THYMOCYTES FROM MICE IMMUNIZED AGAINST AN ALLOGRAFT RENDER BONE-MARROW CELLS SPECIFICALLY CYTOTOXIC*

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After allograft rejection in mice specifically cytotoxic lymphoid cells can be detected throughout the lymphoid apparatus with the notable exception of the thymus (1). This apparent lack of involvement of thymocytes from suitably immunized animals at the effector level of cell-mediated immunity may seem surprising, for thymocytes have been shown to undergo blast transformation in vitro in the presence of foreign lymphoid cells (2, 3), and certain actively cytotoxic cells have been shown both to bear the thymus-specific antigen (4) and to be derived from the thymus (5).

The present study confirms earlier findings that thymocytes from immune animals are not cytotoxic in vitro to specific allogeneic target cells when cells from the spleen and lymph nodes are. However, we found that thymocytes from such immune animals may cooperate with other nonimmune cells in vitro causing specific cytotoxic effects on target cells. Cytotoxic activity of the cooperating cells was measured in a system previously used to assay the direct cytotoxic activity of spleen cells sensitized in vivo to the murine lymphoma L5178Y (6). Earlier studies have reported a synergistic cytotoxic effect on target lymphoma cells by immune lymphoid cells and nonimmune macrophages (7, 8), and it may be that the present investigation represents another aspect of such cell interactions.

Cooperation between thymus-derived cells and bone-marrow cells has been extensively studied and is thought to be necessary for the initiation of both some humoral (9) and cell-mediated (10, 11) immune responses. This report describes cooperation between specifically immune thymocytes and other cells to effect a synergistic cytotoxic reaction in vitro. A distinction must be made between the modes of cell cooperation involving thymic cells that occur at the afferent and the efferent arms of the immune response.

Materials and Methods

Animals.—Specific pathogen-free mice of inbred C57BL strain were obtained from our own breeding colonies. The experimental animals used were of either sex and between 10 and 14 wk of age.

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Tumors.—The L5178Y lymphoma obtained from Dr. Fischer (Yale University, New Haven, Conn.) is of thymic origin, syngeneic in DBA/2 mice. TLX5 (CBA) and TLX9 (C57BL) lymphomas were induced in these laboratories by X-rays and benzpyrene, respectively; the August rat leukemia is spontaneous and myeloblastic. These tumors have all been maintained by serial passage in adult syngeneic animals and regular returns to early passages preserved in a tumor bank have been made.

Immunization of Lymphoid Cell Donors.—C57BL mice were immunized intraperitoneally with a single dose of 10^7 live L5178Y lymphoma cells. In tests for specificity, 10^7 TLX5 lymphoma cells or August leukemia cells or 0.1 ml of heparinized whole sheep blood was substituted.

Preparation of Cell Suspensions.—Spleens, thymuses, and femurs were removed from groups of five mice and pooled cell suspensions prepared by squeezing the excised organs with forceps in tissue culture medium 199 (TC 199).[1] Bone-marrow cells were obtained by syringing the marrow cavities of mouse femurs. To remove the thymuses, lateral incisions were made from diaphragm to clavicle and the resultant flap of rib cage was deflected back. Care was taken to avoid removal of the parathymic lymph nodes.

Culture of L5178Y Lymphoma and Lymphoid Cells.—At weekly intervals, lymphoma cells from the peritoneal cavity of DBA/2 mice were removed and washed twice in TC 199. The cells were maintained in culture for a period of 1 wk in Fischer's medium (obtained in powdered form from Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland). Before use this serum was heat inactivated at 56°C for 90 min. The cells were routinely grown for 24 hr in vitro before use in growth-inhibition tests. McCartney bottles (capacity 15 ml) fitted with silicone rubber stoppers were used as culture vessels. L5178Y cells grow logarithmically as single unattached cells with a doubling time of 12–16 hr to a maximum of 8×10^5 cells/ml. To 1.5×10^5 L5178Y cells were added 3.0×10^7 lymphoid cells (previously mixed at a ratio of one thymocyte to one bone-marrow or spleen cell). The final volume was 3.3 ml and to avoid nutrient depletion in the medium, an equal volume of fresh medium was added at each 24 hr interval during the experiment.

