LYMPHOCYTE TRANSFORMATION INDUCED BY AUTOLOGOUS CELLS

IV. Human T-Lymphocyte Proliferation Induced by Autologous or Allogeneic Non-T Lymphocytes*

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Paul Ehrlich was the first to contrast the ease of inducing immunity to foreign cells with the difficulty of inducing immunity to autologous cells (1). The phenomenon of self-tolerance has remained a fundamental tenet of immunobiology. Burnet postulated that self-tolerance resulted from the loss, during development, of lymphoid cells capable of expressing immunity to autologous antigens (2). This hypothesis has been challenged by the existence of B lymphocytes which bind autologous antigens (3) and the capacity of lymphocytes removed from the original host to react to syngeneic cells and tissues (4, 5). Thus, Cohen and his coworkers demonstrated that lymphocytes sensitized in vitro become cytotoxic to syngeneic cells and can induce a graft-versus-host reaction in syngeneic recipients (4, 5). These observations suggest that lymphocytes in vivo are actively restrained from reacting with certain autologous cells and tissues.

We have previously presented evidence that lymphocytes are stimulated by autologous lymphoblasts. Lymphoblasts established in continuous culture (6) or induced by mitogens (7) stimulate the proliferation of autologous lymphocytes. In the present study we report that human T lymphocytes are stimulated to proliferate by autologous non-T lymphocytes. Opelz et al. (8) have recently presented evidence that such a reaction probably accounts for the background thymidine incorporation in the mixed lymphocyte reaction.

In the present study we have examined the capacity of mononuclear cells in the B-lymphocyte preparation to stimulate autologous T lymphocytes. The population of cells found to be most stimulatory formed rosettes with sensitized human erythrocytes but not with mouse erythrocytes. K lymphocytes have these characteristics (9). We have also shown that non-T lymphocytes stimulate T-lymphocyte proliferation in the allogeneic mixed lymphocyte reaction. The proliferative response of T lymphocytes cultured with autologous non-T lymphocytes may reflect not merely a loss of self-tolerance in vitro but also a mechanism by which T lymphocytes regulate immunity.

Materials and Methods

Preparation of Lymphocyte Suspensions. 50 ml of venous blood from healthy volunteers were drawn into a plastic syringe containing 10 U of heparin (Upjohn Company, Kalamazoo, Mich.) per

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ml of blood. The blood was diluted with an equal volume of calcium and magnesium-free Hanks' balanced salt solution (HBSS,[1] Microbiological Associates, Bethesda, Maryland). 35–40 ml of the diluted blood were layered over 12 ml of a mixture of Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) and sodium diatrizoate (Hypaque, Winthrop Laboratories, New York) in sterile 25 × 150-mm glass screw top tubes (Corning Glass Works no. 9825, Corning, N. Y.). Ficoll-Hypaque mixtures were prepared by mixing one part of 50% Hypaque with four parts of 8% (wt/vol) Ficoll in water. The density of the Ficoll-Hypaque mixture was adjusted to a specific gravity of 1.078–1.080 with distilled water and passed through an 0.45 μm Millipore filter. Tubes containing the diluted blood layered on Ficoll-Hypaque were centrifuged at 400 *g* for 40 min at 20°C. The cells removed from the interface were washed three times with HBSS with 10% heat-inactivated fetal bovine serum (FBS, Microbiological Associates) in 16 × 125-mm plastic tubes (Falcon Plastics no. 2037, Oxnard, Calif.) and collected by centrifugation at 150 *g* for 10 min at 20°C. The cells were then resuspended in RPMI 1640 (Microbiological Associates) with 100 U penicillin/ml, 100 μg streptomycin/ml, L-glutamine 2 mM (Microbiological Associates), and 20% FBS at a concentration of 2 million lymphocytes per ml.

To remove "phagocytic" cells, 1 ml of carbonyl iron (Lymphocyte Separating Reagent, Technicon Instrument Co., Tarrytown, N. Y.) was mixed with 2 ml of the mononuclear cell preparation obtained by the Ficoll-Hypaque technique. The cells and carbonyl iron were agitated at 37°C for 30 min. The cells that had phagocytized the carbonyl iron were removed in a magnetic field. The nonphagocytic cells were washed three times and resuspended as above.

To remove "adherent" cells, venous blood was collected in a plastic syringe and defibrinated by placing it in a sterile 125-ml Erlenmeyer flask containing glass beads. The glass beads and blood were gently swirled 10–15 min until a clot formed. The defibrinated blood was removed from the flask, diluted with an equal volume of HBSS, and the mononuclear cells isolated as described above. These cells were washed once with HBSS containing 10% FBS and resuspended in 10 ml HBSS 10% FBS. This suspension of cells was poured into a 30-ml plastic syringe containing sterile nylon wool (Leuko-Pak, Fenwal Laboratories, Morton Grove, Ill.) which had been prewet with HBSS with penicillin, streptomycin, glutamine, and 15% FBS at 37°C. After an incubation period of 30–40 min at 37°C, 60 ml of HBSS was poured over the column to wash off the nonadherent cells. The nylon wool was then compressed with the syringe plunger. The nonadherent cells were washed three times and resuspended as above.

