Cellular and Humoral Factors Involved in the Mechanism of the Micro-Leukocyte Adherence Inhibition Reaction

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Abstract

To study the cellular basis for specific antigen-induced leukocyte adherence inhibition, enriched populations of B-cells, T-cells, and monocytes were prepared by a two-stage adherence separation procedure from spleen cells of normal C57BL/6J mice and mice bearing progressively growing MCA-38 tumors. The reactor cell undergoing specific antigen-induced adherence inhibition was identified as a monocyte (esterase positive, did not respond to mitogens, and did not bear Thy 1.2 antigen or surface immunoglobulin). Furthermore, an enriched population of MCA-38-sensitized B-cells could program normal monocytes to undergo specific antigen-induced adherence inhibition. In contrast, enriched populations of MCA-38-sensitized T-cells could not program normal nylon wool-adherent cells to undergo antigen-specific adherence inhibition.

Programming of normal monocytes by MCA-38-sensitized B-cells occurs through a soluble mediator and not by direct cell contact. The soluble mediator appears to be immunoglobulin in nature and induced both adherence inhibition and the inhibition of adherence. Thus, in this murine tumor model, leukocyte adherence inhibition appears to be due to programming of monocytes by a secretory product of specifically sensitized B-cells.

Introduction

Over 70 studies by a minimum of 15 different laboratories using 3 different techniques (hemocytometer, test tube, microtest) suggest that the LAI assay can detect the specific presence of different forms of human cancer. While there is disagreement on the nature of the LAI response in patients with large tumor burdens and in patients who have had their tumors surgically resected, these studies indicate with few exceptions that the LAI assay can detect minimal tumor burden in the early stages of tumor growth. Thus, the LAI assay has great potential as an immunodiagnostic test. However, the prognostic and diagnostic potential of this test will not be fully realized until the immunological mechanisms by which the response occurs is understood.

At this workshop, most investigators are of the opinion that multiple mononuclear cell types are involved in the mechanism of reactivity, but they cannot agree on the specific cell types or on their role in antigen-induced adherence inhibition. While most investigators agree on the fact that adherence inhibition reflects changes in monocyte function (2, 4, 5, 8, 16), there is profound disagreement on the following points: (a) whether the tumor antigen(s) directly interacts with the monocyte; (b) whether its activity is dependent upon a soluble mediator that is released after interaction between a sensitized lymphocyte and a tumor antigen(s) (2, 6, 8, 16); (c) whether the lymphocyte is a T-cell (2, 6, 12) or a B-cell (16); or (d) whether the soluble mediator is a lymphokine (LAIF) (2, 6, 12) or immunoglobulin (9). This controversy may be in part due to the fact that most of these studies have been performed with poorly characterized mononuclear populations.

In this study, we have used a 2-stage adherence procedure to isolate enriched fractions of T-cells, B-cells, and monocytes from normal and tumor-bearing mice. These mononuclear cells were characterized by mitogen responses, morphological parameters, and cell surface markers. After the identity of the various cell fractions was established, each was evaluated alone and in combination for specific antigen-induced adherence inhibition. Furthermore, supernatants of biologically active cell fractions were prepared and evaluated for their ability to induce adherence inhibition of normal adherent cells.

Materials and Methods

Mice

Six- to 8-week-old male C57BL/6J mice were obtained from West Seneca Laboratories (West Seneca, N. Y.) and used in all experiments. They were fed a standard diet of pellets and water ad libitum.

Tumors

Murine colon adenocarcinoma cells (MCA-38) were established in culture in this laboratory by Tan et al. (17) in 1976. The original tumor cell line was obtained by chemical induction using 1,2-dimethylhydrazine. B16 murine melanoma cells were established in tissue culture in our laboratory and were originally obtained from The Jackson Laboratory (Bar Harbor, Maine) in 1974.