The method used for determining the cytotoxic effect in mixtures of lymphoid and lymphoma cells has been described (6). Growth inhibition was expressed as the percentage of surviving cells in test cultures compared to the number of cells in control cultures after a 72 hr period.

Irradiation of Cell Cultures.—Cell suspensions were prepared and diluted to 5×10^7 cells/ml with Fischer's medium at 5°C immediately before irradiation with 220 kv X-rays given at 800 R/min.

Assay for Toxicity by Chromium Release.—Following Wigzell (12), L5178Y cells from cultures were incubated at 37°C with ^{51}Cr (as sodium chromate in saline from the Radiochemical Centre, Amersham, England) at 25 μCi/ml for 30 min. After washing twice, the labeled lymphoma cells were resuspended at 2.0×10^5 cells/ml and 4×10^7 lymphoid cells were added. At intervals thereafter 1-ml aliquots were diluted to 3.0 ml with medium 199 and centrifuged at 2000 rpm for 3 min. 1.0-ml samples of supernatant fluid were counted in a Packard gamma counter, Packard Instrument Co., Downers Grove, Ill. Total release was found by freeze-thawing samples three times, spinning, and counting the supernatant.

Stimulation of Nucleic Acid Synthesis in Immune Thymocytes.—Thymocytes (2×10^6 cells/ml) were cultured with 10^4 target cells/ml in Fischer's medium. At intervals 1-ml aliquots of culture were taken and pulsed with uridine-^3H or thymidine-^3H (Radiochemical Centre) at 5 μCi/ml for 60 min. The cells were poured onto filter paper, washed thoroughly with phosphate-buffered saline and with ice-cold 5% trichloroacetic acid, rinsed with ethanol and acetone, and the activity counted in a Packard Tri-Carb scintillation counter. The

[1] *Abbreviations used in paper:* FCS, fetal calf serum; TC 199, tissue culture medium 199.

results were expressed as:

$$\text{Per cent increase in incorporation} = 100 \times \frac{\text{count}/10^6 \text{ thymocytes from immune mice}}{\text{count}/10^6 \text{ thymocytes from nonimmune mice}}$$

Preparation of Thymus Supernatant.—1.0×10^7 thymocytes/ml were incubated for 24 or 48 hr in Fischer's medium containing 1.0×10^5 lymphoma cells/ml (irradiated with 5000 R of X-rays 24 hr previously). The supernatant was separated by centrifugation at 2000 rpm, filtered through a sterile 0.45 μ Millipore filter (Millipore Corp., Bedford, Mass.), and stored at −20°C. For assay 1.5 ml of supernatant was added to 1.5×10^7 bone-marrow cells/flask.

Antisera.—

Rabbit anti-mouse-γ-globulin serum: Serum globulins from old breeding mice were precipitated twice with 50% ammonium sulfate, dissolved in saline, and injected intravenously into rabbits three times at 14-day intervals; venous sera were obtained 10 days later from the ear and were tested for activity by immunodiffusion against normal mouse serum. The serum was adsorbed with L5178Y cells. Activity was detected against IgM but was predominantly to IgG.

Rabbit anti-mouse macrophage serum: DBA/2 peritoneal macrophage cultures were grown for 24 hr in dishes coated with collagen following Ehrmann and Gey (13). Lymphocytes were removed by washing and viable macrophages were obtained by treating the cultures with 0.1% collagenase (Sigma Chemical Co., St. Louis, Mo.) for 20 min. 5×10^6 macrophages were injected intravenously at 10-day intervals and the sera obtained 14 days later. After adsorption the serum was tested in the presence of 3% complement on peritoneal macrophage cultures, spleen cells, and thymocytes. Cell death was determined after a 30 min incubation at 37°C and lysis of 100, 11, and 2% were found, respectively.

All sera (except FCS) were adsorbed on washed L5178Y cells at 4°C for 1 hr at a ratio of 1 vol packed cells: 2 vols serum. After centrifugation the sera were filtered through a sterile 0.45 μ Millipore. Sera were then heat inactivated at 56°C for 45 min.

