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Cell-Populations Involved in the Humoral Leukocyte Adherence Inhibition Reaction. The T8-Suppressor/Cytotoxic Cells are Both Effectors and Responders

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Abstract—*In an attempt to identify the cell subpopulation involved in the humoral leukocyte adherence inhibition (H-LAI) reaction, mononuclear leukocytes were fractionated and the response with the different cell populations was measured in the H-LAI assay using sera from lung cancer patients and a lung cancer associated antigen.*

The monocytes were found to exhibit a suppressive effect on the H-LAI response. The B-lymphocytes seemed not to take an active part in the reaction. Depletion of the T-lymphocytes abolished the reaction. Subsequent studies on the T-lymphocyte subpopulations revealed that removal of the T4-cells had little effect while removal of the T8-cells resulted in complete abrogation of the H-LAI response. Direct blocking by monoclonal antibodies against the T4 and T8 surface determinants gave additional support for the T8-cell dependency of the H-LAI assay.

The cells that specifically were induced to loose adherence in the H-LAI assay upon incubation with serum from a lung cancer patient and lung cancer associated antigen were also analyzed. Compared to the total mononuclear leukocyte population, the relative content of monocytes and B-cells were lower in the non-adherent population. An increase was found in the relative number of T-cells. While the relative number of T4-cells decreased, the content of T8-cells was signficantly increased. Furthermore, a direct correlation was found between the loss of adherence of the T8-subpopulation and the index of the H-LAI response.

The present study indicates the T8-subpopulation of the T-lymphocytes to be involved both in the effector and responder side of the H-LAI reaction and gives implications for an immunological reaction behind the response of the H-LAI assay.

INTRODUCTION

TUMOR immunity can be measured *in vitro* by the leukocyte adherence inhibition (LAI) technique [1, 2]. The original cellular LAI (C-LAI) assay is based on the activation of the patients own leukocytes. The non-adherence of the leukocytes to glass depends on immunological recognition of tumor-associated antigens giving rise to the production of lymphokines which reduces the adherence of the leukocytes. Evidence has been obtained in the C-LAI assay that both T-cells and armed macrophages recognize the tumor-associated antigens [3–6]. In some LAI systems there are indications for a MHC restricted response, and the reaction seems to be confined to a specific T-lymphocyte subset [3, 4, 7].

The humoral leukocyte adherence inhibition (H-LAI) assay is thought to be complementary to the original LAI method [8, 9]. The responses of the H-LAI assay are mediated by a soluble serum factor [10]. Complexes formed between the serum factor and tumor-associated antigen trigger non-adherence of trypsinized indicator leukocytes from healthy persons [10, 11]. The serum factor responsible for the reaction is an antigen specific glycoprotein of about 70–80 kd [12]. This factor appears to be present at an early stage in cancer development as H-LAI response has been observed several years prior to clinical diagnosis of lung cancer [13]. In ovarian cancer, the H-LAI serum factor has been found to recognize a defined purified human tumor-associated antigen [14].

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Information on the various cell populations participating in an immunological test is of importance for the understanding of the mechanisms involved. The purpose of the present study on the H-LAI test has been to identify the reactive subpopulations of the total mononuclear leukocyte population, the indicator cells, taking part in the H-LAI reaction. Furthermore, it was studied whether there are differences in the cells triggered by the tumor-associated antigen—serum factor complex, the effector cells, and the cells that actually lose adherence in the assay, the responder cells.

MATERIALS AND METHODS

Collection of serum

Sera from lung cancer patients were obtained from blood samples drawn 1 day after admittance to the Norwegian Radium Hospital.

H-LAI analyses

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 [15]. The cells were washed and treated with 0.025% trypsin as previously described [8, 9]. The trypsin-treated indicator cells were cryopreserved and thawed before use in the H-LAI assay [16].

Cancer antigens. Potassium chloride (3.5 M) extracts from the cell line Calu-1 were used as lung cancer antigen [8].

H-LAI assay. Details of the procedure have been published elsewhere [8, 9]. In brief, serum (0.5 μl) and antigen (5 μg protein) were incubated in a total volume of 150 μl Eagle's minimum essential medium (EMEM) (Gibco, Paisley, Scotland) at 4° C for 1 hr. Indicator cells (10^6 cells), depleted or enriched for various cell subpopulations, 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. All together 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 [8, 9].