Preparation of Sheep, Mouse, and Sensitized Human Erythrocyte Rosettes: Fractionation of T-, B-, and K-Lymphocyte Populations. Sheep erythrocytes (SRBC) obtained from the New York City Department of Health were washed two times in HBSS, and three drops of packed SRBC were suspended in 10 ml HBSS. Equal volumes of human lymphocyte and SRBC suspensions were combined in 16 × 125 Falcon plastic tubes, and the tubes were centrifuged at 50 *g* for 5 min at 20°C, as described by Steel et al. (10). The cells were incubated overnight at 4°C.

The pellets were then gently resuspended, and 35 ml of the SRBC-lymphocyte suspension were layered over 12 ml of Ficoll-Hypaque in 25 × 100-mm glass tubes. The cells were centrifuged at 400 *g* for 40 min at 4°C. Unrosetted lymphocytes were removed from the interface. The lymphocyte preparation which did not form rosettes with SRBC is referred to as purified non-T-lymphocyte preparation and contains B lymphocytes, K lymphocytes, and possibly other cell types. We will refer to this preparation as non-T lymphocytes or, for convenience, as B lymphocytes, recognizing that it is heterogenous with regard to cell type. Rosetted T lymphocytes in the pellet beneath the Ficoll-Hypaque were washed twice with 0.83% ammonium chloride-0.17 M Tris buffer, pH 7.2, and collected by centrifugation (150 *g* for 10 min at 20°C) to lyse the SRBC (11). The unfractionated, purified T and non-T lymphocytes were washed three times with HBSS with 10% FBS and resuspended at a concentration of 2 million lymphocytes per 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.

In certain experiments, the B-lymphocyte preparation was fractionated. Rosettes formed with human erythrocytes sensitized with Ripley anti-Rh IgG antiserum were prepared to deplete the B-cell preparation of lymphocytes with Fc receptors (12). Mouse erythrocyte rosettes were prepared to deplete the B-cell preparation of surface immunoglobulin-positive lymphocytes (13). In both cases, rosetted lymphocytes were separated from unrosetted lymphocytes as above.

[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution.

Mixed lymphocyte cultures were established in triplicate and consisted of 0.2 ml of final culture medium containing 10^5 responding lymphocytes and 2×10^5 stimulating lymphocytes irradiated with 3,000 R from a cesium source.

All cultures were incubated for 144 h in a 5% CO_2/95% air humidified environment. 24 h before the termination of the incubation period, 1 μCi [*methyl*-^{3}H]thymidine (sp act 2 Ci/mM, New England Nuclear, Boston, Mass.) in 1 μl was added to each culture well. At the end of the incubation period the lymphocytes were aspirated from the wells, transferred to glass fiber filter paper (Reeve-Angel, Inc., Clifton, N. J.), and washed with water using an apparatus based on the design of Hartzman et al. (14). The glass fiber disks were placed into 15 × 45-mm vials and 2½ ml of Hydromix (Yorktown Research Inc., Hackensack, N. J.) were added. These vials were placed inside standard scintillation vials and counted in a Beckman ambient temperature liquid scintillation counter. The average of the counts per minute of the triplicate cultures is given. The counting efficiency for tritium under these conditions was 34%.

Lymphocytes reacting with rabbit antihuman immunoglobulin were identified by incubating at least 5×10^5 lymphocytes with 0.1 ml of a 1:10 dilution of fluoresceinated rabbit antihuman immunoglobulin (Sylvana Laboratories, Millburn N. J., Lot 112471-13) for 15 min at 4°C. The cells were washed three times with PBS and resuspended in a small volume of PBS. The preparation was examined as a suspension under a nail polish-sealed cover slip with a Leitz phase microscope with Ploem fluorescent illumination. 200 lymphocytes were counted under phase illumination and the percentage of fluorescent lymphocytes determined.

Results

Proliferation of Unfractionated and Purified T Lymphocytes Cultured with Allogeneic Non-T Lymphocytes. Mononuclear cells were isolated from human blood by centrifugation over a solution of Ficoll-Hypaque. These cells were mixed with SRBC. T lymphocytes which formed rosettes were separated from unrosetted lymphocytes by centrifugation through the Ficoll-Hypaque solution. The cells that do not form rosettes include monocytes, B, and K lymphocytes. This method yielded a T-lymphocyte preparation in which an average of 7% (range 5–13%) of the lymphocytes reacted with fluoresceinated antihuman immunoglobulin antiserum. 93–98% of the lymphocytes in the non-T lymphocyte preparation reacted with the fluoresceinated anti-immunoglobulin antiserum. As many as 18% (average 15%) of the mononuclear cells in the non-T lymphocyte preparation phagocytized latex particles.

