H-LAI assay. All five supernatants gave response in the H-LAI assay in a similar way as previously found with sera from patients with cancer of the lung. Moreover, the medium itself as well as supernatant from two different T4-cell clones stimulated with BCG and a supernatant from T8-cells stimulated with PHA or supernatant from PBL from a control person stimulated with ConA gave no reaction at all.

The results shown in Tables 2 and 3 were obtained using the C-LAI assay. Table 2 shows the response of leukocytes from five patients with lung cancer and two control persons against lung cancer-associated antigen. Positive C-LAI response were obtained with the lung cancer patients tested, while the two control persons did not respond. When autologous serum were added to the test system, the response of the leukocytes decreased for all five lung cancer patients, while no effect was found when serum from healthy persons were added.

Table 3 shows that the supernatant from oligoclonal cultures of T-cells derived from lung cancer patients blocked the response in a similar way to autologous sera. On the other hand, addition of medium alone had no effect on the response.

The experiments in Tables 1, 2 and 3 give support for the similarity of the factor in the oligoclonal T-cell supernatant and in serum. In order to study the identity of the factors in serum and in the oligoclonal T-cell supernatant further, two experiments were carried out in which the factors were purified.

The results in Fig. 1 show the activity and protein profiles found in FPLC gel filtration of ConA-desorbed fractions from cancer serum and from supernatant of oligoclonal T-cells. The profiles are very similar and, most important, the H-LAI activity which is indicated by the shadowed area appears at the same molecular weight range.

In the second experiment (Fig. 2) serum from a lung cancer patient and oligoclonal T-cell supernatant derived from a patient with lung cancer were passed through a lung cancer-associated antigen-coupled column. The factors desorbed from the column were subjected to FPLC gel filtration. Also in this case similar protein and activity profiles were obtained. Again the LAI activity appeared in the same molecular range.

In the last experiments (Table 4) the binding of antibodies raised against the serum factor from lung cancer patients and against supernatant of oligoclonal T-cell cultures derived from lung cancer patients were analyzed for binding to mononuclear cells from cancer patients. Antiserum was raised against the substances desorbed from the lung cancer-associated column. The extent of binding was similar with antiserum against the serum factor and the oligoclonal T-cell supernatant. Interestingly, the binding was considerably less to cells from a

Table 2. Blocking of the C-LAI response of patients with lung cancer with autologous serum

Group of patients	C-LAI (without serum)	With autologous serum	With normal serum
Ca pulm			
TB	17	8	—
EJT	18	10	21
TA	16	7	13
OGH	28	11	21
OCR	12	9	12
Controls			
AK	2	3	2
GMG	9	10	8

C-LAI was determined with Calu-1 antigen.

Table 3. Blocking of the C-LAI response of patients with lung cancer with supernatants from oligoclonal T-cells

Lymphocytes from	C-LAI	Addition of Supernatant from T-cells	Medium alone
ÅL	23	5	—
MH	21	−1	—
OKS	13	4	13
JLH	37	10	31
KKS	23	10	20

Lymphocytes were obtained from patients with lung cancer and tested with Calu-1 antigen.

patient with breast cancer and cells from two patients with cancer of the rectum. Likewise, the percentage of positive labelling was low in the case of lymphocytes from control persons.

DISCUSSION

The present results show that a factor found in the supernatant of oligoclonal T-cell cultures has similar properties to the serum factor involved in the H-LAI response. Thus, the two factors have the same or similar molecular weight, they give a tumor-specific response in the H-LAI assay and they cause a specific inhibition of the C-LAI response. Moreover, antiserum raised against the two factors seems to have similar specificity in binding to peripheral blood lymphocytes from cancer patients, while binding only to a small fraction of lymphocytes from healthy control persons. On the basis of these results it is suggested that the H-LAI serum factor is synthesized by T-lymphocytes.

Previously we have found that the serum factor responsible for the H-LAI reaction is an antigen-specific glycoprotein of about 70–80 kD [15]. The

