Assessment of the Mechanism of the Leukocyte Adherence Inhibition Test

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Abstract

This study was designed to elucidate the mechanism of the leukocyte adherence inhibition (LAI) test in man. To identify the reactive cell types, enriched leukocyte populations (dextran-separated leukocytes and Hypaque-Ficoll isolated mononuclear cells and neutrophils, as well as rosette-isolated B- and T-lymphocytes) were tested for leukocyte adherence in the absence of serum to tumor-specific antigens. LAI reactivity was not restricted to any of the enriched populations, suggesting the involvement of multiple cell types. Attempts to demonstrate soluble lymphocyte factors in the LAI mechanism have been uniformly negative. In contrast, factors in serum of immune donors were able to arm naive cells to be specifically responsive. This suggests a role for serum factors in the mechanism of LAI reactivity and partially explains the participation of multiple cell types in the responses observed. In additional studies, we could not document a correlation between the magnitude of the dermal test (delayed cutaneous hypersensitivity) and the magnitude of the LAI response in patients with squamous cell carcinoma of the head and neck. In 34 of 54 of these patients, there was agreement between the two tests (both positive, 27 of 54; both negative, 7 of 54). In the remaining 20 patients, the dermal test was >5 mm while the LAI test was negative (<30% inhibition).

Introduction

The LAI test, developed by Halliday and Miller (5) and modified by others (1, 6, 7, 9, 12, 13), apparently measures specific immune responses to tumor extracts in humans. However, there is considerable disagreement among several laboratories concerning the mechanism of the LAI test. Maluish and Halliday (10), Powell et al. (12), and Holt et al. (8) suggest that the LAI test is mediated by a soluble T-cell factor and that the indicator cells include lymphocytes and/or neutrophils. Grosser et al. (4) and others (3, 6, 11) contend that T-cell factors are not involved and that LAI reactivity is mediated by cytophilic antibody. Additionally, their evidence implicates circulating monocytes as both the antigen-responsive and indicator cells.

Burger et al. (1) have utilized the LAI assay to assess tumor-specific immunity in patients with melanoma, squamous carcinoma, and other types of cancer. In the following report, we describe experiments which support the presence of a serum arming factor and the involvement of a variety of cell types in the LAI reaction. Additionally, we present data which examine the relationship between skin test response and LAI reactivity in squamous cell carcinoma patients.

Materials and Methods

Tumor Extract

Tumor tissues were obtained within 4 hr of excision from melanoma, squamous cell carcinoma, and neuroblastoma patients with histologically proven cancer. Tumor antigen extraction with 3 M KCl has been described in detail elsewhere (1).

Experimental Subjects

Experimental subjects were selected according to LAI responses to melanoma, squamous carcinoma, or neuroblastoma extracts. Patients were tumor-free for at least 3 months after therapeutic intervention and were considered immunocompetent by their ability to respond to one or more common microbial skin test antigens or mitogenic agents in vitro and/or in vivo. Nonimmune donors were healthy adults who had no previous contact with tumor extracts or patients and had negative LAI values to the tumor extracts.

Fractionation of Mononuclear and Polymorphonuclear Cells

Heparinized (10 units of heparin per ml) blood (5 ml) was combined with 1 ml of 6% dextran (M. W. > 250,000) and incubated in an inverted plastic syringe for 30 min at 37°. The cell-rich supernatant fluid was centrifuged at 160 × g for 10 min, resuspended in twice the original volume of RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.), and washed in RPMI 1640 at 160 × g. The washed cells were incubated in 2 ml of cold 0.85% NaCl for 10 min at 4° to lyse erythrocytes. Medium (10 ml) was added to the suspension to reestablish isotonicity, and the cells were centrifuged at 160 × g for 10 min and were resuspended at 5 to 10 × 10⁶ cells/ml.

