LYMPHOCYTE TRANSFORMATION INDUCED BY AUTOLOGOUS CELLS
V. Generation of Immunologic Memory and Specificity during the Autologous Mixed Lymphocyte Reaction*

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Lymphocyte proliferation in vitro may follow antigen recognition and serve as a correlate of cell-mediated immunity. Lymphocyte proliferation can also be stimulated by nonimmune mechanisms as, for example, following culture with plant lectins, lipopolysaccharides, or staphylococcal protein A (1). The autologous mixed lymphocyte reaction (MLR) refers to the proliferation of T lymphocytes cultured with autologous non-T lymphocytes (2, 3). The purpose of this study was to determine whether lymphocyte proliferation in the autologous MLR results from immune or nonimmune mechanisms. We have shown that the autologous MLR has two classical attributes of an immune phenomenon: memory and specificity.

Materials and Methods

Preparation of Lymphocyte Suspensions. Venous blood from healthy volunteers was drawn into plastic syringes and diluted with 10 U heparin/ml blood. The T- and non-T-lymphocyte preparations were isolated as previously described (3). Briefly, heparinized blood was diluted with an equal volume of calcium- and magnesium-free Hanks' balanced salt solution (HBSS). 30-40 ml of the diluted blood was layered over 12 ml of a mixture of Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) and Hypaque (sodium diatrizoate), with a sp. gr. of 1.078–1.080, and was then centrifuged at 400 g for 40 min at 20°C. The cells were removed from the interface, washed, and then resuspended in RPMI 1640 containing 20% fetal calf serum at a concentration of 2 × 10^6 cells/ml. Equal volumes of the lymphocyte suspension and a suspension of sheep erythrocytes (1.5 × 10^8 cells/ml) were mixed. The suspension of cells was centrifuged at 50 g for 5 min at room temperature and incubated overnight at 4°C. The pellets were then gently resuspended and 35 ml of the suspension was layered over 12 ml of Ficoll-Hypaque. The cells were centrifuged at 400 g for 40 min at 4°C. Non-T lymphocytes were removed from the interface and rosetted T lymphocytes in the pellet were washed once with a solution of 0.83% ammonium chloride and 0.17 M Tris buffer, pH 7.2. The T- and non-T-lymphocyte preparations were washed three times with HBSS and were resuspended at a concentration of 10^6 lymphocytes/ml in the final culture medium: RPMI 1640 with 100 U penicillin/ml, 100 μg streptomycin/ml, 2 mM glutamine, and 20% heat-inactivated human AB serum.

Preparation of Primary and Secondary Mixed Lymphocyte Cultures. Primary cultures were established in small flasks (model 3018; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and

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consisted of 20 ml of final culture medium containing \(10^7\) responding T lymphocytes and \(10^7\) stimulating, irradiated (3,000 R) non-T lymphocytes. The culture flasks were placed in an upright position at 37°C in a 5%-CO\(_2\)/95%-humidified air environment. At various times, 0.2-ml aliquots of the cell suspensions were removed from culture flasks and placed in round-bottomed microtiter plates (IS-MRC-96 TC; Linbro Chemical Co., New Haven, Conn.). Thymidine incorporation during the subsequent 24-h period was measured by adding 1 \(\mu\)Ci [methyl-\(^3\)H]thymidine (sp act 2 Ci/mM; New England Nuclear, Boston, Mass.) to 1 \(\mu\)l. After the 24-h pulse period, the lymphocytes were aspirated from the wells, transferred to glass fiber filter paper (H. Reeve Angel & Co., Inc., Clifton, N.J.), and then washed with water using an apparatus based on the design of Hartzman et al. (4). The glass fiber disks were placed into 15 \(\times\) 45-mm vials and 2\(1/2\) ml of antireticular cytotoxic serum (ACS; Amersham/Searle Corp., Arlington Heights, Ill.) was added. These vials were placed inside standard scintillation vials and counted in a "Delta 300" model 6890 ambient temperature liquid scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). The average of the counts per minute for triplicate cultures is given. The counting efficiency for tritium under these conditions was 34%.