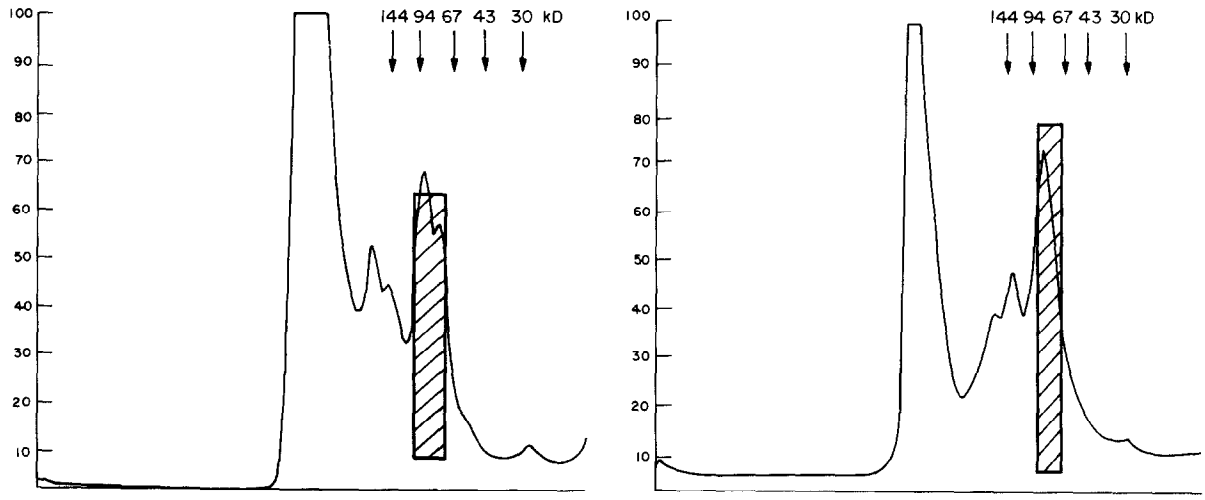

Fig. 1. FPLC gel filtration of ConA-desorbed fraction of serum from a patient with lung cancer (left panel) and supernatant from oligoclonal T-cell cultures (right panel) of a patient with lung cancer. The molecular weights are indicated on the X-axis. The shaded area shows the H-LAI activity.

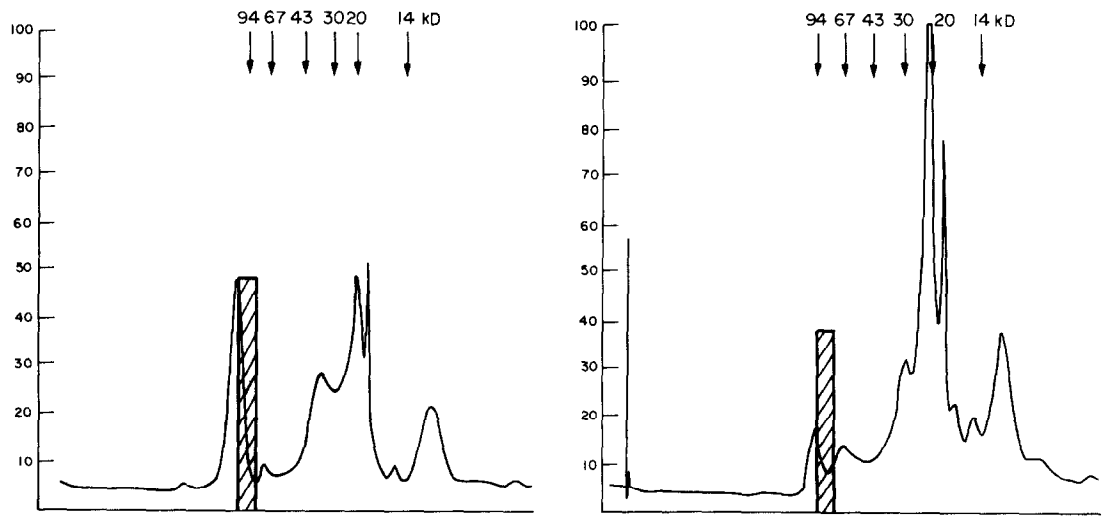

Fig. 2. FPLC gel filtration of desorbed fraction from a lung cancer-associated antigen-coupled column of serum from a patient with lung cancer (left panel) and supernatant from oligoclonal T-cell cultures (right panel) of a patient with lung cancer. The molecular weights are indicated on the X-axis. The shaded area shows the H-LAI activity.

Table 4. Binding of antiserum raised against the H-LAI active fraction of sera from lung cancer patients and a corresponding antiserum raised against the supernatants of oligoclonal T-cells to lymphocytes from cancer patients and controls

Patient	Binding of antiserum against	
	T-Cell supernatant (%)	Lung cancer serum (%)
Ca pulm		
P1	36	25
P2	32	31
P1	47	26
HH	14	28
EJ	30	30
JK	27	34
ON	27	23
OK	29	39
RL	20	23
JHS	26	24
HS	36	37
HSH	24	23
Ca mam		
CB	6	4
Ca recti		
BB	9	7
JK	7	9
Controls		
OK	8	13
PMB	8	9
ORK	5	2
HKK	21	18
WS	6	3
ARH	3	5
AR	6	5
ARI	1	11

factor in the blood of a patient with breast cancer will specifically bind to a tumor-associated cancer antigen from mammary cancer, but will not bind to a lung cancer-associated antigen. Correspondingly, the factor present in serum from a lung cancer patient will bind a lung cancer-associated antigen, but does not affect a breast cancer-associated antigen [20].