Glass Bead Treatment of Cell Suspensions.—Cell suspensions containing 2×10^7 cells/ml were placed in 30-ml tissue culture flasks (Falcon Plastics, Los Angeles, Calif.) containing sterile glass beads (4.5–5.5 mm diameter Hopkins and Williams Ltd., Essex, England). The flasks were incubated for 24 hr with periodic gentle agitation to ensure maximum cell surface and glass contact. The supernatant was removed and the flasks gently washed with TC 199. The cells were concentrated by centrifugation, counted using trypan blue, and then used.

RESULTS

Specific Growth Inhibition by Thymus Cells from Immune Mice in Presence of Normal Bone-Marrow or Spleen Cells.—The growth of L5178Y lymphoma cells (strain of origin DBA/2 mice) in vitro was inhibited in an immunologically specific manner by spleen cells (6) and bone-marrow cells but not by thymocytes from C57BL mice that had been immunized 21 days previously with L5178Y cells. Bone-marrow and spleen cells from nonimmune C57BL mice did not affect the growth of the L5178Y cells and under the conditions used there was no evidence for allogeneic inhibition (6, 14). However, as can be seen from Table I, thymocytes from immunized mice exerted a cytotoxic effect on L5178Y lymphoma when nonimmune bone-marrow or spleen cells were also added.

Specificity.—The cytotoxic action of the mixture of immune thymocytes

from mice immune to DBA/2 lymphoma with nonimmune spleen or bone-marrow cells was tested for specificity by two methods. The cell mixture was cultured with the C57BL mouse lymphoma referred to as TLX9. The growth of TLX9 cells was unaffected under conditions when the growth of L5178Y lymphoma cells was inhibited by 80%. Alternatively, C57BL mice were immunized with 0.1 ml of sheep blood, or 10^7 leukemia cells from a transplanted CBA mouse lymphoma (referred to as TLX5), or 10^7 August rat leukemia cells. The growth inhibitory activity of the thymocytes was then tested with and without bone marrow cells. These results are summarized in Table II.

TABLE I

Effect of C57BL Lymphoid Cells and Cell Mixtures on the Growth of L5178Y Cells In Vitro

Cell suspensions* prepared from		Per cent growth inhibition ± SE at:	
Nonimmune mice	Immune‡ mice	48 hr	72 hr
Spleen	None	8 ± 4	10 ± 3
None	Spleen	91 ± 4	97 ± 3
Bone marrow	None	6 ± 4	5 ± 4
None	Bone marrow	77 ± 5	95 ± 3
Thymus	None	3 ± 2	4 ± 3
None	Thymus	4 ± 4	4 ± 6
Cell mixtures§: thymus + bone marrow		6 ± 2	7 ± 1
Bone marrow	Thymus	61 ± 11	80 ± 8
Thymus + spleen		7 ± 3	9 ± 4
Spleen	Thymus	56 ± 4	68 ± 7

* Total cell to lymphoma ratio 200:1.

‡ C57BL mice immunized 21 days previously with 10^7 live L5178Y cells i.p.

§ Ratio of thymus cells to bone marrow or spleen cells 1:1.

To study the effects of varying the ratio of the two cell populations which cooperate in the cytotoxic reaction, the ratio of "immune" thymocytes to lymphoma cells was varied from 25:1 to 100:1. Nutrient fluctuations in the culture medium due to differing cell concentrations were avoided by maintaining the total number of cells in the system constant by adding nonimmune thymocytes. Table III shows that the cytotoxic effect became greater as the concentration of immune thymocytes was increased. Variation of the bone-marrow cell concentration outside the range shown was not possible, as these cells were found to be metabolically very active, the use of high numbers resulted in nutrient depletion to an extent that would not support lymphoma cell growth.

Changes in the Thymus after Immunization with the Allogeneic Lymphoma Cells.—In a previous study (6) two populations of cytotoxic lymphocytes were distinguished in the spleen of immunized C57BL mice by several criteria, the

most striking being resistance to radiation. The radioresistant cytotoxic cells appeared within 6 days after immunization but were not detected at periods longer than 14 days, whereas the population of radiosensitive cytotoxic cells built up slowly in the spleen with maximum activity between 14 and 21 days

TABLE II

The Specificity of the Synergistic Cytotoxic Reaction

Thymus cells from C57BL mice immunized with	Immunizing dose	Target cell* in vitro	Per cent growth inhibition at 72 hr
L5178Y	1×10^7 cells	L5178Y	80 ± 8
		TLX9	7 ± 6
Sheep red blood cells	0.1ml of heparinized whole blood	L5178Y	9 ± 4
TLX5	1×10^7 cells	L5178Y	0
August rat leukemia	10^7 peripheral blood cells	L5178Y	0

* Cell mixture 100 thymus cells + 100 nonimmune bone marrow cells: one target lymphoma cell.