Depletion or enrichment of cell subpopulations

Depletion of monocytes/macrophages by monolayer cultures. The monocytes were depleted according to the method of Paul [17]. The trypsinized indicator cells (7.5×10^5 cells/cm^2) were incubated for 30 min at 37° C in tissue culture bottles with balanced salt solution and EDTA (BSSE) containing 25% fetal calf serum (FCS) (Gibco, Paisley, Scotland). The non-adherent cells were removed and washed with Hank's balanced salt solution (HBSS) (Gibco, Paisley, Scotland) and EMEM.

T-cell and B-cell enrichment by sheep red blood cells (SRBC)-rosetting technique. Sheep red blood cells were treated with 2-amino-ethyl-isothiouroniumbromide (AET) (Sigma, St. Louis, MO) according to the method of Pellegrino *et al.* [18]. The leukocytes were suspended in AET-treated SRBC in EMEM (4.0×10^6 cells/ml 1.5% SRBC suspension), incubated for 5 min at 0° C and centrifuged (10 min/185 ***g***). The pellets were incubated for 1 hr at 0° C and carefully resuspended and gradient centrifuged on Lymphoprep (25 min/600 ***g***) (Nyco, Oslo, Norway). Both the SRBC-T-cell-rosettes and the un-rosetted B-cells were collected. The sheep erythrocytes were hemolyzed with NH_4Cl-Tris buffer and both T-cell enriched and B-cell enriched populations were washed with HBSS and EMEM.

Depletion of lymphocyte subpopulations by complement mediated cytotoxicity. The cells (1.0×10^6 cells/ml) were incubated for 1 hr with monoclonal antibodies or heteroantiserum at room temperature (2 μg of OKT3, OKT4 or OKT8 and 20 μg of rabbit antihuman F(ab)-IgG) [19]. OKT3, OKT4 and OKT8 were obtained from Ortho Pharmaceutical Corp. (Raritan, NJ), and antihuman F(ab)-IgG from Nordic Immunological Laboratories (Tilburg, The Netherlands). The cells were subsequently washed in HBSS containing 0.2% BSA and resuspended to the original concentration in EMEM containing 20% FCS. Low toxicity rabbit complement (Cedarlane, Ontario, Canada) was added (1 : 5 cell-suspension and complement solution) and the mixture was incubated for 1 hr at 37° C. The viability was controlled by trypan blue exclusion. Viable cells were separated from dead cells by centrifugation on Lymphoprep (1.100 ***g***/15 min). The viable cells were finally washed with HBSS and EMEM.

Blocking of lymphocyte function with monoclonal antibodies

Monoclonal antibodies were added directly to the preincubation mixture in the H-LAI assay. Increasing amount of T4 and T8 were added. T4 and T8 was obtained from Coulter Clone (Hialeah, FL).

Characterization of lymphocyte subpopulations

SRBC-rosetting. The SRBC procedure used for leukocyte fractionation described above was also used for measurement of the amount of T- and B-cells in the leukocyte population. Cells with three or more SRBC attached to the surface were counted as T-cells.

Immunobeads. Cell suspension (100 μl containing 10^7 cells/ml) and reconstituted Immunobeads (BioRad, Richmond, CA) (50 μg) were incubated for minimum 1 hr at 4° C or overnight at room temperature. The suspension was diluted to a total volume of 350 μl in PBS before counting of rosettes. Cells with three or more beads attached to the surface were counted as B-lymphocytes and cells which had phagocytized beads, after overnight incubation, were counted as monocytes/macrophages.

Immunofluorescense with monoclonal antibodies and heteroantisera. The monoclonal antibodies OKT3, OKT4 and OKT8 were obtained from Ortho Pharmaceutical Corp. (Raritan, NJ) and 1D5, a monoclonal antibody against a monocyte-macrophage marker, was provided by Dr. G. Gaudernack (Rikshospitalet, Oslo). Rabbit antihuman F(ab)-FITC-conjugated antiserum was purchased from Dakopatt (Copenhagen, Denmark).