Tumor cell suspensions were obtained by exposure of tumor cell monolayers in culture to a solution of 0.25% trypsin and 0.25% EDTA. The cell suspension was washed, resuspended in RPMI medium 1640 (No. 187-G; Grand Island Biological Co., Grand Island, N. Y.), and 1 x 10⁶ cells were inoculated s.c. in the medial thigh area of C57BL/6J...
Preparation of B-Cells, T-Cells, and Monocytes

The isolation and characterization of enriched populations of B-cells, T-cells, and monocytes has previously been described (15). A 2-stage adherence procedure was used and is outlined in Chart 1. In the initial stage, spleen cells were incubated on nylon wool columns using a procedure similar to Trizio and Gudkowicz (19). This resulted in nylon wool-nonadherent and nylon wool-adherent fraction. The nylon wool-adherent fraction was eluted by agitation and further fractionated on the basis of adherence to a plastic surface in the presence of 10% normal mouse serum. After incubation for 1 hr at 37°, the flask nonadherent cells were removed by decantation. Fresh medium was added to the flask, and the flask nonadherent population was removed with the aid of a rubber policeman. This resulted in a flask-adherent and flask-nonadherent fraction.

The nylon wool-nonadherent and the flask-adherent and nonadherent fractions were characterized for the response to mitogens (LPS, Con A), morphological characteristics (Wright's differential, nonspecific esterase activity), and the presence of cell surface markers (Thy 1.2 antigen and surface immunoglobulin). The nylon wool-nonadherent fraction was positive for cell surface Thy 1.2 antigen, responded to Con A but not to LPS, and had no appreciable esterase activity. These findings are consistent with known properties of monocytes (3). The flask-nonadherent fraction was positive for surface immunoglobulin, responded to LPS but not Con A, and had no appreciable esterase activity. These findings are consistent with the properties of B-cells.

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The nylon wool-adherent fraction was further fractionated on the basis of adherence to a plastic surface in the presence of 10% normal mouse serum. After incubation for 1 hr at 37°, the flask nonadherent cells were removed by decantation. Fresh medium was added to the flask, and the flask nonadherent population was removed with the aid of a rubber policeman. This resulted in a flask-adherent and flask-nonadherent fraction.

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the intermediate nylon wool-adherent fraction consisting of B-cells and monocytes were evaluated for adherence in the presence of tumor antigen alone or in combination with other cells to see if programming could be observed. In the programming experiments, normal and MCA-38-sensitized T-cells were mixed with normal and MCA-38-sensitized nylon wool-adherent cells, where T-cells comprised approximately 50% of the cell population. In these experiments, the B-cells comprised approximately 50% of the cell population.

Experiments Designed to Evaluate the Biological Activity of MCA-38-sensitized B-Cell Supernatants

Generation of MCA-38-sensitized B-Cell Supernatants. Sensitized B-cells (6.5 x 10⁶/ml) and an equal number of normal B-cells were incubated for 1 hr at 37°C with an equal volume of MCA-38 or B16 tumor membrane antigen(s) of 0.01 mg/ml. After centrifugation at 200 x g for 5 min at 20°C, the supernatants were harvested and frozen at −70°C until used. In preliminary experiments, it was observed that a 1:10 dilution of the supernatants resulted in a specific optimal LAI response.

Determination of the Sequence of Reactivity of the B-Cell Supernatants. To determine the sequence in which a B-cell supernatant interacted with the normal peritoneal cells, the following experiment was performed. Single drops of biologically active supernatants were instilled into each well of the experimental quadrants in place of tumor antigen(s) normally used in the micro-LAI assay. Normal peritoneal cells were added as described previously (Test Pattern 1, inhibition of adherence).

Alternatively, the normal peritoneal cells were added first and allowed to adhere by incubating the microtest plates for 1 hr at 37°C. The supernatants were added to the adherent cells to see if they could induce adherence inhibition (Test Pattern 2).

Immunoglobulin Nature of the Active B-Cell Supernatant. To determine if the activity was due to the presence of immunoglobulin, experiments were performed to see if activity could be abolished by incubating an active B-cell supernatant on an affinity column. The IgG fraction of rabbit antimouse whole immunoglobulin was coupled to Affi-gel 10 in the presence of 0.1 M sodium phosphate, pH 7.2, for 2 days at 4°C. Coupling was terminated by the addition of 1 methanolamine, pH 8.0. The slurry (10 ml) was placed into a column (12-ml syringe barrel) and washed extensively. The B-cell supernatants were incubated overnight at 4°C on the column and eluted with 15 ml of RPMI medium 1640 (buffer eluate). The bound fraction was eluted with 0.1 M acetic acid and 1.0 M sodium chloride, pH 2.0. The eluted fraction was immediately brought to pH 4.0 after elution by the use of a hollow-fiber device (Bio-Rad Laboratories, Rockville Center, N. Y.). The absorbed and unabsorbed fractions were evaluated by both Test Patterns 1 and 2.