Unfractionated, purified T or non-T lymphocytes were employed as responding or stimulating populations in the mixed lymphocyte reaction (Table I). Purified T lymphocytes or unfractionated lymphocyte preparations proliferated when cultured with allogeneic lymphocytes. T lymphocytes were more responsive than unfractionated lymphocytes. Cells in the B-lymphocyte preparation did not proliferate when cultured with allogeneic lymphocytes. The survival of the lymphocytes in the T- and B-lymphoid preparations was comparable. After 6 days in culture, 66% of the T cells and 52% of the non-T cells were viable as measured by trypan blue dye exclusion.

In contrast, the irradiated, unfractionated, or B-lymphocyte preparations stimulated allogeneic lymphocyte proliferation while the irradiated T lymphocytes did not. The B-lymphocyte preparation was significantly more stimulatory than were unfractionated lymphocytes. Thus, it appears that T lymphocytes are the responding lymphoid population, and B or K lymphocytes are the stimulating lymphoid population in the allogeneic mixed lymphocyte reaction.

Proliferation of Purified T Lymphocytes Cultured with Autologous Non-T

TABLE I

*Thymidine Incorporated by Unfractionated, Purified T, or Non-T Lymphocytes Stimulated by Allogeneic Lymphocytes**

Responding lymphocytes	Allogeneic irradiated lymphocytes	Thymidine incorporated/culture‡ (cpm × 10^{-3})				
		Experiment 1		Experiment 2	Experiment 3	Mean
	Subjects:	(A)	(B)	(C)	(D)	
Unfractionated lymphocytes	Unfractionated lymphocytes	4.8	4.7	0.8	25.5	8.95
Unfractionated lymphocytes	T lymphocytes	2.5	0.8	0.1	2.1	1.4
Unfractionated lymphocytes	Non-T lymphocytes	22.1	13.7	8.1	23.1	16.8
T lymphocytes	Unfractioned lymphocytes	6.0	10.7	0.7	38.4	9.8
T lymphocytes	T lymphocytes	1.1	1.4	0.0	1.9	1.1
T lymphocytes	Non-T lymphocytes	22.8	23.6	14.0	51.2	27.9
Non-T lymphocytes	Unfractionated lymphocytes	0.7	1.2	0.0	4.6	1.6
Non-T lymphocytes	T lymphocytes	0.7	0.0	0.1	0.4	0.3
Non-T lymphocytes	Non-T lymphocytes	0.9	0.3	0.1	2.6	1.0

* Lymphocytes were isolated from human blood and fractionated into T and non-T-lymphocyte preparations as described in Materials and Methods. Each lymphocyte preparation was cultured, in triplicate, for 6 days with irradiated allogeneic unfractionated, T- or non-T-lymphocyte preparations. The proliferative response was measured by thymidine incorporation during the last 24 h of culture.

‡ The counts per minute/culture indicated is obtained by subtracting the counts per minute in the autologous mixture, AAx (ranging from 500 to 1,500 cpm) from the counts per minute in the allogeneic mixture, ABx.

Lymphocytes. It has previously been shown that B lymphoblasts stimulate the proliferation of autologous lymphocytes (6, 7). We found that a B-lymphocyte preparation also stimulates autologus T-lymphocyte proliferation (Table II). Thus, the irradiated B-lymphocyte preparation stimulated 15 times as much thymidine incorporation by autologous T lymphocytes as did cultures of T lymphocytes mixed with irradiated T lymphocytes. The irradiated B-lymphocyte preparation stimulated significantly more thymidine incorporation by autologous T lymphocytes than did irradiated unfractionated lymphocytes. The purified non-T-lymphocyte preparation was not stimulated to proliferate by any of the autologous lymphocyte preparations tested.

The kinetics of the stimulation of T lymphocytes by autologus B-lymphocyte preparations were followed over the course of 9 days. The amount of thymidine incorporated by T lymphocytes mixed with irradiated autologous non-T lymphocytes increased from 1×10^3 cpm/culture on day 3 to 61×10^3 cpm/culture on day 9. After 6 days of incubation, the stimulation index (TBx divided by TTx) was greatest. For this reason, an incubation period of 6 days was adopted as routine in these studies.

From these studies it appeared that thymidine incorporation by T lymphocytes cultured with autologous irradiated lymphocytes resulted from the presence of B (or K) lymphocytes in the irradiated lymphoid population. This hypothesis was supported by two experiments (Fig. 1) in which various mixtures of the irradiated T- and B-lymphocyte preparations were made and cultured with autologous T lymphocytes. As can be seen, the amount of thymidine

TABLE II

*Thymidine Incorporated by Purified T or Non-T Lymphocytes Stimulated by Autologous Lymphocytes**