TABLE III

Effect of Varying the Ratio of Thymus to Bone-Marrow Cells on Growth Inhibition of the Target Cells

Number of		Growth inhibition of L5178Y cells at 72 hr
Immune thymocytes per L5178Y cell	Nonimmune bone-marrow cells per L5178Y cell	
25*	100	17
50*	100	31
75*	100	73
100	100	80
150	50	56

* To maintain the ratio of lymphoid to L5178Y cells constant at 200:1, thymocytes from nonimmune animals were added at ratios of 75, 50, and 25:1, respectively.

but remaining detectable for periods of up to 4 months. Fig. 1 shows the time sequence for the appearance of cells in the thymus which are cytotoxic when mixed in vitro with bone-marrow or spleen cells from nonimmune animals. This pattern resembles that observed for the appearance of the radiosensitive cytotoxic cells of the spleen cells from immune mice.

Further evidence was sought for changes in the cells of the thymus of C57BL mice after intraperitoneal injection of an immunizing dose of L5178Y cells. Knecht and Ertl (15) have reported that in rats the incorporation of radio-

active thymidine into the DNA of thymus cells was decreased after transplantation of tumor. Fig. 1 shows that after injection of the L5178Y cells there was a transient fall in the incorporation of thymidine by the cells in the thymus but that this level returns to normal at about 7 days. At this time the thymocytes become capable of causing growth inhibition in conjunction with normal bone-marrow cells. The drop in thymidine incorporation was unlikely to be due to the stress involved in the injection for the results were compared with a control group of mice that had been injected intraperitoneally with saline.

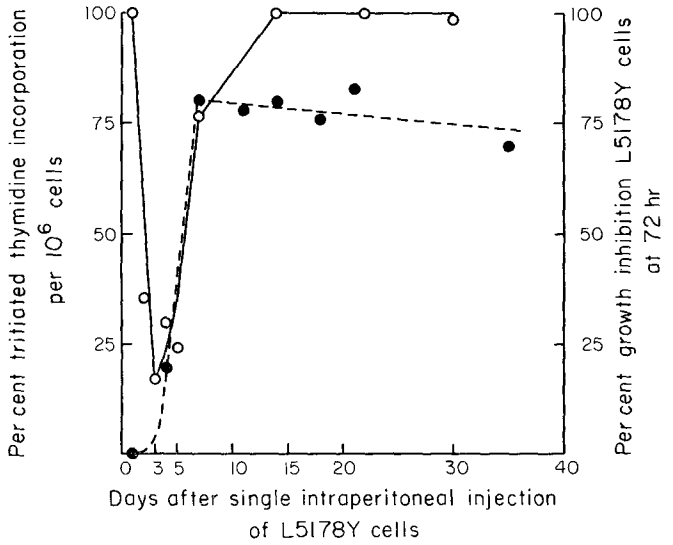

FIG. 1. Appearance after immunization of immunologically active thymocytes and DNA-synthesizing cells. (O—O), Per cent thymidine-^{3}H incorporation per 10^6 thymocytes (compared to control thymocytes from animals injected with saline). (●- - -●), Per cent growth inhibition after 72 hr culture by mixtures of thymocytes from immune mice and normal bone-marrow cells.

Attempt to Assay Cytotoxicity by Chromium-Labeled Target Cells. In the experiments described so far the recorded effect was the inhibition of growth of lymphoma cells after 3 days of culture. These lymphoma cells were found to be of similar size to some of the cells present in the bone marrow, and it was therefore not possible to determine the fate of the lymphoma cells within the first 36 hr or so of contact with immune thymocytes and nonimmune bone marrow by the phase-contrast counting technique. After this time death of the lymphoid cells and dilution of the culture facilitated accurate cell counts of surviving tumor cells. To test if the cooperative growth-inhibition effect was due to lysis of the target cells occurring during the first 24 hr, as was found the case when the effector cells were spleen cells from immune mice (6), or whether

