SYNERGY DURING IN VITRO CYTOTOXIC ALLOGRAFT RESPONSES

I. EVIDENCE FOR CELL INTERACTION BETWEEN THYMOCYTES AND PERIPHERAL T CELLS*

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The importance of cell interaction among lymphoid cells in the development of immune responses is well established. In humoral responses bone marrow-derived (B) lymphocytes are recognized to be precursors of the cells that release serum antibody (1-4). Thymus-derived (T) lymphocytes, on the other hand, do not develop into antibody-forming cells (5) but play an important role in primary antibody responses by cooperating with B cells (6, 7).

In contrast, cellular immunity is thought to be mediated by T lymphocytes, apparently without collaboration with B lymphocytes. However, there is increasing evidence to suggest that cooperative interaction occurs among subsets of T lymphocytes (8-11). For example, Cantor and Asofsky proposed that a T1-T2 cell interaction takes place during the initiation of graft-vs.-host responses in F1 neonates (8, 9).

Cytotoxic in vitro allograft responses have been shown to be mediated by T lymphocytes (12, 13). As such, they provide a convenient tool for analyzing the cellular requirements necessary for such a response to occur. Experiments therefore were designed to evaluate in vitro the concept of T-T cell interaction in cellular immunity against transplantation antigens. The results obtained are compatible with the idea that two cell types interact with each other in the initiation of such a response. Moreover, evidence is provided that one cell type acts mainly as helper cells allowing precursor cells of cytotoxic lymphocytes (CL)† to be immunized effectively.

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† Abbreviations used in this paper: CL, cytotoxic lymphocytes; FCS, fetal calf serum; LD, lymphocyte defined; MLC, mixed lymphocyte culture; PBL, peripheral blood lymphocytes; PEC, peritoneal exudate cells; SD, serologically defined; TDL, thoracic duct lymphocytes.

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**Materials and Methods**

**Mice.**—Female CBA/H/Wehi, BALB/c, (CBA/BALB/c)F<sub>1</sub> hybrids, and AKR/J mice aged about 60 days were used throughout.

**Alloantigen.**—Mitomycin C-treated BALB/c spleen cells (H-2<sup>d</sup>) were used as source of cell-bound alloantigen. Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) treatment was performed at a final concentration of 40 μg/ml as described (14).

**Treatment with AKR Anti-θ C3H Serum.**—Anti-θ C3H serum was raised in AKR/J mice by the method of Reif and Allen (15). After inactivation at 56°C for 30 min, its potency was tested by cytotoxicity against CBA thoracic duct lymphocytes (16) and its specificity by the fact that CBA brain absorbed the whole cytotoxicity. Anti-θ serum was used together with agarose-absorbed guinea pig complement under optimal conditions for killing T cells as described elsewhere (13).

**Peritoneal Exudate Cells.**—CBA mice 6–9 mo of age were injected intraperitoneally with 1 ml of sterile proteose peptone broth (Difco Laboratories, Inc., Detroit, Mich.). 4 days later the peritoneal exudate cells (PEC) were harvested into phosphate-buffered saline. PEC were treated with AKR anti-θ C3H serum plus complement before using them in culture.

**Cell Separation Method.**—CBA thymocytes were separated into low-density and high-density cells by centrifugation on a discontinuous albumin gradient (d 1.077 g/cm<sup>3</sup> to 1.088 g/cm<sup>3</sup>) as described by Shortman et al. (16). The low-density cells that have been shown to be unreactive in in vitro allograft responses<sup>2</sup> were used as “filler cells.”

**Cell Preparations.**—Single-cell suspensions of thymocytes and spleen cells were performed as described (13, 14).

**Peripheral Blood Lymphocytes (PBL).**—PBL were prepared by collecting blood of CBA mice into phosphate-buffered saline containing 100 IU heparin per ml. The cells were spun down at 600 g for 7 min and resuspended in 0.17 M NH<sub>4</sub>Cl. After 5 min at room temperature, 1 ml of fetal calf serum (FCS) was layered below the cell suspension and the cells were centrifuged (600 g, 5 min). The pellet of cells was washed once with culture medium.

LN cells were prepared by dissecting the inguinal and axillary LN of CBA mice and teasing the tissues through an 80 gauge stainless steel sieve into cold culture medium. Thoracic duct lymphocytes (TDL) of CBA mice were obtained by cannulation of the thoracic duct basically according to the method described by Woodruff (18) and Sprent (19).

**Allograft Response against H-2<sup>d</sup>-Transplantation Antigens.**—The culture system used was that of Marbrook (20) as described by Diener and Armstrong (21). In brief 15 × 10<sup>6</sup> H-2<sup>d</sup> lymphocytes (either CBA mouse or AKR mouse derived or a mixture of both) were cultured together with 2 × 10<sup>6</sup> mitomycin C-treated allogeneic BALB/c spleen cells. The cell suspension was placed in a glass tube of a diameter of 1.0 cm, sealed off by a dialysis membrane, and suspended from the stopper of a 125 ml Erlenmeyer flask containing tissue culture medium. The cultures were usually terminated at day 6, which was the optimum for the generation of cytotoxic activity (13). Cultures were set up in triplicate. Eagle’s minimal essential medium with supplementary nonessential amino acids was obtained from Grand Island Biological Co., Grand Island, N.Y. This was supplemented with 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia), 100 μg/ml of streptomycin, 100 U/ml of penicillin G and was buffered with sodium bicarbonate. Cultures were placed in a humidified incubator at 37°C in an atmosphere of 10% CO<sub>2</sub> in air.