The antigen-specific factor appears to be present at an early stage in cancer development as H-LAI responses have been observed several years prior to clinical diagnosis of lung cancer [7]. This suggests that the serum factor is likely to be an immunoregulatory protein. During cancer development, immunoregulatory factors (i.e. the serum factor) specific for organ-specific cancer-associated neoantigens of the particular type of cancer of this person will be produced. In ovarian cancer, the H-LAI serum factor has been found to recognize a defined purified human tumor-associated antigen [8].

The fact that the serum factor and supernatant from the oligoclonal T-cells both will block the C-LAI response (see Tables 2 and 3) and that the supernatant directly will induce a positive H-LAI response (see Table 1) gives valuable information. The protein factor in the sera of the cancer patients seems to have the same specificity for the tumor-associated neoantigens as does the effector cells in the assay. However, equally important is the fact that the protein factor in the oligoclonal T-cell supernatant shares the same specificities against the tumor-associated antigen. This indicates that both the serum factor and the supernatant factor have the same idiotope specificity for the tumor-associated neoantigen as the factor or receptor present on the T-lymphocytes giving the response in the C-LAI. The evidence for shared idiotope specificity is further strengthened by the fact that both types of factor can trigger normal lymphocytes of the CD8 phenotype to give a response in the H-LAI assay.

Further evidence for the identity between the serum factor and the factor produced by oligoclonal T-cells comes from data obtained by gel filtration. Thus, the desorbed fractions of the factor from serum and the T-cell supernatant from a ConA-affinity column as well as from a lung cancer antigen coupled immunosorbent column gave biological activities in the same molecular range when separated on a gel-filtration FPLC system (see Figs. 1 and 2). The finding that the antibodies raised against both factors will label about the same fraction of lymphocytes from lung cancer patients further strengthens the hypothesis that the antigen-specific effector cells of the cancer patient and the possible antigen-specific suppressor factor share similar idiotopes. Tumor-specific idiotopes on suppressor factors and suppressor cells have recently been described by Kuchroo *et al.* [21] using monoclonal anti-idiotope-antibodies in a mouse tumor system.

In early studies on cell-mediated immunity, the group of Hellström addressed the question of blocking factors in migration inhibition studies [22]. Later Halliday *et al.* [23] postulated an MHC-restricted suppressor substance involved in the blocking of the LAI reaction in a mouse tumor system. Other investigators using other mouse model systems reported on antigen-binding molecules from suppresor T-cells with apparent molecular weights of 65,000–70,000 daltons, showing MHC restriction [24, 25]. A corresponding factor in the range of 80,000–90,000 daltons has been isolated from human tonsil cells giving no suppressive activity across the HL-A barrier [26]. In accordance with this information, the results in Table 2 reflect some important findings. In the experiments where autologous serum were added to the system 100% (5/5) of the C-LAI response was blocked (see Table 2). However, only 60% (3/5) of the homologous sera blocked the reaction (data not shown). This could indicate a restriction element

involved in the C-LAI reaction. Furthermore, blocking the indicator cells with monoclonal antibodies against the class I MHC determinants seem to abrogate the H-LAI reaction, while antibodies against class II determinants had little effect (data not shown). Removal of restriction elements could give a possible explanation of why the indicator cells in the H-LAI assay have to undergo a mild trypsinization in order to obtain reproducible results.

Labateya and Thomson [27], using the tube LAI system which measures a cellular response, have found that T8-lymphocytes are involved in the immunological recognition of autologous tumor-associated antigens. Previously it was postulated that all cellular activities in the C-LAI reaction are confined to the Tg^+-subpopulation [28, 29]. Together with the information that there is a CD8 subtype dependency in the H-LAI reaction, these data indicate that the same subclass of T-lymphocytes participates in both the C-LAI and H-LAI reactions.

All data taken together suggest that, while the C-LAI reaction appears to be measuring the factor bound to the T-cells, the H-LAI reaction measures the same factor released to the serum. The hypothesis that the same immunological reaction is involved in both the C-LAI and H-LAI reactions is in accordance with the finding that the responses obtained in studies using C-LAI and H-LAI are very similar.

In conclusion, the serum factor responsible for the H-LAI reaction seems to be produced by T-lymphocytes upon antigen stimulation. It is conceivable that this is an antigen-specific suppressor factor produced by lymphocytes of the CD8 phenotype. Furthermore, the antigen-specific suppressor factor and the tumor antigen-reactive cells of the patient seem to share similar idiotopes. This finding may have an implication for our understanding of cancer immunology and may also be important in the development of immunological tests for early detection of cancer.

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