it was merely an inhibition of cell division (cytostasis), the release of radioactive chromium from labeled lymphoma cells in the presence of lymphoid cell mixtures was studied. As we had found earlier (6), approximately 50% of ^{51}Cr was released spontaneously within 24 hr. During this period there was no detectable increase in free chromium in any cultures when the mixture of immune, or control nonimmune thymocytes and nonimmune bone-marrow cells was added. To eliminate the possibility that there was a latent period during which the cooperating thymocytes and bone-marrow cells had to interact before acquiring the capacity for cell lysis, ^{51}Cr-labeled lymphoma

TABLE IV

Effect of Agitation, Complement, and Anti-Mouse-γ-Globulin Serum

Immune thymocytes + normal bone-marrow cells incubated with	Per cent growth inhibition of L5178Y cells at 72 hr
No agitation	80 ± 8
Agitation after initial 18 hr*	62
" for 72 hr	0
Agitation after 18 hr + complement‡	59
" for 72 hr + "	0
No agitation + normal rabbit serum	81 ± 4
" " + rabbit anti-mouse-γ-globulin serum§	79 ± 6

* Agitation by means of a rotary blood cell separator at 30 rpm.

‡ Normal rabbit serum preadsorbed with packed L5178Y cells final concentration 10%.

§ Final concentration 10%.

cells were added at periods between 12 and 72 hr to a culture of immune thymocytes, nonimmune bone-marrow cells, and nonlabeled L5178Y cells. In no case was any increased release of ^{51}Cr observed. These experiments indicate that the mechanism of growth inhibition by the mixture of immune thymocytes and normal bone-marrow cells differs from that of immune spleen cells as cell lysis could not be demonstrated. We cannot distinguish between the possibility that the primary lesion responsible for growth inhibition is a lytic process which requires more than 24 hr of culture, or alternatively, that the effect results from an inhibition of division.

Requirement for Cell-to-Cell Contact.—The growth inhibition of L5178Y cells obtained by the standard procedure of stationary incubation with immune thymocytes and normal bone-marrow cells was lost if the cells were agitated. Table IV shows that with constant agitation at 30 rpm on a rotary blood cell separator at 37°C there was no growth inhibition; however, if the mixed culture was left stationary for 18 hr and then agitated, growth inhibition occurred.

To test if contact between cells was required for the local production of anti-lymphoma antibody and/or complement in sufficiently high concentrations to affect cell growth, active complement (as rabbit serum) or anti-mouse-γ-globulin serum was added to the cultures at a dilution of 1:10. These sera were adsorbed to remove "anti-mouse activity" as described in Materials and Methods. The results in Table IV show that neither the addition of complement nor anti-globulin sera influenced the synergistic effect obtained with immune

TABLE V

Radiosensitivity of Thymocytes and Normal Bone Marrow in the Synergistic Cytotoxic Test

Cells tested for cytotoxicity*		Per cent growth inhibition at 72 hr
Immune thymocytes	Normal bone marrow	
Not irradiated	Not irradiated	80 ± 8
" "	200 R	76 ± 6
" "	1000 R	60 ± 3
" "	2000 R	42 ± 6
" "	5000 R	0
200 R	Not irradiated	32 ± 5
400 R	" "	0
Thymocytes cultured for 24 hr‡		
(*a*) Without L5178Y		
No irradiation	Not irradiated	54 ± 5
200 R	" "	10 ± 4
(*b*) With L5178Y		
No irradiation	Not irradiated	67 ± 3
200 R	" "	65 ± 4
1000 R	" "	49 ± 2

* Final cell suspension contained 100 thymocytes + 100 normal bone-marrow cells:one lymphoma cell.

‡ Thymocytes incubated for 24 hr in the presence or absence of irradiated L5178Y cells. The thymocytes were then washed by centrifugation and the cells irradiated. Subsequently they were added to a normal bone marrow and fresh L5178Y cell mixture.

thymus cells and normal bone-marrow cells. These results indicate that although cell-to-cell contact was required to inhibit the growth of lymphoma cells in vitro, complement and antibody of the immunoglobulin classes present in mouse serum (see preparation of antiserum in Materials and Methods) were not involved.