The leukocytes ($0.5–1.0 \times 10^6$) were centrifuged through 3 ml HBSS containing 0.2% BSA and 15 mM NaN_3) (HBSS-BSA) and incubated for 30 min at 4° C with monoclonal antibodies or heteroantiserum (5 μg OKT3, OKT4 or OKT8; 50 μl FITC-conjugated rabbit antihuman F(ab); 50 μl ID5 diluted 1 : 150 from acites), all in a total volume of 100 μl HBSS-BSA according to the manufactor's instruction. After incubation the cells were washed twice with HBSS-BSA. Cells treated with unconjugated antibodies were subsequently incubated for 30 min at 4° C in the dark with goat-antimouse–IgG-FITC (Nordic Immunology, Tilburg, The Netherlands). The cells were washed twice in HBSS-BSA before counting in a fluorescense microscope.

The manual fluorescense counting was also compared with flow cytophotometric counting on an EPICS cell sorter (Coulter Electronics, Hialeah, FL). The scores for the cell sorter were usually about 3% higher when compared to the manual counting.

RESULTS

In order to identify the cell population involved in the H-LAI reaction, the response obtained with different leukocyte cell subpopulations was determined with serum from lung cancer patients and a lung cancer associated antigen. The responses were compared with the results of the total leukocyte population, the indicator cells. In the first experiments the indicator leukocytes were separated into a T-cell and a B-cell enriched fraction by the use of the SRBC-rosetting technique. A significantly higher LAI index was obtained with the T-cell enriched fractions than with the B-cell enriched fractions. When the fractions were remixed at various proportions, a nearly linear response relationship was obtained between the H-LAI index and the T-cell content of the indicator cell population (Fig 1).

In the subsequent experiments, techniques like immunosorbent columns, panning and depletion by complement-mediated cytotoxicity and monoclonal antibodies were used for purification of subpopulations of lymphocytes. A technical problem which had to be resolved, was that the purification procedure should not affect the function—or preactivate the indicator lymphocytes. This was controlled by comparing the adherence of the subpopulation relative to that of the unfractionated cells, in the absence of antigen. Populations with a relative loss of more than 10% in adherence were discharged. The viability of the used cells was always greater than 90%. Complement-mediated cytotoxicity with subpopulation specific monoclonal antibodies was found to be the most reliable method.

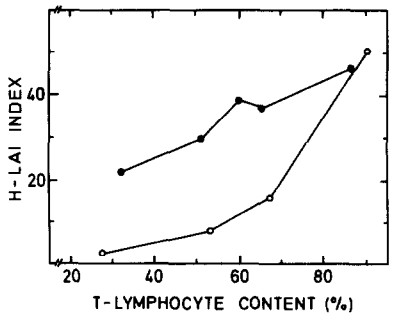

Fig. 1. H-LAI index as a function of the relative content of T-lymphocytes. The T-lymphocytes were removed from the indicator cell population by SRBC-rosette formation and mixed back to the indicator cells of the same donor at various proportions. Results from two different experiments are presented. The T-lymphocyte content was calculated from the scores obtained by Immunobeads. The H-LAI indices were assayed with serum from a lung cancer patient and Calu-1 antigen.

An enhancement in the H-LAI activity occurred when the macrophages/monocytes (MØ) were removed. The LAI index increased by a factor of two when the percentage of cells binding the ID5 monoclonal antibody (marker for MØ) decreased from 8 to 2% (Table 1). Depletion of non-target cells will always give an increase in the reaction index, but the enhancement was greater than could be accounted for by removal of monocytes alone.

In the second experiment shown in Table 1, B-lymphocytes were depleted by the use of anti-Fab-antibodies and complement. While the binding of the F200 marker for B-cells decreased from 23 to 3%, the LAI index increased from 30 to 51. Concurrently, the ID5 marker for macrophages/monocytes decreased from 16 to 1%. The observed enhanced effect of the H-LAI index may thus be caused by the effect of simultaneous removal of the monocytes. The results suggest that B-cells are of little importance in the H-LAI assay.