**Cell-Mediated Cytotoxic Assay.**—The <sup>51</sup>Cr assay used was a modification of that described by Brunner et al. (22) and was performed as described previously in detail (13, 23).

**Labeling of Target Cells.**—As BALB/c (H-2<sup>b</sup>) and DBA/2 (H-2<sup>d</sup>) mice are of identical H-2 specificity, the DBA/2-derived mastocytoma line P815X-2 was used as target cells in the

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<sup>2</sup>Wagner, H., and K. Shortman, manuscript in preparation.
cytotoxicity assays. This cell line was kept in continuous exponential culture by Dr. Alan W. Harris. Usually $5 \times 10^6$ cells were labeled with 100 $\mu$Ci of chromate ($^{32}$Cr) (CEA, Gif-Sur_Yvette, France) in a final volume of 1 ml of fortified Eagle's medium (IFEM) Grand Island Biological Co.) for 30 min at 37°C in an incubator gassed with 10% CO$_2$ in air. The cells were washed twice through FCS and adjusted to a concentration of $10^6$ cells per 100 $\mu$l.

**Assays.** At termination of the culture, cells from triplicate cultures were harvested, pooled, and washed twice. The viability of the cultured cells in an arbitrarily chosen reference group (usually LN-derived CL) was determined by the eosin dye exclusion method and the cell concentration adjusted to 0.66 $\times$ 10$^6$ medium-to-large-sized viable lymphocytes/ml.

Usually a dilution of 1 to 6 (3), and 1 to 36 (18) was performed and triplicates of 1 ml of each cell dilution were assayed for cytotoxicity against $5 \times 10^4$ target cells. In actual fact 1 ml of the respective cell suspension was pipetted to 35 $\times$ 10 mm Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and 50 $\mu$l of target cells ($5 \times 10^4$) were added, resulting in a ratio of cultured viable cells to $^{41}$Cr-labeled target cells given. Each of the assays was performed in triplicate. Similarly to the cells of the reference group, cells of the other cultures were harvested, pooled and adjusted to the same volume as the reference cells, and diluted in equal manner in order to compare on a culture basis the cytotoxic activity generated relative to that obtained in the reference culture. The dishes were placed in an airtight box, gassed with 10% CO$_2$, and rocked on a platform for 200 min. The cells were then harvested and transferred into plastic tubes; a drop of 20% sheep erythrocytes solution was added; the cells were centrifuged; and the supernatant was separated from the pellet. The radioactivities of the supernatant and pellet were determined in an automatic well-type radiation counter and the results expressed as per cent of maximal $^{32}$Cr release as determined by freezing and thawing $5 \times 10^6$ labeled target cells four times (13).

**X-Irradiation.** Thymocytes were irradiated with 800 rad in a Philips (RT 250) X-ray machine. Details of the irradiation technique for cells have been given by Miller and Sprent (24).

**Adult Thymectomy.** Adult thymectomies of CBA mice were performed according to the method of Miller (25).

**RESULTS**

**Heterogeneity among T Lymphocytes in their Capacity to Mount a Cell-Mediated Immune Response (CMI) In Vitro.**—The generation of CL in in vitro mouse allograft responses has been shown to be a function of thymus-derived (T) lymphocytes (12, 13). It was of interest therefore to investigate whether there exists a heterogeneity within T lymphocytes as obtained from different tissues in regard to their capacity to differentiate into cytotoxic effector cells. First, the number of mitomycin C-treated allogeneic BALB/c spleen cells that elicited in $15 \times 10^5$ CBA mouse-derived responder cells (H-2k) optimal cytotoxic allograft responses was determined. It was found that $2 \times 10^6$ mitomycin C-treated allogeneic BALB/c spleen cells were optimal (Fig. 1). In a second step, a quantitative comparison of the cytotoxic responses obtained with different responder cells was performed. In these experiments thymocytes, splenic lymphocytes, LN cells, PBL, and TDL were tested. The respective cell populations ($15 \times 10^6$) were cultured with cell-bound alloantigen ($2 \times 10^6$ mitomycin C-treated BALB/c spleen cells) for 6 days and the cytotoxic response generated was compared on a culture basis relative to that obtained with LN
cells. The results of a typical experiment are illustrated in Fig. 2. Two points are to be discussed. First, there existed a linear relationship between percentage of lysis and the ratio CL to target cells used, if the latter was plotted in a log scale. Consequently this linear relationship allowed the determination of the ratio CL to target cells necessary to obtain 50% lysis. Since the overall number of responder cells at the initiation of the culture was kept identical, the precursor cell potential for CL of the different cell populations could thus be compared quantitatively in terms of the ratio CL to target cells that resulted in 50% lysis. The reciprocal value of this ratio was defined as lysis unit. Second, within thymus-processed lymphocytes, there existed a heterogeneity in their capacity to differentiate in vitro into cytotoxic effector cells. Thus LN cells, PBL, and TDL (which generated CL able to lyse 50% of the target cells at a ratio of about 1:1) were found to be four times more reactive than splenic