Responding lymphocytes	Autologous irradiated lymphocytes	Thymidine incorporated/culture (cpm × 10^{-3})							
		Experiment I	Experiment II			Experiment III		Experiment IV	Mean
	Subjects:	(A)	(B)	(C)	(D)	(E)	(F)	(G)	
T lymphocytes	Unfractionated lymphocytes	8.1	1.0	18.1	3.6	–	–	11.1	8.4
T lymphocytes	T lymphocytes	0.2	0.2	3.7	0.5	0.6	0.1	0.8	0.9
T lymphocytes	Non-T lymphocytes	16.4	11.1	32.3	16.4	10.5	1.4	17.6	15.1
Non-T lymphocytes	Unfractionated lymphocytes	0.8	–	–	–	–	–	–	0.8
Non-T lymphocytes	T lymphocytes	0.8	2.3	–	–	1.0	0.5	–	1.2
Non-T lymphocytes	Non-T lymphocytes	1.1	1.4	–	–	1.3	0.3	–	1.0

* T or non-T-lymphocyte preparations isolated from human blood were cultured in triplicate for 6 days with irradiated autologous unfractionated, T- or non-T-lymphocyte preparations. The proliferative response was measured by thymidine incorporation during the last 24 h of culture.

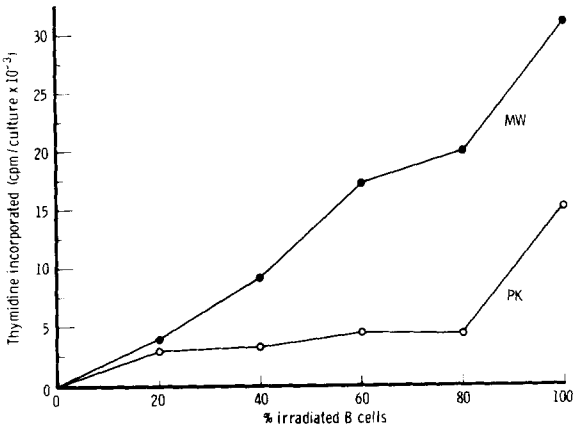

FIG. 1. T lymphocytes isolated from human blood were cultured in triplicate with irradiated autologous T- or B-lymphocyte preparations. In addition, T lymphocytes were cultured with irradiated lymphocytes consisting of 20, 40, 60, or 80% of the B-lymphocyte preparation. The proliferative response was measured by thymidine incorporation during the last 24 h of a 6-day incubation period. The thymidine incorporated by cultures containing T lymphocytes mixed with autologous irradiated T lymphocytes (MW cpm/culture, 398; PK cpm/culture 348) was subtracted from that incorporated by cultures of T lymphocytes mixed with irradiated autologous B-lymphocyte preparations.

incorporated by T lymphocytes was directly related to the presence of non-T lymphocytes in the irradiated cell mixture. The greatest amount of thymidine was incorporated by T lymphocytes cultured with the purified B-lymphocyte preparation.

The proliferative response of T lymphocytes stimulated by the autologous or by the allogeneic B-lymphocyte preparations was directly compared (Table III).

TABLE III

*Thymidine Incorporated by Purified T Lymphocytes Stimulated By Irradiated Autologous or Allogeneic Lymphocytes**

Irradiated lymphocytes	Thymidine incorporated/culture (cpm × 10^{-3})					
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Mean
Subjects:	A	B	C	D	E	
Autologous T lymphocytes	0.8	0.3	0.2	2.7	0.2	0.8
Autologous non-T lymphocytes	17.6	3.5	16.4	19.8	11.1	13.7
Allogeneic non-T lymphocytes	40.9	14.3	51.4	46.9	68.2	44.3

* T lymphocytes isolated from human blood were cultured, in triplicate, with irradiated autologous T- or non-T-lymphocyte preparations or an allogeneic non-T-lymphocyte preparation. The proliferative response after 6-day culture was measured by thymidine incorporation during the last 24 h of incubation.

In each of five experiments, allogeneic non-T lymphocytes stimulated more than twice the amount of thymidine incorporation by T lymphocytes than did similarly prepared autologous lymphocytes. Thus, the proliferative response observed in the allogeneic mixed lymphocyte culture appears to be made up of two components: the response to determinants displayed by lymphocytes in the autologous B lymphocyte preparation and the response to foreign histocompatibility determinants. In contrast, autologous B-lymphoblastoid cells stimulated as much lymphocyte proliferation as did allogeneic B-lymphoblastoid cells (Table IV). As was observed in the allogeneic mixed lymphocyte reaction (Table I), allogeneic T-lymphoblastoid cells did not stimulate lymphocyte proliferation, whereas allogeneic B-lymphoblastoid cells did stimulate lymphocyte proliferation.

The capacity of the B-lymphocyte preparation to stimulate the proliferation of autologous T lymphocytes was not due to the gamma irradiation used to prevent non-T-lymphocyte proliferation (Table V). When stimulating lymphoid preparations were not irradiated, more thymidine was incorporated when autologous T and non-T lymphocytes were cultured together than when the same total number of lymphocytes were cultured alone (Table II). Although the non-T lymphocytes were not irradiated, they neither respond to allogeneic or autologous lymphoid cells nor incorporate large amounts of thymidine when cultured alone (Tables I and II). As was seen earlier, the mixture of a B-lymphocyte preparation with T lymphocytes resulted in more thymidine incorporation than occurred when T lymphocytes were mixed with the unfractionated lymphoid preparation.