Results

Role of T-Cells in the Micro-LAI Assay. Spleen cells from normal mice bearing 0.3- to 0.50-cm MCA-38 tumors were initially fractionated on nylon wool columns into a T-cell fraction and a nylon wool fraction consisting of B-cells and macrophages. Both fractions were evaluated individually and in combination with complementary normal cells to identify what fraction is undergoing specific antigen-induced adherence inhibition.

The results of Chart 2 indicate that the LAI activity of the whole spleen cell population was present in the nylon wool-adherent fraction and not in the T-cell fraction. Furthermore, MCA-38-sensitized T-cells could not program normal nylon wool-adherent cells to undergo adherence inhibition. The activity observed in this experiment was due to the presence of MCA-38-sensitized nylon wool-adherent cells.

Role of B-Cells in the Micro-LAI Assay. The biologically active MCA-38-sensitized nylon wool-adherent fraction was further fractionated into a B-cell fraction and a monocyte fraction. Both of these fractions were tested alone or in combination for antigen-specific adherence inhibition activity.

The results on Chart 3 indicate that the LAI activity of the
nylon wool-adherent fraction was present in the macrophage fraction and not in the B-cell fraction. However, when MCA-38-sensitized B-cells were mixed with normal macrophages, the MCA-38-sensitized B-cells could program the normal macrophages to undergo MCA-38 antigen-induced adherence inhibition.

**Nature of B-Cell Programming.** To determine if programming of normal adherent cells occurred through a soluble mediator or involved direct cell contact, MCA-38-sensitized B-cells were incubated with the MCA-38 tumor membrane extract or control B16 extract for 1 hr at 37°C. The supernatants were harvested and were evaluated for their ability to induce adherence inhibition of normal adherent cells. The MCA-38-sensitized B-cell supernatants were used in the place of tumor antigen, and the test was performed as described previously (Test Pattern 1). The results on Chart 4 indicate that MCA-38-sensitized B-cell supernatants (unabsorbed) could induce adherence inhibition of normal adherent cells.

To determine if the activity was due to immunoglobulin or to a lymphokine, the MCA-38-sensitized B-cell supernatants were incubated on an affinity column consisting of anti-mouse whole immunoglobulin coupled to Affi-gel 10. The absorbed B-cell supernatants were evaluated along with the unabsorbed B-cell supernatants for their ability to induce adherence inhibition of normal adherent cells. The results in Chart 4 indicate that MCA-38-sensitized B-cell supernatant activity was lost after passage over a solid immunoadsorbent that specifically deleted mouse immunoglobulin (IgG, IgA, IgM).

With the experimental format used in these supernatant studies, it is impossible to determine if the biologically active B-cell supernatants prevent normal cells from initially adhering (inhibition of adherence) or induced previously adherent normal cells to undergo adherence inhibition. To evaluate the latter possibility, normal macrophages were incubated for 1 hr at 37°C and allowed to adhere to the bottom of the microtest wells. The unabsorbed and absorbed B-cell supernatants were then added to the adherent cells and incubated for 1 additional hr (Test Pattern 2). The results in Chart 4 indicate that the unabsorbed MCA-38-sensitized B-cell supernatant was capable of inducing adherence inhibition of previously normal adherent cells.

**Discussion**

In this study, we observed that the cell that underwent antigen-specific adherence inhibition adhered to nylon wool and "coated plastic" surfaces; furthermore, it was esterase positive, it had no surface immunoglobulin or Thy 1.2 antigen, and the majority of these cells were 14.4 μm in size. These characteristics are common properties of macrophages. Thus, it would appear that the macrophage is the cell that is undergoing antigen-specific adherence inhibition. While our recently reported study ruled out B- and T-lymphocytes as indicator cells (16), it does not preclude the possibility that small numbers of other cell types [K-cells (1), tightly adherent cells (11), or null cells (20)] may be present in the macrophage fraction and may act as indicator and/or rector cells in this assay. This finding is in agreement with results of Holáñ et al. (5) and Thomson et al. (4, 9), who observed that antigen-specific adherence inhibition was due to the interaction between tumor antigen and a sensitized monocyte or macrophage.