Radiosensitivity of the Thymocytes and Bone-Marrow Cells.—Thymocytes and bone-marrow cell suspensions were each exposed separately to doses of X-rays as shown in Table V. After irradiation the suspensions were mixed at a ratio of 100 thymocytes + 100 bone-marrow cells to one lymphoma cell and the cultures assayed 72 hr later for cytotoxic activity. While the thymus cells

were extremely radiosensitive, a dose of 400 R X-rays was sufficient to ablate the cytotoxic activity, bone-marrow cells were found to be more radioresistant, and 2000 R was required to reduce the cytotoxicity of the system by 50%.

Change in Radiosensitivity of Thymocytes.—The cytotoxic action of spleen cells from mice immunized 21 days previously with L5178Y was found to be very radiosensitive, but if these immune spleen cells were exposed to the antigen, they transformed into radioresistant cytotoxic cells (6). A similar change occurred with the radiosensitive immune thymocytes (see Table V). The immune thymocytes were cultured with specific antigen (i.e. L5178Y cells) previously rendered incapable of division by exposure to 5000 R X-rays) for 24 hr, centrifuged, washed, and resuspended before irradiation with X-rays. After

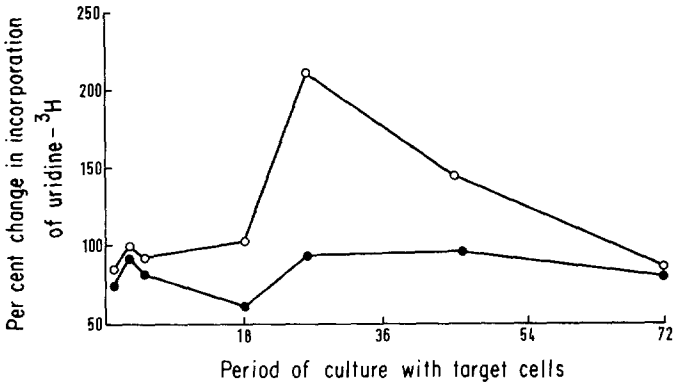

FIG. 2. Increase in RNA synthesis in thymocytes from mice immunized with L5178Y cells after confrontation with irradiated L5178Y cells: (○—○), immune thymocytes + L5178Y cells; (●—●), immune thymocytes. Results expressed relative to uridine incorporation by nonimmune thymocytes cultured with or without L5178Y cells, respectively.

irradiation fresh bone-marrow and live L5178Y cells were added and the mixture assayed for cytotoxic activity 72 hr later. The results in Table V show that the thymus cells cultured without antigen and then irradiated with 200 R X-rays lost their cytotoxic potential but cells cultured in the presence of antigen did not. Further, the majority of the activity was retained after a dose of 1000 R X-rays.

Increase in RNA Synthesis of Immune Thymocytes in Contact with Antigen.—In view of the increase of radioresistance of immune thymocytes after culture with irradiated lymphoma cells, the effect of culturing with irradiated lymphoma cells on RNA synthesis in immune thymocytes was studied (see Fig. 2). To reduce synthesis of nucleic acids by the lymphoma cells, these cells were irradiated with 5000 R of X-rays 24 hr earlier. This dose reduced the capacity of the irradiated cells to incorporate uridine-^{3}H by >90%. At various

times the mixed cell cultures were pulsed for 1 hr with tritiated uridine and the incorporation into acid-insoluble material was determined. The results in Fig. 2 show that thymocytes from immunized mice in the presence of antigen incorporate uridine at an increased rate after the first 20 hr.

Release of an Immunologically Specific Active Factor from Immune Thymocytes in Presence of Antigen.—The finding that RNA synthesis in immune thymocytes was stimulated on coming into contact with specific antigen and that the cytotoxic potential of the thymocytes became radioresistant, suggested that the growth inhibition by immune thymocytes and normal bone-marrow cells occurred in several stages. The first and radiosensitive phase probably involved stimulation of the immune thymocytes by the specific antigen after which the cells become radioresistant. To test if after contact with antigen the

TABLE VI

Capacity of Supernatants from Cultures of C57BL Thymocytes Incubated with Irradiated Lymphoma Cells to Render Normal Bone-Marrow Cells Specifically Cytotoxic