The third experiment clearly shows an obligate dependency of T-lymphocytes in the H-LAI assay. When T-lymphocytes were depleted by complement-mediated cytotoxicity with the anti-T-cell monoclonal antibody (OKT3), the LAI response was reduced to about 10% of that obtained with the unfractionated cells. When corresponding techniques were used to remove T4 (OKT4) and T8 (OKT8) cells, it was found that removal of the T4-cells did not affect the H-LAI index, while removal of the T8-cells reduced the response by more than 90%.

The differences in the influence on the H-LAI reaction of the two subsets of T-lymphocytes were further studied by direct blocking of the cells by addition of monoclonal antibodies to the T4- and T8-surface determinants into the reaction mixture. Addition of anti-T4 had no effect on the LAI index, while anti-T8 blocked the reaction (Fig. 2). These results show that the T8-cells are essential in the H-LAI assay.

In order to determine which type of cells will lose adherence upon reaction with the serum factor and antigen complex, experiments with more than 200 hemocytometers were conducted. The cells that specifically were induced to lose adherence were collected from the rinsing solution by centrifugation and analyzed. Table 2 shows the individual results from four different experiments. The same trend in the response pattern was obtained in all cases. This is summarized in Fig. 3. It is apparent that while the relative contents of B-cells and monocytes were lower in the non-adherent population a small increase occurred in the relative number of T-cells. This increase could be accounted for by an increase of T8-cells. Thus, while the relative number of T4-cells decreased by about 20%, the relative content of T8-cells was doubled and significantly increased ($P = 0.03$) (Student t-test).

Table 1. Cell populations involved in the H-LAI responses

Leukocyte population removed	H-LAI index	Characterization of subpopulations
Experiment 1		
Total population	32	OKT3: 71%, F200: 18%, ID5: 8%
monocytes	60	82% 2%
Experiment 2		
Total populations	30	OKT3: 47%, F200: 23%, ID5: 16%
B-lymphocytes	51	82% 3% 1%
Experiment 3		
Total population	23	OKT3: 79%, F200: 11%, ID5: 8%
T-lymphocytes	2	*
Experiment 4		
Total population	13	OKT4: 48%
T4-lymphocytes	15	*
Experiment 5		
Total population	13	OKT8: 28%
T8-lymphocytes	1	*

The H-LAI index was determined with sera from lung cancer patients and Calu-1 antigen. The monocytes/macrophages were removed by plastic adherence. B-lymphocytes were destroyed by anti-Fab antibodies and complement. The T-lymphocytes and its T4 and T8 subclasses were depleted by complement mediated cytotoxicity using the monoclonal antibodies OKT3, OKT4 and OKT8, respectively.

*The effect of complement mediated cytotoxicity was examined by trypan blue incorporation. Generally more than 90% of the cell subpopulation was found dead.

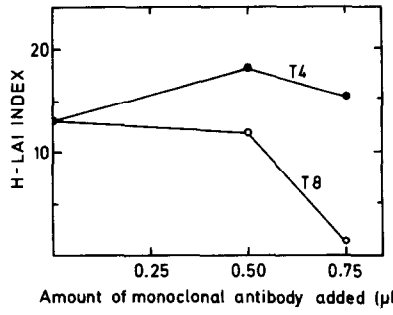

Fig. 2. H-LAI index as a function of added T4 and T8 monoclonal antibodies. The H-LAI response was determined with serum from a lung cancer patient and Calu-1 antigen.

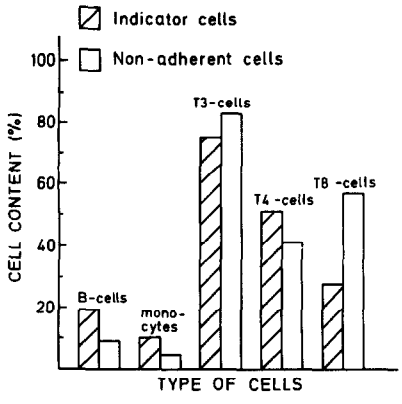

Fig. 3. Cell types in the total indicator cell population and in the population of non-adherent cells. The data is based on the results in Table 2.