This study could not detect a programming role for T-lymphocytes in antigen-specific adherence inhibition as an enriched population of MCA-38-sensitized T-cells could not program normal nylon wool-adherent cells to undergo antigen-specific adherence inhibition. In the above experiments, MCA-38-sensitized T-cells comprised approximately 50% of the cell population. These experiments were also performed at other ratios of MCA-38-sensitized T-cells to normal wool-adherent cells, and uniformly negative results were observed (unpublished observations). Furthermore, we have previously demonstrated that anti-Thy 1.2 serum and complement could not abolish antigen-specific adherence inhibition of the whole spleen cell population or nylon wool-adherent fraction (15). This latter experiment indicates that the activity of the sensitized nylon wool-adherent fraction is not due to a nylon wool-adherent T-cell subset as recently described by Moorhead (10). This finding directly contrasts with the observations of Holt (6) who worked with a *Bacillus Calmette-Guérin*-purified protein derivative, Cremers (2) who worked with a mammary tumor virus system, and Powell (12) who utilized classical hapten conjugates. In Holt's and Cremers' studies, anti-Thy 1.2 serum and complement did abolish antigen-specific adherence inhibition. Several factors may account for the discrepancy between our results and the results of Holt, Cremers, Powell, and their coworkers. This difference may be reconciled on the basis that the nature of sensitization to the test antigen in the respective systems is fundamentally different. In our tumor model, the host is continually sensitized to the tumor antigen by virtue of the presence of a progressively growing tumor and possibly a tumor antigen(s) that is being shed from the cell surface. In the other nontumor system, the host is intermittently immunized with the antigen (2). Thus, the nature of antigenic stimulation may result in
Mechanism of Micro-LAI

different lymphoid cells being involved in antigen-specific adherence inhibition.

While this study was performed in mice with progressively growing tumors, the potential role of T-cells in this assay should also be studied in mice in different states of tumor-genesis. Perhaps the tumor load may be suppressing the T-cell response, suggesting that studies in mice that have had their tumors resected or that are hyperimmunized to the tumor may indicate a potential role of T-cells in this model.

Furthermore, in our tumor system, MCA-38-sensitized B-cells could program normal monocytes to undergo tumor-specific adherence inhibition in the presence of tumor membrane antigen(s). We have also demonstrated previously that specific programming by MCA-38-sensitized B-cells could be abolished if these cells were pretreated with anti-mouse immunoglobulin reagents and complement using an indirect cytotoxicity method (15). Furthermore, pretreatment of the sensitized monocyte fraction alone with anti-mouse immunoglobulin reagents and complement did not abolish LAI activity, indicating that the activity of the sensitized monocyte fraction was not due to the possible presence of small numbers of B-cells contaminating the sensitized monocyte fraction. Thus, in the MCA-38 system, antigen-induced specific adherence inhibition is due to programming of monocytes by sensitized B-cells. However, in vitro, antigen-induced specific adherence inhibition may manifest itself both through the presence of monocytes sensitized in vivo and by normal monocytes programmed by sensitized B-cells.

To determine if programming of normal adherent cells (macrophages) by MCA-38-sensitized B-cells was due to a soluble mediator or involved direct contact between these 2 cell types, supernatants of MCA-38-sensitized B-cells incubated with specific (MCA-38) and nonspecific (B16) tumor antigen were prepared and tested in the micro-LAI assay. Supernatants from MCA-38-sensitized B-cells incubated with specific antigen could both prevent normal leukocytes from adhering (inhibition of adherence) and reverse the adhesion process of leukocytes that were firmly attached to a coated plastic surface (adherence inhibition). Thus, in the MCA-38 system, antigen-induced specific adherence inhibition is due to a soluble mediator and not due to direct cell contact between the sensitized B-cell and the normal adherent cell.