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E-Rosette Separation Procedure

Fresh SRBC (Prepared Media Inc., Tualatin, Oreg.) were washed 3 times immediately before use and adjusted to a final concentration of 1% (v/v) in RPMI 1640. E-rosettes were prepared by mixing equal volumes of 1% SRBC and mononuclear cells at 5 x 10^6/ml in a 50-ml conical tube as described by Dean et al. (2). The mixture was immediately centrifuged at 200 x g for 5 min and incubated at 4° for 1 hr. After gentle resuspension, the reaction mixture was gently layered over a Ficoll-Hypaque gradient at 4° and centrifuged at 400 x g for 30 min. Both the unrosetted interphase and the rosetted pellet fractions were washed again and resuspended at 5 x 10^6/ml. The rosetted lymphocytes were recovered after incubation in 2 ml of 0.85% NH₄Cl for 10 min at 4° to lyse erythrocytes. Medium (10 ml) was added to the suspension to reestablish isotonicity, and the cells were washed twice at 160 x g for 10 min and resuspended at 5 x 10^6 cells/ml in RPMI 1640.

EAC-Rosette Separation Procedure

EAC-rosettes were prepared by a modification of the procedure described by West and Herberman (14). Sheep erythrocytes were washed 3 times and resuspended at a concentration of 1% (v/v) in RPMI 1640. An equal volume of RPMI 1640 containing trypsin at 10 mg/100 ml was added, and trypsinization was carried out at 37° for 30 min. The trypsinized SRBC were washed 3 times and resuspended at the original volume in PBS. An equal volume of 195 rabbit anti-SRBC at a 1:20 dilution (courtesy of Dr. Gerrie Leslie, Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oreg.) was added, and the mixture was incubated for 30 min at 37°. The erythrocyte antibody mixture was washed twice with PBS at 160 x g for 10 min. After resuspension to the original volume, an equal volume of human AB serum at a concentration of 1% (v/v) in RPMI 1640 was added, and the mixture was incubated with appropriate concentrations of 0.15 ml antigen and medium as described in the preincubation step above. Supernatant fluids of these mixtures were obtained by centrifugation at 200 x g for 10 min. Control supernatants were generated in a similar manner by incubating immune cells without antigen. The supernatants were transferred to plastic tubes containing 0.15 ml recipient cells and further incubated for 20 min at 37°. The incubated mixtures were then treated in the same manner as described in the adherence step above to determine the percentage of LAI of recipient cells.

Leukocyte Adherence Inhibition

The LAI assay used was a variation of the method described by Halliday and Miller (5) and modified by others (1, 6, 7, 9, 12, 13).

Preparation of Donor Cells. LAI was carried out using dextran-sedimented leukocytes and mononuclear and PMN fractions, as well as T- and B-enriched and -depleted fractions as prepared by E- and EAC-rosettes (described above).

Preincubation Step. For each antigen or antigen dilution to be tested, 1.5 to 3.0 x 10^6 cells in 0.15 ml were added to a plastic tube (Falcon 2054) containing 0.15 ml of antigen and 0.3 ml of medium, and the suspension was incubated at 37° for 30 min. As a negative control, 2.5 x 10^6 cells in 0.25 ml were combined with 0.75 ml medium in a plastic tube and incubated under the same conditions.

Adherence Step. The preincubated cell suspensions were transferred with plastic-tipped Eppendorf pipets (Brinkman, Westbury, N. Y.) to wettable plastic tubes (Falcon 3033; Bioquest, Cockeysville, Md.) to test for leukocyte adherence. Four replicates of control cells and 2 replicates of test cells (0.2 ml/tube) were prepared from each preincubated tube. The number of cells in 2 of the control tubes were determined on a Coulter particle counter (Coulter Electronics Inc., Hialeah, Fla.) after residual erythrocytes were eliminated by lysis. This count established the total number of cells before the adherence process began. The total remaining control and test samples were further incubated for 1 to 2 hr at 37° to allow adherence. These samples were then diluted in 10 ml Isoton II (Coulter Diagnostics, Hialeah, Fla.), and the cells were counted as above. The LAI was calculated as follows:

% inhibition
= Nonadherent cells (test - incubated control samples) × 100
Total control cells - nonadherent cells (incubated control)

Inasmuch as LAI values were usually less than 20% in control subjects and in patients with tumors of different histological types, responses of 20 to 30% were considered questionable, and those greater than 30% were considered positive.

Quest for Soluble Mediators of LAI Reactivity

To look for mediators released by immune cells, 0.15 ml immune cells was incubated with appropriate concentrations of 0.15 ml antigen and medium as described in the preincubation step above. Supernatant fluids of these mixtures were obtained by centrifugation at 200 x g for 10 min. Control supernatants were generated in a similar manner by incubating immune cells without antigen. The supernatants were transferred to plastic tubes containing 0.15 ml recipient cells and further incubated for 20 min at 37°. The incubated mixtures were then treated in the same manner as described in the adherence step above to determine the percentage of LAI of recipient cells.