Removal of Phagocytic or Adherent Cells in the B-Lymphocyte Preparation Increases Its Capacity to Stimulate Autologous T-Lymphocyte Proliferation. 82–95% of the cells in the B-lymphocyte preparation had morphological characteristics of small lymphocytes. The remaining cells were mononuclear, the majority of which were phagocytic. The capacity of the non-T lymphocytes to stimulate autologous T-lymphocyte proliferation was attributable to the lymphocytes and not to phagocytic or adherent mononuclear cells in the B-lympho-

TABLE IV

*Thymidine Incorporated by Lymphocytes Stimulated by Irradiated Autologous or Allogeneic Cultured Lymphoblastoid Cells**

Irradiated lymphoid cells	Thymidine incorporated/culture (cpm × 10^{-3})				
	Exp. I	Exp. II	Exp. III	Exp. IV	Mean
Autologous lymphocytes	0.7	0.6	1.2	0.6	0.8
Autologous B lymphoblastoid line	45.6	112.6	82.6	56.7	74.4
Allogeneic B lymphoblastoid line (HH)	49.6	96.2	76.4	70.2	73.1
Allogeneic T lymphoblastoid line (Molt 4)	1.6	0.9	1.8	0.9	1.3

* Lymphocytes were isolated from human blood and cultured in triplicate for 6 days with irradiated autologous or allogenic cells. The proliferative response was measured by thymidine incorporation during the last 24 h of culture.

cyte preparation (Table VI). Thus, removal in a magnetic field of mononuclear cells which had phagocytized iron filings or depletion of adherent cells by defibrination of blood, followed by passage of the mononuclear cells over nylon fibers, did not reduce the capacity of the B-lymphocyte preparation to stimulate autologous T-lymphocyte proliferation. In fact, both methods that depleted the B-lymphocyte preparation of mononuclear cells augmented its capacity to stimulate autologous T lymphocytes. It is possible that surface immunoglobulin-positive cells are to some degree retained on the nylon fibers (9). This would result in a preparation not only depleted of monocytes and B lymphocytes, but also enriched with K lymphocytes. Cells so prepared were more stimulatory than were preparations depleted of phagocytic cells (Table VI). Defibrination and filtration over nylon fibers yielded a preparation consisting of 98–99% small lymphocytes. Supernatant culture medium obtained after 6 days of incubation of T lymphocytes with an irradiated autologus B-lymphocyte preparation did not stimulate autologous lymphocyte proliferation.

Fractionation of the B-Lymphocyte Preparation. There is evidence that surface immunoglobulin-positive human lymphocytes form rosettes with mouse erythrocytes (13). Froland and Natvig have offered evidence that surface immunoglobulin-negative cytotoxic human lymphocytes, K cells, bind to sensitized human erythrocytes (9). B-lymphocyte preparations were separated into populations that did and did not form rosettes with mouse erythrocytes or sensitized human erythrocytes. For ease of presentation, those cells that formed rosettes with mouse erythrocytes will be referred to as the B-enriched population and those cells that formed rosettes with sensitized human erythrocytes as the K-enriched population. K-enriched populations obtained by either technique stimulated far more thymidine incorporation by autologous T lymphocytes than did the B-enriched populations (Table VII). Rosette formation could not explain the difference in the stimulatory capacities of the B- and K-enriched populations, as in the mouse erythrocyte technique, B lymphocytes formed rosettes, whereas in the sensitized human erythrocyte technique, K lymphocytes formed rosettes.

Effect of Autologous Serum on the Autologous Mixed Lymphocyte Reaction. Cohen and Wekerle have found that autologous serum inhibits the

TABLE V

*Thymidine Incorporated by Purified T Lymphocytes Stimulated by Irradiated or Unirradiated Autologous Lymphocytes**

Treatment of stimulating lymphocytes	Stimulating lymphocytes	Thymidine incorporated/culture (cpm × 10^{-3})		
		Experiment I		Experiment II
		Subject A	Subject B	Subject C
3,000 R	T lymphocytes	1.6	1.3	4.5
3,000 R	Unfractionated lymphocytes	12.4	7.0	20.1
3,000 R	Non-T lymphocytes	27.5	10.8	30.9
None	T lymphocytes	7.3	0.5	11.8
None	Unfractionated lymphocytes	24.7	11.0	25.4
None	Non-T lymphocytes	32.7	13.5	31.9

* T lymphocytes isolated from human blood were cultured in triplicate with irradiated or unirradiated autologous lymphocyte preparations. The proliferative response after 6-day culture was measured by thymidine incorporation during the last 24 h of incubation.

reactivity of rat lymphocytes with autologous cells (15). For this reason, we examined whether serum drawn from the lymphocyte donor would inhibit the autologous mixed lymphocyte reaction. The autologous mixed lymphocyte reaction in three subjects was not inhibited by autologous serum. In two of three subjects tested, thymidine incorporation by T lymphocytes stimulated by the autologous B-lymphocyte preparation was greater in the presence of autologous serum than in the presence of allogeneic (AB) serum. Thymidine incorporation by lymphocytes incubated with autologous T lymphocytes was not increased in the presence of autologous serum. In preliminary studies, it was found that serum from some pregnant women inhibited the autologous mixed lymphocyte reaction.