Fig. 1. Dose-response relationship between cytotoxic allograft response generated and number of allogeneic stimulator cells used. CBA mouse-derived LN cells (15 × 10⁶) were cultured for 6 days together with a graded number of mitomycin C-treated BALB/c spleen cells. In vitro-generated cytotoxicity was compared relative to that obtained when 2 × 10⁶ allogeneic stimulator cells were used. Single stars represent background lysis of P815 target cells in the presence of normal LN cells.
lymphocytes (50% lysis at a ratio of about 4:1) and 20-fold more reactive than thymocytes. That in all cell populations the responding cells were \(\theta\)-positive cells was strongly suggested by the fact that treatment of the reactive cells before culture with AKR anti-\(\theta\) C3H serum plus complement abolished their capacity to generate CL in vitro and that in vitro-generated CL were susceptible for lysis by such a treatment (12, 13 and Tables III and V).

**Limiting Dilution of Responder Cells.**—The effect of "limiting" dilution of responder lymphocytes per culture upon the magnitude of cytotoxic allograft response generated was investigated. In these experiments CBA mouse-derived LN cells (CBA LN cells) were used (containing about 80% of peripheral T lymphocytes). Since macrophages have been shown to be essential in the induction phase of T cell-mediated in vitro allograft responses (23) and since PEC could substitute for macrophages, 0.5 \(\times\) 10⁶ CBA mouse-derived PEC devoid of T cells (23) were added per culture to provide a sufficient number of macrophages. To keep the cell density per culture of CBA-derived lymphocytes constant, advantage was taken of the fact that high density (1.077 g/cm³) CBA thymocytes obtained from cell fractions of a continuous albumin gradient were found to be unresponsive in in vitro allograft responses.³ Thus high-

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density CBA thymocytes were used as a source of inert CBA lymphocytes in order to keep the number of CBA lymphocytes (15 × 10⁶) per culture constant. The results of a representative experiment are given in Fig. 3. There was a sigmoidal relationship noted between the number of reactive LN cells and the cytotoxic response generated. For example, 1–5 × 10⁶ CBA LN cells resulted in poor cytotoxic responses. About 15 × 10⁶ reactive LN cells per culture were found to generate optimal cytotoxic allograft responses. Similar results were obtained using X-irradiated CBA thymocytes as source of filler CBA lymphocytes.

Fig. 3. Effect of limiting dilution of responder cells upon cytotoxic response generated. A graded number of LN cells (from 1–15 × 10⁶) was cultured together with 2 × 10⁶ mitomycin C-treated allogeneic BALB/c spleen cells. In order to keep the cell density in all cultures constant (15 × 10⁶) varying numbers of high-density thymocytes were used as filler cells. In vitro-generated cytotoxicity was compared on a culture basis relative to that obtained with 15 × 10⁶ LN cells. The results are expressed in lysis units.

Synergy among Thymocytes and LN Cells.—Since few CBA LN cells (1–5 × 10⁶) in the presence of high-density or X-irradiated thymocytes yielded poor cytotoxic allograft responses when cultured together with cell-bound allo-antigen (Fig. 3), the cytotoxic response obtained in a mixture of few CBA LN cells (1.5 × 10⁶) and normal CBA thymocytes (14 × 10⁶) was investigated. Surprisingly it was found that such a cell mixture yielded cytotoxic allograft responses the magnitude of which equaled or exceeded that obtained using 15 × 10⁶ CBA LN cells as responder cells (Fig. 4). Since 15 × 10⁶ thymocytes alone and 1.5 × 10⁶ CBA LN cells (in the presence of 14 × 10⁶ high-density
or X-irradiated thymocytes) generated poor cytotoxic activity, the magnitude of cytotoxic response obtained with a mixture of both was about 20-fold greater than one would predict. Similar types of synergy could be demonstrated using mixture of $1.5 \times 10^6$ CBA mouse-derived PBL or TDL and syngeneic thymus cells (Tables I and II).