Thymocytes from	Irradiated lymphoma present	Incubation period	Per cent growth inhibition at 72 hr produced by normal bone-marrow cells plus	
			Incubated thymocytes	Supernatant from thymocyte culture
		hr		
Immune	None	24	60	0
Immune	L5178Y	24	66	24
"	"	48	12	57
Nonimmune	L5178Y	48	0	0
Immune	TLX9	48	0	0

thymocytes produced an active factor which interacted with the bone-marrow cell, the supernatant from cultures containing immune thymocytes and antigen were tested for the capacity to render bone-marrow cells cytotoxic. Thymocytes were incubated for 24 and 48 hr with irradiated L5178Y cells at a ratio of 100:1. The supernatant (removed by centrifugation and passed through a 0.45 μ Millipore filter) was added to nonimmune bone-marrow cells, and the capacity of the bone-marrow suspensions to inhibit the growth of the lymphoma cells assessed. Table VI shows that cell-free supernatants from immune thymocytes and antigen rendered the bone-marrow cells cytotoxic but that similar supernatant from nonimmune thymocytes and antigen or from immune thymocytes alone was quite ineffective. Thymocytes cultured for 24 hr had produced an active supernatant and yet the cells were still active when added to normal bone-marrow cells; cells incubated for 48 hr were inactive. The release of the soluble factor was found to be immunologically specific because thymocytes from mice immunized with L5178Y cells did not give an active supernatant on incubation with TLX9 cells.

Nature of the "Cooperating" Cells.—To test the hypothesis that the cells in the bone marrow which are rendered specifically cytotoxic by immune thymocytes belong to the monocyte-macrophage series, the normal bone-marrow cells were pretreated by culturing with either glass beads to remove glass adherent cells or anti-macrophage serum. After treatment the viable cells were assayed by exclusion of 0.10% trypan blue and cell concentrations were corrected to the ratios described elsewhere. Table VII shows after glass bead treatment which removes approximately 20–30% of all the bone-marrow cells, these were no longer cytotoxic in the presence of immune thymocytes. On the other hand, incubation of the immune thymocytes with glass beads did not reduce their capacity to cause cytotoxicity in conjunction with normal bone-marrow cells. Rabbit anti-mouse macrophage serum was adsorbed with lymphoma cells, heat inactivated, and subsequent treatment of thymocyte

TABLE VII

Effect on Cytotoxicity of Immune Thymocytes + Bone-Marrow Cells after Incubation with Glass Beads or Anti-Macrophage Serum

Treatment of*		Per cent growth inhibition at 72 hr
Immune thymocytes	Nonimmune bone-marrow cells	
None	None	69
Glass beads	None	62
None	Glass beads	7
Anti-macrophage serum	None	63
None	Anti-macrophage serum	13

* Incubation of cells with glass beads or 10% anti-macrophage serum as described.

and bone-marrow cell suspensions (in the presence of complement for 30 min) resulted in the death of <5% of the thymocyte suspensions and 35–50% of the bone-marrow cell suspensions. Pretreatment of the thymocytes did not prevent cytotoxic activity whereas bone-marrow cells pretreated in a similar manner were inactivated.

DISCUSSION

The effector mechanisms in allograft immunity are varied and complex. In immune mice both circulating antibody and two classes of directly cytotoxic lymphocytes have been demonstrated (6). Evidence is now accumulating (7, 8) for a third type of lymphoid cell which has the capacity to render monocytes and/or macrophages specifically cytotoxic but by itself has no effect on the target cells.

Soon after immunization cytotoxic cells having the characteristics of immunoblasts can be found in blood, lymph, and the draining node (or spleen). These cells do not persist and are rarely found more than 10 days after appli-