Mixing of Leukocytes from Immune and Nonimmune Donors

Mononuclear cells from immune and nonimmune donors were prepared by Ficoll-Hypaque fractionation as described. After reconstitution of the cells in medium, they were mixed in the following proportions of immune to nonimmune cells: 100:0, 90:10, 75:25, 50:50, 25:75, 10:90, 1:99, and 0:100. Each mixture was independently tested for LAI reactivity.
Evaluation of Serum Factors for Effects on LAI Reactivity

Serum was evaluated for either arming or blocking effects on LAI reactivity using a preincubation step. To assess arming, serum (in a final dilution of 1:2) was incubated with LAI nonreactive cells for 30 min at 37° prior to the adherence step in the standard LAI procedure. The incubation mixture was washed (160 x g for 10 min), and the cells were tested for LAI reactivity as described. To assess blocking, serum (in a final dilution of at least 1:2) was incubated with LAI-responsive cells as described for arming experiments. The cells were washed, and the LAI test was conducted as described above.

Results and Discussion

Cells Involved in the LAI Test. Although tumor-specific LAI reactivity can be demonstrated in both tumor patients and cancer-free immune donors by the modified LAI assay, the nature of the responding/reacting cells involved is still a controversial issue. In the following experiments, enriched cell populations from previously identified immune donors were used to attempt to define the reactive cell types. Responses from 3 donors to melanoma extract were measured using dextran-separated leukocytes and Ficoll-Hypaque-fractionated mononuclear and polymorphonuclear cells. The mean LAI response (±S.E.) of each fraction from at least 4 concomitant determinations is presented in Chart 1. In each cell fraction tested, LAI reactivity to melanoma was retained. However, the Ficoll-Hypaque-separated mononuclear cells elicited higher mean LAI reactivities (p < 0.05 in Donors 1 and 2) than did the dextran-separated leukocytes. Mean responses of the polymorphonuclear fractions, on the other hand, were consistently lower than those of unfractonated leukocytes, but this difference was only significant (p < 0.05) in Donor 1. When mononuclear and polymorphonuclear cells were reconstituted according to their respective percentages after Ficoll-Hypaque fractionation, the LAI reactivity observed in unfractonated leukocytes was restored. In all 3 experiments, the differences in LAI reactivity in the reconstituted and unfractonated cells were not statistically significant. Antigen specificity was retained by all of the cell fractions tested, since no significant responses were detected to the control antigen, a squamous carcinoma extract.

Since the mononuclear cell fractions appeared to produce an enhanced LAI response, we examined the LAI reactivity of subpopulations which had been depleted of T- or B-cells. The results of 1 of 4 such studies using cells from a melanoma-immune donor are presented in Chart 2. Depletion of T-cells by E-rosettes (Bar E) or of B-cells by EAC-rosettes (Bar G) did not reduce or abolish LAI responsiveness. The LAI response was retained in the rosetted B-cell populations (Bar F) and was significantly elevated (p < 0.05) in the E-rosetted T fraction. However, there was no corresponding elevation in LAI response in the T-enriched fraction after B-cells were removed by EAC rosetting (Bar G). These results cannot clearly distinguish whether the increased response by E-rosetted T-cells implicates T-cells as indicator/responder cells in this assay or simply represents artifacts created by the E-rosetting procedure.

Soluble Mediators in the LAI Test. With the observations above that (a) LAI reactivity is observed in all of the cell populations examined and (b) greater than 40% of the cells participated in the glass-adherence inhibition phenomenon (data not shown), it seemed likely that the LAI response involved more than a single type of responder-indicator cell. To determine whether soluble mediators which could activate bystander cells were involved in the reaction, supernatants obtained after preincubating immune cells with the appropriate antigens were assessed for adherence inhibition activity on nonimmune cells. In 5 experiments using supernatants from melanoma-immune cells, little or no change in adherence inhibition of normal cells was observed (Table 1). These data suggested that the LAI...
% LAI reactivity to