Secondary cultures were established after lymphocytes had been incubated for 10 days. The precultured cells were washed twice with HBSS and resuspended at \(10^6\) viable lymphocytes/ml of fresh culture media. Viability of lymphocytes was determined by trypan blue exclusion. Non-T lymphocytes used as a secondary stimulus were freshly isolated from the blood of the original non-T-lymphocyte donors. The freshly prepared non-T lymphocytes were resuspended at \(10^7\) cells/ml in final culture medium and irradiated. 0.1 ml of culture medium containing fresh stimulating cells was mixed with \(10^6\) viable lymphocytes which had been taken from the primary culture flasks and resuspended in 0.1 ml of fresh final culture medium. Lymphocyte cultures were set up in triplicate in Linbro round-bottomed microtiter plates. Replicate plates were pulsed with \([\text{H}]\)thymidine on days 2-6. Thymidine incorporation by such cultures was assayed as described above.

**Results**

*Kinetic Analysis of the Primary and Secondary MLR.* The proliferative response of T lymphocytes cultured with irradiated allogeneic or irradiated autologous non-T lymphocytes was measured by thymidine incorporation after 3–9 days of culture. After 9 days of mixed lymphocyte culture, we attempted to stimulate a secondary response by adding fresh, irradiated non-T lymphocytes from the donor of the original stimulating cells to the cultured lymphocytes. Lymphocyte proliferation in the secondary response was similarly assayed. Thymidine incorporation during the primary and secondary response by T-lymphocytes cultured with autologous or allogeneic non-T lymphocytes is shown in Fig. 1. The data presented are the average of four independent cultures using different donors for the primary response and 10 different donors for the secondary response. The peak in thymidine incorporation occurred on the 5th day of culture in the primary allogeneic MLR and on the 7th day of culture in the primary autologous MLR. The amount of thymidine incorporated during the allogeneic MLR was usually, but not invariably, greater than that in the autologous MLR. Thymidine incorporation in autologous MLR averaged 55% of that incorporated in the allogeneic MLR. After 9 days of incubation, the MLR had subsided. At this time, the cells were washed and resuspended in fresh medium at a concentration of \(10^6\) viable lymphocytes/ml. These cells were rechallenged with the same stimulator cells used in the primary MLR. The secondary response differed from the primary response in the kinetics of thymidine incorporation. Peak thymidine incorporation in the secondary MLR occurred 2 days after rechallenge with allogeneic non-T lymphocytes, and 3 days after rechallenge with autologous non-T lymphocytes. The
FIG. 1. Primary and secondary response of lymphocyte cultured with allogeneic or autologous non-T lymphocytes. Purified T-lymphocytes were cultured with irradiated autologous or allogeneic non-T cells. Cultures were incubated for various lengths of time with thymidine incorporation measured during the last 24 h of culture. After 9 days of incubation, the cultured cells were washed and resuspended in fresh medium at a concentration of 10^6 viable cells/ml. Those cells were then rechallenged with freshly prepared, irradiated non-T cells from the original donors of stimulating cells. These secondary cultures were maintained for 1-5 days with thymidine incorporation measured during the last 24 h of culture. The mean thymidine incorporation of four autologous and four allogeneic primary and secondary MLR are given. MLC-mixed lymphocyte culture.

magnitude of the secondary allogeneic MLR was greater than that of the secondary autologous MLR.

Specificity of the Secondary MLR. The specificity of the secondary proliferative response following a primary autologous or allogeneic MLR was assessed. T lymphocytes incubated with autologous or allogeneic non-T lymphocytes for 9 days were rechallenged with the original stimulating cell, or challenged for the first time with allogeneic or autologous non-T lymphocytes. Thymidine incorporation was measured during the 2nd or 3rd day of culture (the peak of the secondary response), and during the 6th day of the culture (to assess the primary response) after challenge. The results of eight such experiments are presented in Table I. When T lymphocytes were cultured alone, challenge with either allogeneic or autologous cells resulted in a response typical of a primary response with more thymidine incorporated on day 6 than on day 2 or 3 of culture. In contrast, responding cells rechallenged in secondary culture with the same non-T lymphocytes present in the primary culture clearly showed a secondary response, manifested by a greater thymidine response on day 3 (autologous) or day 2 (allogeneic) of culture than on day 6. It should be noted that challenge with a different cell in the secondary reaction lead to a response with kinetics typical of the primary reaction with peak incorporation on day 6. To assess the effect of the primary cellular interaction upon the amount of thymidine incorporated during the secondary response, thymidine incorporated
Table I