The Cellular Requirements for the Synergistic Effect.—CBA LN cells (or PBL or TDL) represent a mixture of about 70–80% peripheral T cells and 20% B lymphocytes (26). It was therefore of interest to determine the cell type responsible within LN cells for the synergistic phenomena described. The fact that treatment of LN cells (or TDL) with AKR anti-$\theta$ C3H serum plus complement before culture abolished the capacity of the remaining cells to mount cytotoxic allograft responses and to yield synergism with thymocytes strongly suggested that they were T cells in type (Table III). In addition, mitomycin C treatment of both thymocytes or LN cells abrogated the synergistic effect indicating that both cell populations must be able to undergo cell proliferation. This conclusion was supported by the finding that (CBA × BALB/c) $F_1$
### TABLE I

**Synergy between Thymocytes and PBL in Cytotoxic Allograft Responses**

| Responder cells (H-2*) | % Lysis (%Cr assay)* | Ratio CL to target cells |
|-----------------------|----------------------|--------------------------|
|                       | 12:1                 | 2:1                      | 0.3:1                    |
| 15 × 10^6 PBL         | 100                  | 75 ± 2.8                 | 30 ± 1.2                 |
| 15 × 10^6 thymocytes  | 42 ± 1.5             | 20 ± 0.4                 | 12 ± 2.0                 |
| 1.5 × 10^6 PBL plus 14 × 10^6 irradiated thymocytes | 24 ± 2.1 | 13 ± 1.7 | 12 ± 1.9 |
| 1.5 × 10^6 PBL plus 14 × 10^6 high-density thymocytes | 27 ± 0.6 | 15 ± 2.1 | 11 ± 3.6 |
| 1.5 × 10^6 PBL plus 14 × 10^6 thymocytes | 100 | 82 ± 0.6 | 33 ± 1.5 |

* Responder cells (H-2*) were cultured together with 2 × 10^6 mitomycin C-treated allogeneic BALB/c (H-2*) spleen cells. Cytotoxic activity generated in vitro was assayed in a 51Cr test for 200 min. Cytotoxicity obtained was compared on a culture basis relative to the cytotoxicity generated by PBL-derived CL. Background lysis of P815 target cells (in the presence of normal lymphocytes) was 12 ± 1.3%.

### TABLE II

**Synergy between Thymocytes and TDL in Cytotoxic Allograft Responses**

| Responder cells (H-2*) | % Lysis (%Cr assay)* | Ratio CL to target cells |
|-----------------------|----------------------|--------------------------|
|                       | 12:1                 | 2:1                      | 0.3:1                    |
| 15 × 10^6 TDL         | 98 ± 1.9             | 64 ± 2.4                 | 36 ± 1.8                 |
| 15 × 10^6 thymocytes  | 24 ± 0.3             | 13 ± 1.5                 | 9 ± 2.1                  |
| 1.5 × 10^6 TDL plus 14 × 10^6 irradiated thymocytes | 21 ± 1.4 | 11 ± 1.6 | 10 ± 0.9 |
| 1.5 × 10^6 TDL plus 14 × 10^6 thymocytes | 100 | 61 ± 1.7 | 35 ± 1.2 |

* Responder cells (H-2*) were cultured with 2 × 10^6 mitomycin C-treated allogeneic BALB/c (H-2*) spleen cells. Cytotoxic activity generated in vitro was assayed in a 51Cr test for 200 min. Cytotoxicity obtained was compared on a culture basis relative to the cytotoxicity generated by PBL-derived CL. Background lysis of P815 target cells (in the presence of normal lymphocytes) was 9 ± 1.7%.
## TABLE III

**Cellular Requirements for Synergistic Effect**

| Responder cells (H-2k)                                                                 | % Lysis (%Cr Test)* | Ratio CL to target cells |
|--------------------------------------------------------------------------------------|---------------------|--------------------------|
| 15 × 10⁶ CBA LN cells                                                                 | 100                 | 79 ± 1.9                 |
| 1.5 × 10⁶ CBA LN cells plus 14 × 10⁶ thymocytes                                        | 100                 | 72 ± 2.3                 |
| 1.5 × 10⁶ CBA LN cells treated with anti-θ serum and C plus 14 × 10⁶ thymocytes        | 29 ± 1.2            | 14 ± 2.7                 |
| 1.5 × 10⁶ CBA LN cells plus 14 × 10⁶ thymocytes mitomycin C treated                   | 24 ± 2.4            | 13 ± 1.4                 |
| 1.5 × 10⁶ CBA LN cells mitomycin C treated plus 14 × 10⁶ thymocytes                   | 31 ± 1.1            | 15 ± 1.8                 |
| 1.5 × 10⁶ F₁(CBA × BALB/c) LN cells plus 14 × 10⁶ thymocytes                          | 28 ± 1.7            | 14 ± 0.9                 |
| 1.5 × 10⁶ CBA LN cells plus 14 × 10⁶ F₁(CBA × BALB/c) thymocytes                      | 21 ± 1.3            | 13 ± 2.8                 |

*C = complement.

*Responder cells were cultured together with 2 × 10⁶ mitomycin C-treated allogeneic BALB/c (H-2k) spleen cells. Cytotoxic activity generated was assayed in a %Cr test for 200 min. Cytotoxic activity generated was compared on a culture basis relative to the cytotoxicity generated by 15 × 10⁶ CBA LN cells. Background lysis of P815 target cells (in the presence of normal lymphocytes) was 10 ± 1.0%.