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<th>Melanoma extract</th>
<th>Squamous carcinoma extract</th>
<th>Neuroblasticoma extract</th>
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<td></td>
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<td>+ nonimmune cells</td>
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<td>2</td>
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The involvement of many cell types and the participation in the LAI test by 40 to 75% of the total added cells in all of the enriched fractions examined do not support the contention of Marti et al. (11) that the monocyte is the sole reactive cell type. Contrary to reports demonstrating the presence of soluble mediators from immune lymphocytes (8, 10, 12), our experiments even after T- or B-cell depletion. The involvement of many cell types and the participation in the LAI test by 40 to 75% of the total added cells in all of the enriched fractions examined do not support the contention of Marti et al. (11) that the monocyte is the sole reactive cell type. Contrary to reports demonstrating the presence of soluble mediators from immune lymphocytes (8, 10, 12), our experiments
using immune supernatants or direct mixtures of immune with nonimmune cells did not result in detection of mediators of LAI reactivity.

**Serum Factors in the LAI Tests.** Due to the participation of a high percentage of cells of multiple types in the LAI reaction, we investigated the possibility of serum factors (antibody) in the mechanism of adherence inhibition. Serum from LAI-responsive subjects armed cells from LAI-negative individuals to respond to tumor antigens in a specific fashion (Table 2). The arming factor in some sera was shown to be relatively potent since dilutions as high as 1:100 were still effective. Since the serum arming effects appeared to be specific, it is likely that immunoglobulin is involved in the LAI mechanism. This factor may be similar to the arming factor previously described by Marti et al. (11). In addition, Howell and Goldrosen (personal communication) found that “arming” of normal mouse peritoneal cells by “hyperimmune” antisera led to strong tumor-specific LAI responses. Together with the observations above concerning multiple cell types and the lack of soluble factors, a mechanism similar to the phenomenon now referred to as antibody-dependent cellular cytotoxicity (in that null cells, monocytes, and lymphocytes become “armed” and can act as effector cells) is suggested.

Although serum from responsive patients routinely armed normal cells to be specifically responsive, serum from LAI-negative patients did not. Moreover, serum from these patients inhibited LAI reactivity of responsive cells. The specificity of the LAI-blocking sera was investigated by using cells which were responsive to both melanoma and squamous cell carcinoma antigens. The blocking sera inhibited LAI reactivity of both autologous and heterologous cells to squamous antigen, but not melanoma antigen (Table 3). This serum and all other LAI-blocking sera tested did not inhibit mitogen-induced lymphocyte proliferation (data not shown).

The role of blocking factors in the mechanism of the LAI test may be an important consideration. It seems reasonable that because of technological differences between laboratories some investigators may be measuring the combined effects of cells and serum factors, whereas others could be measuring cellular effects alone. This could partially account for discrepancies between laboratories on the question of the persistence or decline of LAI responses in various clinical settings.

**Correlations of LAI with Dermal Testing.** Burger et al. (1) previously published a strong correlation between LAI reactivity and dermal testing to melanoma antigen (1). In patients with squamous cell carcinoma of the head and neck, although both DCH reactions and LAI tests appeared to reflect tumor specificity, a correlation between the magnitude of the dermal test (DCH) and the magnitude of the LAI response could not be documented. In addition, in 34 of 54 of these patients there was agreement between the 2 tests (both positive, 27 of 54; both negative, 7 of 54). In the remaining 20 patients, the dermal test was >5 mm while the LAI test was negative (<30% inhibition). This is not completely surprising since this is an attempted correlation between an in vivo (DCH) and an in vitro (LAI) assessment of reactivity. One consideration should be that the positive dermal tests in the patients with negative LAI's may be false positives. An additional consideration is that the LAI test may not be measuring a cell-mediated phenomenon. If this is the case, absolute agreement with DCH reactions or in vitro correlates of DCH reactivity (leukocyte migration inhibition, etc.) could not be expected.

### References

5. Halliday, W. J., and Miller, S. Leukocyte Adherence Inhibition: A Simple...
Discussion

Dr. Halliday: Getting back to the point of the soluble factor and 3 dilution experiments where you diluted out your active cells with normal cells, I am afraid I just cannot appreciate the mathematics of this or the principle of it. It seems to me that, if you have a very small number of specifically reacting cells, most of the cells that you have, in any case, are normal cells. Adding increasing amounts of normal cells is only going to dilute out your activity, which is what you have demonstrated. It does not show how those active cells work, how they react with antigen, or whether they produce a factor or not.