Discussion

These studies have demonstrated that a preparation of human non-T lymphocytes, isolated from blood, stimulated the proliferation of autologous T lymphocytes. The cell in this non-T lymphocyte preparation that stimulates autologous lymphocyte proliferation forms rosettes with sensitized human erythrocytes, but not with mouse erythrocytes. K lymphocytes have these characteristics. Furthermore, preparations depleted of adherent cells by filtration over nylon fibers are more stimulatory than are preparations depleted of phagocytic cells. Nylon columns may remove B lymphocytes, as well as granulocytes and monocytes (9). Consequently, the observed increase in stimulatory capacity of nylon fiber filtered cells may be due either to more complete removal of monocytes or to enrichment with K cells. The capacity of the irradiated non-T-lymphocyte preparation to stimulate autologous T lymphocytes is not attributable to radiation damage, as an unirradiated non-T lymphocyte preparation also stimulated

TABLE VI

*Thymidine Incorporated by T Lymphocytes Stimulated By Autologous Lymphocytes Depleted of Phagocytic or Adherent Cells**

Stimulating lymphocytes	Treatment of stimulating cells					
	Untreated cells		Removal of phagocytic cells		Removal of adherent cells	
	Thymidine incorporated/culture (cpm × 10^{-3})					
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
T lymphocytes	0.6	0.3	0.3	1.1	0.9	0.4
Unfractionated lymphocytes	11.1	1.4	11.1	1.8	16.4	3.7
Non-T lymphocytes	17.6	3.5	27.7	5.7	65.2	13.2

* T lymphocytes isolated from human blood were cultured in triplicate with irradiated autologous lymphocyte preparation. In certain cases the stimulating cell preparation was mixed with iron filings before irradiation. Cells that had phagocytized the iron filings were removed in a magnetic field. In other cases stimulating cells were prepared from defibrinated blood. The isolated lymphocytes were subsequently passed over nylon fibers to remove adherent cells. The proliferative response after 6 days in culture was measured by thymidine incorporation during the last 24 h of culture.

autologous lymphocyte proliferation. No evidence was found that mitogenic factors were released into the culture medium during the autologous mixed lymphocyte culture. Serum from the lymphocyte donor did not inhibit autologous mixed lymphocyte reactivity.

An autologous human mixed lymphocyte reaction was probably observed by Etheredge et al. (16) and has recently been reported by Opelz et al. (8). In contrast to our studies, Opelz et al. found that a B-cell-rich preparation responded to autologous lymphocytes. In the light of our data (Table II), this is probably explained by the presence of T lymphocytes (an average of 29 ± 5% in 11 experiments) in their B-lymphocyte-rich preparation. A syngeneic mixed lymphocyte reaction has also been found when adult murine lymphoid cells were cultured with neonatal thymus cells (17–19). Von Boehmer (20) identified the stimulating cell in the adult lymphoid preparation as a B lymphocyte. Ponzio et al. (21) have recently demonstrated an autologous mixed lymphocyte reaction in adult mice in which splenic B lymphocytes stimulate autologous lymph node cells.

In addition, our studies defined the stimulating and responding lymphoid populations in the allogeneic mixed lymphocyte reaction: human non-T lymphocytes stimulate allogeneic T-lymphocyte proliferation. It is reasonably clear from previous studies that the proliferative response in the human allogeneic mixed lymphocyte reaction depends upon the presence of T lymphocytes (22, 23). In the absence of T lymphocytes, no proliferation occurred. The identification of proliferating chromosomally marked T lymphocytes was the strongest evidence that only T lymphocytes proliferate in the allogeneic mixed lymphocyte reaction (22). Some investigators have presented evidence that non-T lymphocytes may also proliferate in the allogeneic mixed lymphocyte reaction (24). This may result from their recruitment by T lymphocytes in the responding lymphoid

TABLE VII

*Thymidine Incorporated by Purified T Lymphocytes Stimulated By B- or K-Enriched Lymphoid Populations**

Irradiated autologous lymphocytes	Treatment of stimulating cells						
	Mouse rosette				Sensitized human rosette		
	Thymidine incorporated/culture (cpm × 10^{-3})						
Subjects:	A	B	C	D	B	C	D
T lymphocytes	7.2	0.4	0.7	1.6	0.4	0.7	1.6
B-enriched population	15.9	7.7	0.2	0.7	1.9	1.4	0.3
K-enriched population	50.3	44.2	12.4	30.8	24.1	3.7	7.8

* T- and non-T-lymphocyte preparations were isolated from human blood. The non-T-lymphocyte preparation was then rosetted with either mouse erythrocytes or sensitized human erythrocytes. Rosetted and nonrosetted cells were isolated by centrifugation over Ficoll-Hypaque.

population (25) or even from an influence of inactivated T lymphocytes in the stimulating lymphoid populations (26).