In subsequent experiments it was studied whether there was any relation between the H-LAI response and the relative number of T8-cells in the indicator cell population or to the T8-cell content of the non-adherent cell population. In the experiments shown in Fig. 4, indicator cells from different donors were used. No direct relation was observed between the total T-cell content or the content of T8- and T4-subpopulations and the H-LAI index. The H-LAI reaction was, however, found to be directly correlated to the relative fraction of T8-cells that were induced to lose adherence. These results suggest that the T8-lymphocyte subpopulation is both the effector cells and the main responder cells in the H-LAI assay.

DISCUSSION

The present results show that depletion of T-lymphocytes from the indicator cell population of the H-LAI assay nearly abolished the response. In studies on subpopulations of the T-cells it was found that depletion of T4-lymphocytes had little effect, while the T8-cells seemed to be essential in the assay.

Depletion of monocytes from the indicator cell population resulted in an enhanced H-LAI reaction. The enhancement was larger than could be accounted for by the removal of the monocytes alone. While Thomson and Grosser [6] have found the monocytes to be the effector cells in the tube-LAI assay, Morizane and Sjøgren [5] give evidence for both an early T-cell dependent response and a delayed, more unspecific, response of the monocytes in a radioimmune microplate version of the LAI assay. Suppressor effects of monocytes have also been reported by Fritze *et al.* [20] in the C-LAI assay. Similar enhancement of response upon removal of monocytes has been observed in several other immunological systems [21, 22].

Removal of about 90% of the B-lymphocytes also gave an enhancement of the reaction. This enhancement could be due to the concomitant removal of monocytes. Experiments with purified B-cell populations did not give activity in the H-LAI test (data not shown). It can not be excluded, however, that monocytes and B-lymphocytes are unimportant in the H-LAI reaction, as the lower limit of cells that can be discriminated, with the techniques used, is about 1%. Reports have been published claiming that as little as 0.2% monocytes

Table 2. Identification of cellpopulations induced to loose adherence in the H-LAI assay

Experiment	H-LAI index	Indicator cells Subpopulation (%)					Non-adherent cells Subpopulation (%)				
		ID5	F200	OKT3	OKT4	OKT8	ID5	F200	OKT3	OKT4	OKT8
1	39	3	14	74	54	40	—	—	80	—	70
2	27	12	14	79	50	45	5	—	86	35	69
3	19	11	21	75	55	25	14	9	84	50	46
4	29	14	26	67	51	17	—	20	81	41	45

The H-LAI index was determined with serum from a lung cancer patient and Calu-1 antigen. ID5: marker for monocytes, F200: marker for B-lymphocytes, OKT3: marker for T-lymphocytes, OKT4: marker for T-helper/inducer lymphocytes, OKT8: marker for T-suppressor/cytotoxic lymphocytes. The subpopulations have been determined both by cell sorting and manual immunofluorescense.

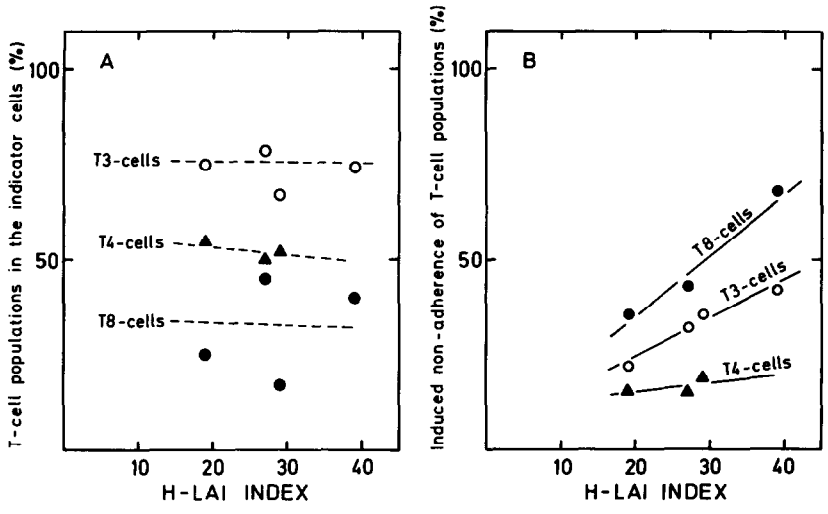

Fig. 4. Influence on the H-LAI index of the content of different T-cells in the indicator cells and in the population of non-adherent cells. Panel A gives the relation between the subpopulation content in the indicator cells and the index in the different experiments. Panel B gives the H-LAI index as a function of specific loss of adherence of the T-lymphocyte subpopulations. The percentage of induced non-adherence is calculated from the particular subpopulation content of the indicator cells in conjunction with the corresponding content of the non-adherent cells and the actual percent of non-adherent cells in each experiment. Cells from 4 different donors were analyzed. The cell types have been determined by immunofluorescense.