Dr. Burger: If you have 2000 antigen-reactive cells and dilute that by 50% with normal cells, and then you use the same number of cells in your assay, you will have half the number of reaction cells; therefore, half the effect. The amount of LAI that you can demonstrate will be half what you demonstrated initially. This does not prove that a factor is not produced, but it makes it unlikely. When this same approach was used with migration inhibition, it was one of the first indications that there were factors released by lymphocytes that affected macrophages because as one diluted the number of immune cells with normal cells, one did not proportionally dilute the activity.

Dr. Halliday: I thought it just meant that one could still detect activity or, in other words, that the original population was very active.

Dr. Burger: I guess what you are telling me is that you think the supernatant activity is directly proportional to the number of reactive cells detected in a one-to-one ratio. Our preliminary experiments suggest that this is not the case.

Dr. Powell: I do not know whether or not I can illuminate this point. Let us suppose for a moment that we have an antigen-reactive cell that is going to produce a supernatant. And let us suppose that cell produces enough supernatant to affect, for example, 10 or 100 normal cells which are present. As long as this material is produced once and only once, then it seems to me that Dr. Halliday is perfectly right, unless the material is utilized in an autocatalytic manner, in which case he would not be right. Now, I think we do have some evidence bearing on that in the sense that, if we treat cells with puromycin, we do seem to get LAIF produced, and this makes it a very different system than the migration inhibition factor system in which puromycin destroys the production of migration inhibition factor. So it would appear that, at least in our experiments (which are not really as complete as they should be), we have prepackaged material which is secreted once and used only once. The point is that if there is a direct relationship between the number of cells and the packets of LAIF produced and the number of cells, you are reducing the amount of LAIF that is present, and you should have a straight-line relationship in your dilution curve, just exactly as you showed.

Dr. Burger: I do not think that is precisely the question. You can, of course, dilute out the activity from a small number of cells producing a material, antibody, or whatever. We were very impressed by the realization that the percentage of LAI was exactly proportional to the number of immune cells. I guess it is very hard for me to see that these cells had precisely the amount of packaged factor to affect just that limiting dilution of cells. Using labeled immune cells, preliminary experiments suggest that nonimmune cells are not recruited into the LAI reactions.

Dr. McCoy: We have talked the whole day about the mechanism, and even once in a while someone throws out the term LAIF. Very, very little that is concrete has been said about LAIF. Does anyone care to say something a little more concrete about the nature of LAIF other than the fact that it has a molecular weight between 35,000 and 50,000?

Dr. Halliday: These are very old data, so I hesitate to mention them because we have done nothing recent on the nature of LAIF. But it was found previously by Annette Maluish that the material was heat sensitive, very similar to other known lymphokines, and it was not affected by antimmunoglobulin antisera.

Dr. Takasugi: Then these people are not across-the-board reactors; rather, they are somewhat selective in their reactivity?

Dr. Burger: The reactivity in the controls is selective in that these individuals only respond to the tumor antigen to which they had exposure (by prolonged contact with a patient). It is definitely not across-the-board reactivity.

Dr. Takasugi: Are you assuming that this reactivity is tumor associated?

Dr. Burger: Yes.

Dr. Thomson: We routinely test personnel in the laboratory, and most of them had been in contact with breast cancer or serum or all of these products for about 3 or 4 years. We looked at all family members of people who were at that time in the hospital with breast cancer, and none of these people reacted to the breast cancer antigen(s).

Dr. Burger: Families of neuroblastoma patients made up the highest percentage of contacts that showed reactivity. Greater than 90% of the family contacts, mothers and fathers, showed reactivity. There was also a high percentage with melanoma and a lower percentage with squamous cell carcinoma, both still positive.
Dr. Thomson: The epidemiology, I think, suggests that many solid tumors are really carcinogen induced. That is, cancers of the bowel or squamous cell carcinoma are not virally induced tumors but are the result of contact with carcinogens. Neuroblastoma may very well be an exception, however.

Dr. Powell: Dr. Thomson made a remark about which I think we have got to be very careful. This is particularly true when we look at close-contact sensitization. I must point out that the immune reactions and the immune reactivity of cancer patients or anybody else tells you absolutely nothing about their levels of resistance. So when you are exposed and you give a positive immune response, this has no known relationship to resistance or susceptibility.
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