Hybrid mouse-derived thymocytes or LN cells, which are genically tolerant of the allointigen used, could not generate the synergistic effect. In one out of five experiments, however, a mixture of (CBA × BALB/c) F₁ mouse-derived thymocytes and CBA LN cellsgenerated enhanced cytotoxic allograft responses. No synergy with either thymocytes or LN cells was observed when CBA spleen cells were used as reacting lymphocytes (Table IV). In contrast subpopulations of CBA spleen cells separated on a continuous albumin gradient produced synergy together with peripheral T cells. The results given in Table IV il-

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4 Wagner, H., and K. Shortman, manuscript in preparation.
**TABLE IV**

Failure of Synergy between Thymocytes and Spleen Cells

| Responder cells (H-2k) | %-Lysis (%Cr test)* | Ratio CL to target cells |
|------------------------|---------------------|-------------------------|
| **12:1**               |                     |                         |
| 15 × 10⁶ LN cells      | 100                 | 82 ± 1.4                |
| 15 × 10⁶ AT-LN cells   | 100                 | 56 ± 4.1                |
| 15 × 10⁶ thymocytes    | 26 ± 1.4            | 11 ± 1.6                |
| 1.5 × 10⁶ LN cells plus 14 × 10⁶ thymocytes | 100 | 53 ± 2.3 |
| 1.5 × 10⁶ AT-LN cells plus 14 × 10⁶ thymocytes | 100 | 86 ± 1.8 |
| 15 × 10⁶ spleen cells  | 82 ± 1.1            | 43 ± 1.5                |
| 1.5 × 10⁶ AT-LN cells plus 14 × 10⁶ spleen cells | 80 ± 2.8 | 45 ± 1.0 |
| 1.5 × 10⁶ LN cells plus 14 × 10⁶ spleen cells | 84 ± 0.8 | 40 ± 2.3 |
| 1.5 × 10⁶ spleen cells plus 14 × 10⁶ thymocytes | 39 ± 1.6 | 14 ± 3.3 |

*AT-LN = LN cells of adult thymectomized mice 6 wk after operation. Responder cells were cultured together with 2 × 10⁵ mitomycin C-treated allogeneic BALB/c (H-2d) spleen cells. Cytotoxic activity generated was assayed in a %Cr test for 200 min. Cytotoxicity was compared on a culture basis relative to the cytotoxicity generated by 15 × 10⁶ CBA LN cells. Background lysis of P815 target cells was 10 ± 2.3%.

Intricate also that LN cells of adult thymectomized CBA mice were found to produce a high degree of synergy together with CBA thymocytes.

**Limiting Dilution of LN Cells in the Presence of Normal Thymocytes.**—The effect of a limiting dilution of LN cells in regard to the synergy of thymocytes and LN cells during cytotoxic allograft responses was investigated. A graded number of CBA LN cells (from 2 × 10⁶ to 0.1 × 10⁶) was mixed with 14 × 10⁶ CBA thymocytes and the cell mixture cultured together with cell-bound alloantigen. The results given in Fig. 5 demonstrate that as few as 0.1 × 10⁶ LN cells, a number far too small to produce any response alone (Fig. 2), resulted in significant enhancement of the cytotoxic response generated. When for each group the ratio CL to target cells was determined that resulted in 50% lysis and the lysis units were plotted against the respective number of LN cells...
Fig. 5. Effect of limiting dilution of LN cells upon the synergistic interaction between thymocytes and LN cells. □-□-□, thymocytes (15 × 10⁶); ●-●-●, mixture of LN cells (2 × 10⁶) plus 14 × 10⁶ irradiated thymocytes. The cell concentration indicates the number of LN cells cultured together with 14 × 10⁶ thymocytes. Each of the respective lymphocyte populations was cultured together with 2 × 10⁶ mitomycin C-treated BALB/c spleen cells. In vitro-generated cytotoxicity was compared on a culture basis relative to that obtained with a mixture of LN cells (2 × 10⁶) and thymocytes (14 × 10⁶). ● represents background lysis of P815 target cells.

Effect of Graded Numbers of Thymocytes in the Presence of a Constant Number of LN Cells.—Since the magnitude of cytotoxic responses generated by a mixture of 14 × 10⁶ thymocytes and limited numbers of LN cells appeared to correlate to the number of LN cells present (Fig. 6), it was of interest to perform the reciprocal experiment. Assuming that the majority of precursor cells of CL was LN cell derived and the thymocytes provided a source of “helper cells,” one would predict that a critical number of thymocytes was sufficient to allow all potential reactive LN cells to be activated. Further increase of the number of thymocytes beyond optimal concentration should not alter markedly the final magnitude of the cytotoxic response obtained. The experiment to be performed involved three cell populations: a constant number of CBA LN cells (1.5 × 10⁶), a graded number of CBA thymocytes, and 2 × 10⁶ mitomycin C-treated allogeneic BALB/c spleen cells. The results of a typical experiment are illustrated in Fig. 7. It is immediately apparent that in the

used, a linear relationship between cytotoxic activity generated and the number of reactive LN cells was noted (Fig. 6). This finding suggested that the magnitude of the enhanced cytotoxic activity generated in a mixture of thymocytes and LN cells correlated to the number of reactive LN cells present.