cation of the antigen. Free or cell-bound immunoglobulins have been implicated in their activity (16, 17). A second population of cytotoxic lymphoid cells is found for long periods after immunization in nodes and spleen but to a much lesser extent in the circulation. These cells have some properties associated with memory cells and it appears that before they acquire the capacity to kill target cells, they undergo a biochemical (and possibly morphological) transformation in response to contact with the antigen, which results in their becoming in some respect like immunoblasts. Cells taken from the thymus of suitably immunized mice do not contain these directly cytotoxic lymphoid cell types, but as has been shown here they may still be involved in the effector arm of the immune response. Unexpectedly these thymocytes confer cytotoxic properties onto normal bone-marrow or spleen cells. The cooperative effect of immune thymocytes is abrogated by 400 R or X-rays, whereas the normal bone-marrow cells in the cytotoxic mixture are resistant to 1000 R. Incubation in the presence of lymphoma cells for 24 hr causes them to become less radiosensitive and results in increased RNA synthesis. Hence some form of transformation is induced in the thymocytes by contact with antigen. The cooperative cytotoxic effect requires intimate cell-to-cell contact between immune thymocytes and the cooperating cell as was shown by agitation of the cultures. However, when the immune thymus cells are transformed by contact with antigen, the decrease in radiosensitivity and increasing RNA synthesis are accompanied by the release of a supernatant factor capable of conferring cytotoxic properties onto normal bone-marrow cells. The supernatant alone was not cytotoxic either with or without additional complement which would indicate that the factor is not a cytotoxic antibody. Furthermore, the addition of rabbit anti-mouse-γ-globulin serum did not inhibit cooperative cytotoxicity.

Cells from the bone marrow lose their capacity to cooperate with immune thymocytes after incubation with glass beads and anti-macrophage serum, and it is therefore likely that they are of the monocyte-macrophage series. The suggestion that immune thymocytes release a factor cytophilic for macrophages is in accord with other studies in which peritoneal macrophages were rendered specifically cytotoxic both by contact with immune lymphoid cells and by supernatants from immune lymphoid cells cultured with the specific target cells (8). The release of this putative macrophage arming factor occurs when lymphoid cells from immune animals are exposed to the specific antigen and this material may be related to the migration-inhibition factor (18) with the proviso that it carries the ability to recognize specific antigens. Amos and Lachmann (19) have demonstrated that a material causing inhibition of macrophage migration forms complexes with specific antigens. Chemical investigations are in progress to determine whether the material is an immunoglobulin or an immunologically specific factor having a different constitution.

We consider it unlikely that the class of immune lymphoid cells which are not cytotoxic by themselves but cooperate with nonimmune bone-marrow cells occur only in the thymus. The uniqueness of the thymus as far as effector cells are concerned is that it does not contain either of the types of immune lymphoid cells that are directly cytotoxic and consequently the existence of a cell type which is only cytotoxic in cooperation with another cell can be readily shown. Although the majority of recirculating lymphocytes are in dynamic equilibrium with cells in the lymph nodes and spleen but do not enter the thymus (20, 21), the possibility is not excluded that there may exist a small subpopulation of immune lymphocytes that do pass through the thymus in the course of recirculation. The finding that after immunization with allogeneic lymphoma cells in the thymus there is a phase of rapid depletion (as measured by thymidine incorporation) of dividing cells which is followed by a similarly rapid recovery may be of relevance. This recovery in the DNA synthesis of cells in the thymus is accompanied by the appearance of immune cells in that organ.

Finally, it must be stressed that the cell cooperation effects considered here refer to the process of inhibition of target cells and remain apparently unconnected with cell cooperation between thymus-derived and bone marrow-derived lymphocytes which are involved in the initiation of an immune response.

SUMMARY

Thymocytes from C57BL mice immunized with the DBA/2 lymphoma L5178Y exert in vitro an immunologically specific cytotoxic action against the target cells in the presence of bone-marrow cells. Neither the nonimmune bone marrow nor the immune thymocytes are by themselves cytotoxic. The cells in the bone marrow which take part in the cytotoxic action adhere to glass and are sensitive to anti-macrophage serum. These bone-marrow cells can also be rendered specifically cytotoxic by exposure to the supernatant obtained from a culture of immune thymocytes with the specific target cells. The thymocytes before they are confronted with the specific target cells are very radiosensitive; however, on coming into contact with the target cells, an immunologically specific increase in RNA synthesis occurs and thereafter the thymocytes' capacity to render bone-marrow cells cytotoxic is relatively radioresistant.

Two classes of immune lymphocytes occur in mice immunized with allogeneic cells, those that are capable of killing target cells directly and those that produce a factor capable of rendering macrophages (or monocytes) specifically cytotoxic. In the thymus of immune animals only the latter are found while both categories are present in the spleen and lymph nodes of immune animals.

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