On the other hand, the subpopulation of lymphoid cells which stimulates the human allogeneic mixed lymphocyte reaction has been uncertain. Some investigators believed that T and non-T lymphocytes stimulate allogeneic lymphocyte proliferation equally (27). However, new evidence suggests that the Ia-like human B-cell antigens are the dominant stimulating components in the human mixed lymphocyte reaction (28–31). Our data support the concept that non-T cells stimulate allogeneic T-lymphocyte proliferation. This interpretation is supported by the observation that cultured B lymphoblast lines, but not cultured T lymphoblast lines, stimulate allogeneic lymphocyte proliferation (32, 33). We would suggest that the proliferation of T lymphocytes in the allogeneic mixed lymphocyte culture is probably stimulated by both foreign histocompatibility (LD) determinants and by non-T lymphocyte-specific determinants.

The full biological significance of the mixed lymphocyte reaction is uncertain. Some investigators have suggested that this reaction reflects a mechanism of immune surveillance in vivo (6, 34). This hypothesis has been supported by the finding that leukemic lymphoblasts (35), mitogen-induced lymphoblasts (7), and B-lymphoblast cell lines (6) stimulate autologous lymphocyte proliferation. Recently, however, the importance of immune surveillance as a biologic defense mechanism against neoplasia has been questioned (36, 37). For this reason, we have considered an alternate role for the mixed lymphocyte reaction in immunobiology. As T lymphocytes are recognized to exert positive and negative influences on immunity, it is possible that the autologous mixed lymphocyte reaction reflects a mechanism by which T lymphocytes regulate lymphocyte function.

Summary

An autologous mixed lymphocyte reaction was demonstrated between T and non-T lymphocytes. Sheep erythrocyte rosetting was used to separate human lymphocytes into T and non-T-lymphoid preparations. Non-T lymphocytes stimulated the proliferation of autologous T lymphocytes. The cell in this prepara-

tion that was most stimulatory had the characteristics of a K lymphocyte. The allogeneic mixed lymphocyte reaction was also shown to reflect the proliferation of T lymphocytes stimulated by allogeneic non-T lymphocytes. Proliferation of T lymphocytes in the allogeneic mixed lymphocyte culture probably reflects a response to both foreign histocompatibility determinants and determinants present on non-T lymphocytes. It is suggested that the proliferative response of T lymphocytes to autologous non-T lymphocytes may be a step in the process by which T lymphocytes regulate immunity.

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References

1. Ehrlich, P. 1956. The Collected Papers of Paul Ehrlich. Pergamon Press, London, England.
2. Burnet, M. 1959. The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, Tennessee.
3. Allison, A. C. 1975. Interactions of T and B Lymphocytes in Self-Tolerance and Autoimmunity. *In* Immunological Tolerance. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York.
4. Cohen, I. R., A. Globerson, and M. Feldman. 1971. Autosensitization in vitro. *J. Exp. Med.* 133:834.
5. Cohen, I. R., and H. Wekerle. 1972. Autosensitization of lymphocytes against thymus reticulum cells. *Science (Wash. D. C.).* 176:1324.
6. Weksler, M. E., and G. Birnbaum. 1972. Lymphocyte transformation induced by autologous cells: stimulation by cultured lymphoblast lines. *J. Clin. Invest.* 51:3124.
7. Weksler, M. E. 1973. Lymphocyte transformation induced by autologous cells. II. Stimulation by mitogen-induced lymphoblasts. *J. Exp. Med.* 137:799.
8. Opelz, G., M. Kiuchi, M. Takasugi, and P. I. Terasaki. 1975. Autologous stimulation of human lymphocyte subpopulations. *J. Exp. Med.* 142:1327.
9. Froland, S. S., and J. B. Natvig. 1973. Identification of three different human lymphocyte populations by surface markers. *Transplant. Rev.* 16:114.
10. Steel, C. M., J. Evans, and M. Smith. 1974. The sheep-cell rosette test on human peripheral blood lymphocytes: an analysis of some variable factors in the technique. *Br. J. Hematol.* 28:245.
11. Boyle, W. 1968. An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation (Baltimore).* 6:761.
12. Froland, S. S., F. Wisloff, and T. E. Michaelsen. 1974. Human lymphocytes with receptors for IgG: a population of cells distinct from T and B lymphocytes. *Int. Arch. Allergy.* 47:124.
13. Gupta, S., and M. H. Grieco. 1975. Rosette formation with mouse erythrocytes: probable marker for human B lymphocytes. *Int. Arch. Allergy Appl. Immunol.* 49:734.
14. 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.