Specificity of Thymidine Incorporation during Secondary MLR*

| Primary stimulus (No. of experiments) | Secondary stimulus ([H]thymidine incorporation) | Autologous non-T cells | Allogeneic non-T cells |
|---------------------------------------|-----------------------------------------------|------------------------|-----------------------|
|                                       | Day 2/3                                       | Day 6                  | Day 2/3               | Day 6                |
| None (8)                              | 2.8                                           | 5.1                    | 3.3                   | 33.9                 |
| Autologous non-T cells (8)            | 11.6                                          | 2.3                    | 7.2                   | 43.7                 |
| Allogeneic non-T cells (8)            | 6.7                                           | 7.3                    | 16.9                  | 9.1                  |

* Human T lymphocytes were cultured alone, with irradiated autologous or with allogeneic non-T lymphocytes for 9 days. The cells were then washed, and 10⁶ viable cells were incubated with irradiated autologous or allogeneic non-T lymphocytes for 2, 3, or 6 days. Thymidine incorporation was measured during the last 24 h of culture. Peak thymidine responses to autologous or allogeneic cells are underlined.

Fig. 2. Magnitude of secondary mixed lymphocyte reaction. 10⁶/ml purified T lymphocytes were cultured alone, and with autologous or allogeneic non-T cells for 9 days. All cultures were then challenged with freshly prepared and irradiated autologous or allogeneic non-T cells. Thymidine incorporation was measured at the peak (day 2 or 3) of the secondary MLR. The data shown are the increment in thymidine incorporation of T cells incubated a second time with non-T cells, above thymidine incorporation by T cells incubated for the first time with non-T cells. The mean of 12 independent experiments is presented.

by T cells cultured alone was subtracted from the amount of thymidine incorporated on day 2 or 3 by T cells challenged with non-T cells for a second time. As can be seen in Fig. 2, after a primary autologous MLR, addition of autologous non-T lymphocytes stimulated four times as much thymidine incorporation as did the addition of allogeneic non-T lymphocytes. This is particularly significant since allogeneic non-T lymphocytes stimulate more thymidine incorporation than do autologous non-T lymphocytes in the primary MLR. In contrast, after a primary allogeneic MLR, addition of allogeneic non-T lymphocytes stimulated 4.5 times more thymidine incorporation than did the addition of autologous non-T lymphocytes. These results indicate considerable specificity in the secondary response for the primary cell. Primed lymphocytes respond
more vigorously to cells allogeneic to the original stimulators than do unprimed lymphocytes. This suggests a degree of cross-reactivity between autologous and allogeneic non-T lymphocytes. Another way of assessing cross-reactivity between non-T cells is to analyze the kinetics of thymidine incorporation during the secondary MLR. A secondary response may be defined by the greater incorporation of thymidine by lymphocytes on the 2nd or 3rd day of culture as compared to the 6th day of culture. In 12 experiments, the rechallenge of primary mixed lymphocyte cultures with the same stimulating cells always led to a secondary MLR by this criterion. Challenge of primary autologous mixed lymphocyte cultures with allogeneic non-T cells led to typical secondary MLR kinetics in six cases, and primary MLR kinetics in six cases. When primary allogeneic mixed lymphocyte cultures were challenged with autologous non-T lymphocytes, a secondary response, by the kinetic criterion, was observed in 88% of the experiments.