While these results are consistent with the idea that the soluble mediator inducing antigen-specific adherence inhibition is due to antibody, a B-cell-secretory product, we cannot rule out lymphokines (LAIF), because it is known that B-cells can produce lymphokines such as migration inhibition factor (14). However, the ability of a solid immunoabsorbent directed to mouse immunoglobulin of the IgG, IgA, and IgM classes to remove the LAI activity of a biologically active supernatant makes it less likely that specific antigen-induced adherence inhibition is due to a lymphokine and more probably due to immunoglobulin.

These results directly contrast with the supernatant studies in the mammary tumor virus system by Creemers (2), who observed that supernatants of sensitized T-cells (LAIF) could induce LAI of normal and adherent cells; furthermore, pretreatment of these sensitized T-cells with anti-Thy 1.2 serum abolished the biological activity of these supernatants. However, it is important to note that, in Creemers' study, supernatants were harvested after 24 hr, whereas in our study, supernatants were harvested after 1 hr. It would appear that in our study we are evaluating a factor in the supernatant that is preformed, whereas in the Creemers' study the evaluated factor is being synthesized over a long period of time. These results are consistent with the observations of Thomson et al. (4, 9) and Burger et al. (18) who have demonstrated that normal monocytes could be programmed to undergo adherence inhibition if they were exposed to a specific tumor antigen(s) and serum (antibody) from a patient bearing a tumor of the same tissue type as the tumor antigen(s). Studies to characterize the immunoglobulin class of the MCA-38-sensitized B-cell supernatants are currently in progress.

Acknowledgments

We gratefully acknowledge Joel Zalin, Jean Donovan, and Norman Paolini for their technical assistance and Joann Vandrei for the preparation of this manuscript.

References

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Discussion

Dr. Catanzaro: When you talk about spleen monocytes, these are actually spleen macrophages which may be very different from blood monocytes. This should be kept in mind for people who are looking at this test with peripheral blood.

Dr. Goldrosen: That is a very good point. I should not have called them monocytes in the first place. They are tissue macrophages.

Dr. Catanzaro: The first paper in this pair used peritoneal exudate cells. How were they raised?

Dr. Goldrosen: We harvested the peritoneal cells without any stimulation.

Dr. Catanzaro: Did you sacrifice the animals, or was this a repeated phenomenon?

Dr. Goldrosen: Animals were sacrificed and used.

Dr. Holt: Obviously, we disagree radically about Thy 1.2 antiserum, so I have just a couple of technical questions. First, did you have some sort of a positive control?

Dr. Goldrosen: Yes, our antisera, at a 1:10 dilution, kill 95% of the thymocytes and less than 5% of bone marrow cells.

Dr. Holt: One point that has been derived from studies in leukocyte migration inhibition is that very small percentages of T-cells seem to be able to control the whole system. In fact, there are some reports where people claim that as low as between 1 and 3% of T-cells in a mixture let the system operate in leukocyte migration inhibition, as if the original whole-T-cell population was there.

Dr. Goldrosen: I can give you an idea of how enriched our populations are. The T-cell population is at least 80% enriched. It is contaminated primarily with granulocytes. Our B-cell population is greater than 95% enriched, and our tissue macrophage population is also greater than 95% enriched. So of the 3 populations, our least enriched fraction is the T-cell fraction. But the crucial issue is whether or not there are T-cells in the B-cell fraction or the macrophage fraction that are giving us our activity. We have performed experiments where we diluted the B-cells to the point where the number of T-cells would be minimal, and I cannot see how they could account for the activity.

Dr. Holt: With what did you dilute them out?

Dr. Goldrosen: We used varying ratios of normal and MCA-38-sensitized B-cells and monocytes.

Dr. Thomson: I think that even macrophage migration inhibition is not fully defined with respect to the mechanism. Certainly there are mediators, but I can recall papers which show that you can have the macrophage migration inhibition mediated by antigen-antibody complexes. Again, according to the way that people look at microcytotoxicity they find different mechanisms and mediators. You have killer cells being armed with cytphilic antibody, and you have T-cells which are directly mediating the effect. So I think in a number of different assay systems there seem to be several possible mechanisms.
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