15. Cohen, I. R., and H. Wekerle. 1973. Regulation of autosensitization: the immune activation and specific inhibition of self-recognizing thymus-derived lymphocytes. *J. Exp. Med.* 137:224.
16. Etheredge, E. E., A. R. Shons, K. L. Hohenthaner, and J. S. Najarian. 1973. Mitomycin C inactivation of leukocytes in the mixed leukocyte culture. *Transplantation (Baltimore).* 15:331.
17. Llacer, V., and E. M. Uyeki. 1969. Interaction of spleen and thymic cells in vitro. *Int. Arch. Allergy.* 35:88.
18. Howe, M. L., A. L. Goldstein, and J. R. Battisto. 1970. Isogenic lymphocyte interaction: recognition of self antigens by cells of the neonatal thymus. *Proc. Natl. Acad. Sci. U. S. A.* 67:613.
19. Von Boehmer, H., and P. B. Adams. 1973. Syngeneic mixed lymphocyte reaction between thymocytes and peripheral lymphoid cells in mice: strain specificity and nature of the target cell. *J. Immunol.* 110:376.
20. Von Boehmer, H. 1974. Selective stimulation by B lymphocytes in the syngeneic mixed lymphocyte reaction. *Eur. J. Immunol.* 4:105.
21. Ponzio, N., J. H. Finke, and J. Battisto. 1975. Adult murine lymph node cells respond blastogenically to a new differentiation antigen on isologous and autologous B lymphocytes. *J. Immunol.* 114:971.
22. Lohrmann, H. D., and J. Whang-Peng. 1974. Human mixed lymphocyte culture: identification of the proliferating lymphocyte subpopulation by sex chromosome markers. *J. Exp. Med.* 140:54.
23. Chess, L., R. P. MacDermott, and S. Schlossman. 1974. Immunologic functions of isolated human lymphocyte subpopulation. *J. Immunol.* 113:1122.
24. Perkins, W. D., L. C. Robson, and M. R. Schwarz. 1975. Immunoglobulin-positive cells: their appearance in the mixed lymphocyte reaction. *Science (Wash. D. C.).* 188:365.
25. Piguet, P. F., and P. Vassalli. 1972. Thymus-independent (B) cell proliferation in spleen cell cultures of mouse radiation chimeras stimulated by phytohemagglutinin or allogeneic cells. *J. Exp. Med.* 136:962.
26. Harrison, M. R., and W. E. Paul. 1973. Stimulus-response in the mixed lymphocyte reaction. *J. Exp. Med.* 138:1602.
27. Smith, R. W., and I. Royston. 1975. Human lymphocytes: participation of T and B cells in mixed lymphocyte reaction. *Transplant. Proc.* 7:(Supp. 1):63.
28. Lohrmann, H. P., L. Novikovs, and R. G. Graw. 1974. Stimulatory capacity of human T and B lymphocytes in human mixed leukocyte cultures. *Nature (Lond.).* 250:144.
29. Wernet, P., R. Winchester, H. G. Kunkel, D. Wernet, M. Giphart, A. van Leeuwen, and J. J. van Rood. 1975. Serological detection and partial characterization of human MLC determinants with special reference to B-cell specificity. *Transplant. Proc.* 7(Suppl. 1):193.
30. Cresswell, P., and S. S. Geier. 1975. Antisera to human B lymphocyte membrane glycoproteins block stimulation in mixed lymphocyte culture. *Nature (Lond.).* 257:147.
31. Winchester, R. J., S. M. Fu, P. Wernet, H. G. Kunkel, B. Dupont, and C. Jersild. 1975. Recognition by pregnancy serum of non-HL-A alloantigens selectively expressed on B lymphocytes. *J. Exp. Med.* 141:924.
32. Royston, I., R. B. Pitts, R. W. Smith, and P. R. Graze. 1975. *In Vitro* immunization against cultured human B and T lymphoblastoid cell lines. *Transplant. Proc.* 7(Suppl. 1):531.
33. Weksler, M. E. 1976. Lymphocyte transformation induced by autologous cells. III. Lymphoblast induced lymphocyte stimulation does not correlate with EB viral

antigen expression or immunity. *J. Immunol.* **116**:310.
34. Ling, N. R. 1973. Immune surveillance of lymphoid tissue. A biological role for the mixed lymphocyte reaction. *Immunol. Commun.* **2**:119.
35. Fridman, W. H., and F. M. Kourilsky. 1969. Stimulation of lymphocytes by autologous leukaemic cells in acute leukaemia. *Nature (Lond.).* **224**:277.
36. Prehn, R. T. 1974. Immunological surveillance: pro and con. *In* Clinical Immunobiology. F. H. Bach and R. A. Good, editors. **2**:191.
37. Schwartz, R. S. 1975. Another look at immunological surveillance. *N. Engl. J. Med.* **293**:181.