Effect of Graded Numbers of Thymocytes in the Presence of a Constant Number of LN Cells.—Since the magnitude of cytotoxic responses generated by a mixture of 14 × 10⁶ thymocytes and limited numbers of LN cells appeared to correlate to the number of LN cells present (Fig. 6), it was of interest to perform the reciprocal experiment. Assuming that the majority of precursor cells of CL was LN cell derived and the thymocytes provided a source of “helper cells,” one would predict that a critical number of thymocytes was sufficient to allow all potential reactive LN cells to be activated. Further increase of the number of thymocytes beyond optimal concentration should not alter markedly the final magnitude of the cytotoxic response obtained. The experiment to be performed involved three cell populations: a constant number of CBA LN cells (1.5 × 10⁶), a graded number of CBA thymocytes, and 2 × 10⁶ mitomycin C-treated allogeneic BALB/c spleen cells. The results of a typical experiment are illustrated in Fig. 7. It is immediately apparent that in the
presence of less than $2 \times 10^6$ CBA thymocytes a poor cytotoxic response was obtained. There was a sharp rise of the cytotoxic response when $2-4 \times 10^6$ thymocytes were cultured together with $1.5 \times 10^6$ LN cells. More than $6 \times 10^6$ thymocytes yielded only a limited further increase of the cytotoxic response.

![Graph showing linear relationship between lysis units and LN cells](image1)

**Fig. 6.** Linear relationship between lysis units calculated from Fig. 5 to the number of LN cells used.

![Graph showing effect of limiting dilution of thymocytes](image2)

**Fig. 7.** Effect of limiting dilution of thymocytes in the presence of a constant number of LN cells ($1 \times 10^6$) upon the magnitude of cytotoxic response generated. △-△-△, response of a mixture of a graded number of thymocytes in the presence of a constant number ($1 \times 10^6$) of LN cells; ○-○-○, response of thymocytes alone. Each of the respective lymphocyte populations was cultured together with $2 \times 10^6$ mitomycin C-treated BALB/c spleen cells. In vitro-generated cytotoxicity was compared on a culture basis relative to that obtained with a mixture of LN cells ($1 \times 10^6$) and thymocytes ($14 \times 10^6$). The results are expressed in lysis units.
Evaluation of the Respective Contribution of Thymocytes and LN Cells to the Final Cytotoxic Activity Generated.—AKR mice and CBA mice are of identical H-2 specificity (27) but differ in the ϕ-antigen phenotype (26). The possibility was therefore tested of producing synergy in a mixture of AKR thymocytes and CBA LN cells (or vice versa) and of using AKR anti-ϕ C3H serum in order to kill out the CBA mouse-derived ϕ-positive CL before the 51Cr cytotoxicity assay. It was argued that the remaining CL might then be identified as being AKR thymocyte derived (or vice versa). The experiments given in Table V.

| Responder cells (H-2^k) | % of Lysis (%Cr test)* | Ratio CL to target cells |
|-------------------------|------------------------|-------------------------|
| 1.5 × 10^6 AKR-LN cells plus 14 × 10^5 CBA thymocytes | 100 | 83 ± 2.3 | 41 ± 0.4 |
| 15 × 10^6 CBA thymocytes | 38 ± 2.1 | 23 ± 1.4 | 10 ± 0.8 |
| 15 × 10^6 AKR thymocytes | 19 ± 1.9 | 10 ± 2.2 | 9 ± 2.1 |
| 1.5 × 10^6 AKR-LN cells plus 14 × 10^5 irradiated CBA thymocytes | 21 ± 2.0 | 11 ± 1.5 | N.D. |
| * 1.5 × 10^6 AKR-LN cells plus 14 × 10^5 AKR thymocytes | 100 | 72 ± 2.4 | 34 ± 1.2 |
| Cytotoxic activity after AKR anti-ϕ C3H serum treatment plus C | 100 | 69 ± 1.6 | 33 ± 0.7 |
| ‡ 1.5 × 10^6 CBA LN cells plus 14 × 10^5 CBA thymocytes | 99 ± 2.9 | 56 ± 1.6 | 23 ± 2.5 |
| Cytotoxic activity after AKR anti-ϕ C3H serum treatment plus complement | 10 ± 2.1 | 9 ± 1.4 | 9 ± 3.1 |

N.D. = not done.