Discussion

A more rapid proliferative response of lymphocytes follows rechallenge with allogeneic cells. Hayry et al. (5) used this kinetic criterion to define a secondary MLR by murine lymphocytes in culture. The secondary MLR, like the primary MLR, was activated by lymphocyte determinants distinct from the serologically defined H-2^d or H-2^k histocompatibility determinants. In addition, specificity generated during the primary MLR was clearly demonstrated in the response to secondary challenge. The specificity of the murine MLR was also demonstrated by Binz and Wigzell (6) in experiments in which cells with receptors for lymphocytes from a particular strain were eliminated from the responder cell donors. Lymphocytes from such donor animals did not respond in MLR to lymphocytes from this strain, but they did respond normally to lymphocytes from another strain. A secondary allogeneic MLR, characterized by an accelerated pace of lymphocyte proliferation, has also been demonstrated with human blood lymphocytes. This reaction has been used to distinguish LD determinants on lymphocytes from family members (7). Thus, during the primary human allogeneic MLR, lymphocytes are generated which possess both immunologic memory and specificity.

The goal of the studies reported here was to determine if there is an immunologic basis for the autologous MLR. We have shown that the autologous MLR possesses the typical attributes of an immune response, which are also seen in the allogeneic MLR. During the primary autologous MLR, a population of lymphocytes was generated which exhibited immunologic memory and specificity. Moreover, the same subpopulation of lymphocytes stimulated the autologous and allogeneic MLR (3). Recently, immunological memory and specificity have been demonstrated in the in vitro reaction of rat lymphocytes with syngeneic testicular cells (8).

After a primary culture of lymphocytes with syngeneic dissociated testicular cells, a secondary proliferative response is induced by syngeneic but not allogeneic testicular cells. Of particular relevance to our studies was the discovery that a secondary proliferative response could be elicited not only by the testicular cell preparation but also by syngeneic lymph node cells. These results imply that the determinants on non-T lymphocytes which elicit the
MLR are shared by cells in the testicular preparation but not by fibroblasts. It is possible that sperm cells (which carry Ia-like determinants) are present in the testicular cell preparation and are the stimulating cells.

The existence of autoreactive antibodies and lymphocytes has been related to the disorganization of the immune system which occurs in certain diseases and also accompanies aging. Although it is possible that autoreactive lymphocytes may damage autologous cells, the presence of autoreactive lymphocytes in healthy individuals indicates they need not cause disease. Preliminary studies suggest that subjects with autoantibodies have a marked impairment of the autologous mixed lymphocyte reaction (MLR). In contrast, a vigorous autologous MLR occurs between lymphocytes obtained from umbilical cord blood. Thus, autoreactive lymphocytes do not appear as forbidden clones during aging. On the contrary, autoreactive lymphocytes rather appear to be normal components of the immune system. Autoreactive lymphocytes may be an important component of a system for immune surveillance.

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References

1. Cunningham-Rundles, S., J. A. Hansen, and B. Dupont. 1976. Lymphocyte transformation in vitro in response to mitogens and antigens. In Clinical Immunobiology. F. H. Bach and R. A. Good, editors, Academic Press, Inc., New York. 151.

2. Opelz, G., M. Kuichi, M. Takasugi, and P. I. Terasaki. 1976. Autologous stimulation of human lymphocyte subpopulations. J. Exp. Med. 142:1327.

3. Kuntz, M. M., J. B. Innes, and M. E. Weksler. 1976. Lymphocyte transformation induced by autologous cells. IV. Human T-lymphocyte proliferation induced by autologous or allogeneic non-T lymphocytes. J. Exp. Med. 143:1042.

4. Hartzman, R. J., M. Segall, M. L. Bach, and F. H. Bach. 1971. Histocompatibility matching. VI. Miniaturization of the mixed leukocyte culture test: a preliminary report. Transplantation (Baltimore). 11:268.

5. Hayry, P. 1976. Anamnestic responses in mixed lymphocyte culture-induced cytolysis (MLC-CML) reaction. Immunogenetics. 3:417.

6. Binz, H., and H. Wiegzell. 1976. Specific transplantation tolerance induced by autoimmunization against the individual's own, naturally occurring idiotypic, antigen-binding receptors. J. Exp. Med. 144:1438.

7. Sheey, M. J., P. M. Sondel, M. L. Bach, R. Wank, and F. H. Bach. 1975. HL-A LD (lymphocyte defined) typing: a rapid assay with primed lymphocytes. Science (Wash. D. C.). 188:1308.

8. Werkerle, H. 1977. In vitro induction of immunological memory against testicular autoantigens. Nature (Lond.). 267:357.