are necessary for the antigen-presenting cell function [23]. In some systems the B-lymphocytes can also serve as antigen-presenting cells [24]. Although removal of the T4-cells did not affect the response, it cannot be excluded that the T4-cells are without importance in the H-LAI reaction, since some cells (1–2%) with the T4 marker will remain after depletion by cytotoxicity. Nonetheless, depletion of T8-cells in the same manner had a profound impact on the response.

Antibodies against the T3 surface determinants are found to inhibit several T-lymphocyte dependent functions *in vitro* [25–28]. In studies where these antibodies were used in blocking experiments by direct addition to the H-LAI reaction mixture, anti-T3 was found to induce direct loss of adherence of the T-lymphocytes (data not shown). Thus, blocking with anti-T3 could not give further information except for the important fact that stimulation of T-lymphocytes through the T3 receptor-complex give rise to inhibition of adherence. Monoclonal antibodies against the T4 and T8 determinants can also interfere with certain lymphocyte functions [29, 30], but blocking experiments of this type can be difficult to evaluate [25]. Nonetheless, blocking of the T4 and T8 surface determinants gave results complementary to those achieved by the depletion experiments (see Fig. 2). This gives further evidence to the finding that the T8 subclass is essential in the H-LAI assay.

The results in Fig. 1 indicated that the H-LAI index increases with the total number of T-lymphocytes within indicator cells from the same donor in a system with fixed serum factor and antigen concentrations. Although it is conceivable that this increase might be caused by the increase in the number of T8-cells, the information in this study is not sufficient to conclude whether the quantitative value of the index will depend directly on the T8-cell content of the indicator population or not (see Fig. 4). However, there was a positive correlation ($r = 0.98$) between the loss of adherence of the T8-subpopulation and the index. Thus, the H-LAI index will depend both on the serum factor and on the ability of the T8-cells to respond.

The T8 surface marker is associated with T-lymphocytes with cytotoxic and suppressor functions, while the T4 glycoprotein is supposed to be a general surface marker for helper/inducer cells. Recently, it has been found that both cells with the T8 and T4 marker are functionally heterogeneous [31, 32]. The implication of these observations are that T8 and T4 antigens are markers for the specificity of T-cell subsets for different accessory cells instead of being functional markers. More recently, however, there has been evidence that these determinants possible have a functional relevance [33].

Cells with a surface receptor for the Fc-portion of IgM (Tg^+-cells) and cells with surface receptors for the Fc-portion of IgG (Tg^+-cells), have also been attributed to subset of cells with helper and suppressor functions, respectively [34]. Whether these markers represent better functional markers for the helper and suppressor function is not known [35]. Data from Heijnen *et al.* [36] indicate a functional relationship between suppressor effector cells and Tg^+-cells. Possibly, the Tg^+-lymphocytes represent a cell type that is present in immunized individuals and that will react directly with specific

antigens. Imboden and Stobo [37] have recently showed that subfractions of pre-stimulated T-lymphocytes could react directly on second challenge of a soluble antigen and, also, stimulated T8-cells have been shown to interact directly with antigen [35, 38].

The present experiments indicate the T8 subpopulation of the T-lymphocyte to be involved both in the effector and the responder side of the H-LAI reaction. Previously it has been found that all cellular activities in the original hemocytometer LAI reaction are confined to the Tg^+-subpopulation [3, 4]. Within the tube LAI system the T8-lymphocytes seem to be involved in the immunological recognition of autologous tumor-associated antigen [39]. Together with the information of a T8 subtype dependency of the H-LAI reaction, these data, could be indications for both the C-LAI and the H-LAI reaction to be attributed to the same subclass of T-lymphocytes.

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