* Responder cells (H-2^k) were cultured together with 2 × 10^6 mitomycin C-treated allogeneic BALB/c spleen cells. In the case of groups * and ‡, nine cultures were set up instead of three. After 6 days culture cells per group were harvested. Each cell pool of group * and ‡ was divided into three equal parts. One part remained untreated, one part was treated with normal AKR serum plus complement, and one part was treated with AKR anti-ϕ C3H serum plus complement. Cytotoxic activity of each cell pool was then assayed in a 51Cr test for 200 min and compared relative to the cytotoxic activity obtained with a mixture of AKR-LN plus CBA thymocytes. Background lysis of P815 target cells was 9 ± 1.7%.
demonstrate that synergy occurred within a mixture of AKR thymocytes and CBA LN cells or CBA thymocytes and AKR LN cells, provided the cell mixtures were cultured together with alloantigen. In addition, evidence was obtained that AKR mouse-derived CL, unlike CBA mouse-derived CL, were not killed by treatment with AKR anti-$\phi$ C3H serum. Therefore, AKR anti-$\phi$ CBA serum could be used in order to discriminate between CBA $\phi$-positive cells within a CL cell population. Fig. 8 a illustrates the results of a quantita-

Fig. 8. Discrimination between thymus or T cell-derived CL. (a) $\triangle-\triangle-\triangle$, cytotoxic allograft response obtained by a mixture of AKR mouse thymocytes (14 X 10$^6$) and 1.5 X 10$^6$ CBA mouse-derived LN cells; $\bigcirc-\bigcirc-\bigcirc$, residual activity after treatment with AKR anti-$\phi$ C3H serum plus complement. $\square-\square-\square$, response of 15 X 10$^6$ AKR thymocytes. $\ast-\ast-\ast$, response of 1.5 X 10$^6$ CBA LN cells in the presence of 14 X 10$^6$ high-density thymocytes. (b) $\triangle-\triangle-\triangle$, cytotoxic allograft response obtained by a mixture of CBA mouse thymocytes (14 X 10$^6$) and 1.5 X 10$^6$ AKR mouse LN cells. $\bigcirc-\bigcirc-\bigcirc$, residual activity after treatment with AKR anti-$\phi$ C3H serum plus complement; $\ast-\ast-\ast$, response of 1.5 X 10$^6$ AKR LN cells in the presence of high-density thymocytes; $\square-\square-\square$, response of 15 X 10$^6$ CBA thymocytes.

of the contribution of 14 X 10$^6$ AKR mouse-derived thymocytes within a mixture of AKR thymocytes and 1.5 X 10$^6$ CBA LN cells to the final cytotoxic activity. It is apparent that about 70% of the overall cytotoxicity was specifically lost after treatment with AKR anti-$\phi$ C3H serum. The results of the reciprocal experiment given in Fig. 8 b suggest that 1.5 X 10$^6$ AKR mouse-derived LN cells when cocultivated with 14 X 10$^6$ CBA thymocytes contributed about 70% to the final cytotoxic activity. These experiments suggested that within a mixture of thymocytes and LN cells the majority of precursor
cells of CL was LN derived. However, also the cytotoxic response of the thymocytes was increased in the presence of reacting LN cells. Thus, whereas thymocytes when cultured alone with alloantigen yielded CL able to lyse 30–35% of the target cells at a ratio of 12:1 (Fig. 8 b), in the presence of responding LN cells up to 70% of the target cells were lysed by CBA mouse thymus-derived CL.

DISCUSSION

This paper describes a synergistic cell interaction between murine thymocytes and peripheral T lymphocytes during in vitro cytotoxic allograft responses. Synergy was demonstrated by combining T cells from tissues comparatively active in mounting in vitro cytotoxic allograft responses such as lymph node cells, PBL, or TDL with cells from a tissue with less activity, such as the thymus. Evidence was obtained that the bulk of the cytotoxic activity generated was mediated by CL derived from peripheral T cells. Yet peripheral T cells required the presence of immune reactive thymocytes in order to be optimally immunized in vitro.

Cytotoxic in vitro allograft responses are mediated by T lymphocytes (13) and thus provide a convenient tool for analyzing the concept of T-T cell interactions (11) during cell-mediated immune responses. It was noted that T-cell populations as obtained from lymph nodes or the thoracic duct of nonimmunized mice were able to mount a good cytotoxic allograft response in vitro. Therefore it was concluded that if T-T cell interactions occurred during in vitro cytotoxic allograft responses, then both T-cell subsets must be present in the responder T-cell population. Consequently, we first screened different T-cell sources and assessed their relative capacities to mount a cytotoxic allograft response on their own. It was found that lymphocytes obtained from tissues containing about 80% (25) of recirculating T cells such as LN cells, PBL, or TDL generated up to 20–30-fold more cytotoxic activity than did thymocytes. Spleen cells were found to be three to four times more active (Fig. 2). In order to test for the concept of T-T cell interaction in the in vitro allograft response, $1.5 \times 10^6$ LN cells, numbers too small to yield good cytotoxic effector cells (Fig. 3), were mixed with syngeneic thymocytes ($14 \times 10^6$) and cultured in vitro together with allogeneic lymphoid cells. Such cell mixtures proved to yield cytotoxic responses that exceeded by far the expected sum of the control cultures and that were comparable in magnitude to those obtained with $15 \times 10^6$ LN cells (Fig. 3, Tables I and II).

The fact that the reactive cells within LN cells were $\phi$-positive suggested strongly that they were T lymphocytes (Table III). As synergy between thymocytes and T cells was inhibited by treatment of either cell population with mitomycin C, an agent which inhibits cell proliferation, it appeared that for synergy to occur, both thymocytes and T cells had to be able to undergo cell proliferation. Further support for this notion was obtained by the finding that
lymphocytes derived from (CBA × BALB/c) F1 hybrid mice, which for genetic reasons are unresponsive against BALB/c alloantigens, were ineffective in producing synergy (Table III). No explanation can be offered at present, as to why in one experiment out of five, thymocytes derived from F1 hybrid mice produced synergy with CBA LN cells in the response to H-2^K alloantigen. It was also found that spleen cells, either mixed with LN cells or with thymocytes, failed to produce synergy (Table IV), although spleen cells of mice treated in vivo with moderate doses of antithymocyte serum produced synergy with peripheral T cells. Moreover, when spleen cells were separated on a continuous albumin gradient, distinct cell fractions were obtained that yielded synergy with peripheral T cells. Finally, as TDL obtained from adult thymectomized mice 6 wk after operation were efficient in producing synergy together with thymocytes, it appeared that long-lived, recirculating T lymphocytes were interacting with thymocytes in the in vitro system used (H. W., unpublished observations).

Limiting dilution experiments performed with both thymocytes and recirculating T cells suggested that the magnitude of the cytotoxic response obtained correlated to the number of peripheral T cells present rather than to the number of thymocytes (Fig. 6 and 7). This notion was supported by the fact that the bulk (about 70%) of the cytotoxic activity generated within a mixture of thymocytes (14 × 10^6) and LN cells (1 × 10^6) was LN cell derived (Fig. 8). Therefore it appeared that thymocytes were mainly acting as helper cells. The results reported are similar to those of Cantor and Asofsky (8, 9) as obtained in a graft-vs.-host system, with the exception that under the in vitro conditions used here the major source of precursor cells of effector cells appeared to be provided by peripheral T cells.

At present the mechanism of the T-T cell synergism during in vitro allograft responses is not understood. Any interpretation should take into account (a) that the allograft system used is modeled on a "one-way" mixed lymphocyte culture (MLC) using allogeneic lymphoid cells as stimulator cells and consider (b) the recent demonstration of a third locus (MLC locus) in the genetic region of the major histocompatibility complex (MHC) (28-30). Unlike the two loci (H-2K and H-2D in the mouse), which control serologically defined (H-2) antigens, the third locus (MLC locus) appears to be responsible for allogeneic differences that result in cell proliferation in the MLC, yet are not detectable serologically (lymphocyte-defined [LD] antigens). In addition, all evidence available suggests that the cytotoxic action of CL is directed against serologically defined (SD) (H-2) antigens of the target cells (29, 31). Thus the paradoxical situation has to be considered that two "antigens" (SD and LD) appear to be responsible for the MLC reaction, however, that the cytotoxic effector phase is directed only against SD antigens. Therefore it is proposed that during the in vitro response against allogeneic lymphocytes,

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functional distinct T-cell populations are stimulated by different "antigens." T1 cells (thymocytes or short-lived T cells) respond mainly against allogeneic LD differences; T2 cells (long-lived, recirculating T cells) react immunologically specific against allogeneic SD (H-2) antigens and differentiate into cytotoxic effector cells. The proliferative response of T1 cells to allogeneic LD differences results in an amplification of a concomitant T2 anti-SD (H-2) reaction. The mechanism of amplification may be mediated by a soluble mediator produced by T1 cells.

The above hypothesis predicts that allogeneic cells, such as fibroblasts, which do not display LD antigens, will not provoke the T1 cell-derived helper mechanisms for the stimulation of SD antigen-reactive T2 cells. This might be the explanation why allogeneic fibroblasts do not cause as great cytotoxic allograft responses as do allogeneic lymphoid cells (32). Since mouse strains are now available differing only either in SD or LD antigens (30, 33), the validity of the proposed concept can soon be tested.

SUMMARY

A mouse in vitro allograft system was used to evaluate the concept of T-T interaction in T cell-mediated cellular immunity. In analyzing the responsiveness of thymus-processed lymphocytes as obtained from different tissues, a heterogeneity within T cells was found in regard to their capacity to be immunized in vitro against transplantation antigens. Recirculating T cells were 10-20-fold superior to thymocytes, splenic T cells being intermediate. When few (1.5 × 10⁶) peripheral T cells, in numbers too small to yield good cytotoxic responses, were mixed with 14 × 10⁶ thymocytes and the cell mixture immunized in vitro against cell-bound alloantigens, cytotoxic activity was generated exceeding about 10-20-fold the values that could be explained by a pure additive effect. Synergy occurred also in a mixture of responder T cells derived from CBA (H-2k) and AKR (H-2k) mice. Thus AKR anti-C3H serum could be used for discriminating between thymus-derived and peripheral T cell-derived cytotoxic lymphocytes (CL). Cytotoxic activity produced during the synergistic interaction between thymocytes and peripheral T cells was about 70% T cell derived, the remainder being thymus derived. The synoptic interpretation of this finding and "limiting dilution" experiments of the responder cells suggested strongly that peripheral T cells provide the major source for precursor cells of CL, thymocytes acting mainly as helper (amplifier) cells.

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