

# GENERATION OF CYTOTOXIC LYMPHOCYTES IN MIXED LYMPHOCYTE REACTIONS

## I. SPECIFICITY OF THE EFFECTOR CELLS\*

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The mixed lymphocyte reaction (MLR)<sup>1</sup> represents an in vitro system for the induction of specific immune reactions against the major histocompatibility antigens of the species. In the one-way MLR, where parental cells respond to semisyngeneic F<sub>1</sub> hybrids (1), it has been found that thymus-derived (T) cells are needed both for the development of the proliferative stage of the reaction and for the generation of effector cells capable of being specifically cytotoxic (2-6). Furthermore, cytotoxicity itself appears to be a property of such T cells (5).

Several authors have demonstrated specificity in these in vitro reactions (5, 7, 8), whereas others have found that nonspecific killing of "innocent bystander" cells can occur both in vivo and in vitro (9-12). This effect is best observed when there is close contact between the innocent bystander cell and the specific target undergoing the homograft reaction. Under such conditions, it is difficult to ascertain whether killing of the innocent bystander is a result of direct action of the effector cell or is caused by various indirect effects. Thus, it has not been critically excluded that binding of the effector cell to the specific target may cause the release of short-lived toxic products from either the effector or the damaged target cells, which secondarily may damage the innocent bystander cells in close proximity. Nor is it known whether there is a requirement for the effector cell to bind to the specific target in order for these non-specific effects to occur.

In this report we demonstrate that agglutination of specifically activated effector cells, generated in a unidirectional H-2-incompatible MLR, to H-2-identical targets results in target cell killing. This indicates that the thymus-derived effector cell population activated in a MLR can directly kill innocent

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<sup>1</sup> Abbreviations used in this paper: BSS, balanced salt solution; Con A, concanavalin A; [<sup>125</sup>I]UdR, <sup>125</sup>I-labeled iododeoxyuridine; MLR, mixed lymphocyte reaction(s); PHA, phytohemagglutinin.

bystanders provided the effector cells are brought into close contact with the targets. Furthermore, effector cells need not bind specifically to the targets by antigen-binding receptors for killing to occur.

### *Materials and Methods*

*Animals.*—Mice of the following inbred strains and their F<sub>1</sub> hybrids were used: A, CBA, C57Bl, (A × CBA)F<sub>1</sub>, and (A × C57Bl)F<sub>1</sub>. All mice were aged-matched and of the same sex in individual experiments.

*Target Cell Tumor.*—The YAC (*H-2<sup>a</sup>*) Moloney virus-induced ascites leukemia was obtained from the Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden.

*Isotope.*—<sup>125</sup>I Iododeoxyuridine (<sup>125</sup>I UdR) was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England.

*Mitogens.*—Phytohemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, England. Concanavalin A (Con A) was the same as has been described previously (13). Both mitogens were dissolved in balanced salt solution (BSS), and 0.1 ml of various concentrations was added to the appropriate MLR cultures at the same time as the labeled target cells were added.

*Isotope Labeling of Tumor Cells.*—YAC cells were removed from A/Sn mice between the 6–8th day after intraperitoneal (i.p.) inoculation of 10<sup>6</sup> cells. The tumor cells were washed in BSS and then cultured in minimal essential medium with supplements and 20% fetal calf serum (14) at a concentration of 1.0–1.5 × 10<sup>6</sup> cells/ml. <sup>125</sup>I UdR was added so that the concentration of the isotope in the solution was 0.07 μCi/ml. After 6 h, the suspension was centrifuged and washed before use.

*Immunizations.*—Mice were immunized by one i.p. inoculation of 10<sup>7</sup> spleen cells. 7 days later, the animals were sacrificed and their spleens were excised using sterile technique. The cells were pooled within groups and a cell suspension was made in medium, as described by Mishell and Dutton (15), supplemented with 10% heat-inactivated fetal calf serum. Viable cells were determined by trypan blue staining.

*Mixed Lymphocyte Cultures.*—Spleens were removed from mice and cell suspensions were prepared in BSS. After washing, the cells were resuspended in media with heat-inactivated fetal calf serum and adjusted to a concentration of 10 × 10<sup>6</sup> viable cells/ml. Mixed cultures were obtained by adding 0.5 ml of parental and semisyngeneic F<sub>1</sub> cell suspensions to a 35 mm Petri dish (A/S Nunc, Roskilde, Denmark). Control nonmixed cultures consisted of 1 ml of an individual suspension. Each group contained three replicate cultures and the dishes were kept in a 10% carbon dioxide atmosphere and placed on a rocking platform. Each day the dishes were fed with a nutritional cocktail (16).

*Cytotoxic Assay.*—At varying times after initiation of the MLR, labeled YAC cells (usually 10<sup>5</sup>) were added to the cultures in a volume of 0.1 ml. Unless otherwise indicated, the cultures were incubated with the target cells for 16 h. After that time, cultures were harvested and the amount of <sup>125</sup>I released from the tumor cells was determined as described previously (17). There was no reutilization of the label released from the target cells, since addition of 50 μg/ml of cold thymidine to the culture dishes along with the tumor cells did not alter the percent of isotope release. Spleens taken from mice that had been immunized *in vivo* were added to the Petri dishes together with the YAC cells and incubated and harvested as described for the MLR cultures.

*Preparation of Nonadherent Cells and Supernatants from MLR.*—To obtain nonadherent cells, MLR were cultured for 5 days. After that the nonadherent cells from individual Petri dishes were poured into new dishes and then tested for cytotoxic activity against YAC target cells. Supernatants were taken from MLR by pooling cultures within groups after 5 days, centrifuging for 10 min at 1,500 rpm, and carefully removing the supernatant fluid. The

supernatant was then tested immediately for its cytotoxic effect against YAC. In some cultures we added fresh spleen cells taken from CBA mice to cultures that contained MLR supernatants and YAC cells. The fresh spleen cells were suspended in BSS at  $100 \times 10^6$  cells/ml and 0.1 ml was then added to each culture.

#### RESULTS

*Cytotoxic Effect of Lymphocytes Sensitized by a MLR.*—It has been previously shown that a MLR results in a proliferative response of thymus-derived cell populations (2-5). One-way MLR cultures have been demonstrated to generate a population of cells that are cytotoxic against target cells sharing all or some of the antigens against which the responding cells have been sensitized (5). In the present experiments we mixed parental and semisyngeneic  $F_1$  hybrid lymphocytes together in equal proportions. In such combinations only the parental lymphocytes respond against the foreign histocompatibility antigens of the  $F_1$  hybrid. Thus, the MLR is unidirectional (2).  $[^{125}\text{I}]\text{UdR}$ -labeled YAC cells were added to unidirectional MLR cultures between 4 and 7 days after initiation, and 16 h later the cultures were harvested and the isotope release was measured. The peak cytotoxic effect was noted when labeled target cells were added between 4 and 6 days after the start of the culture (Fig. 1), the

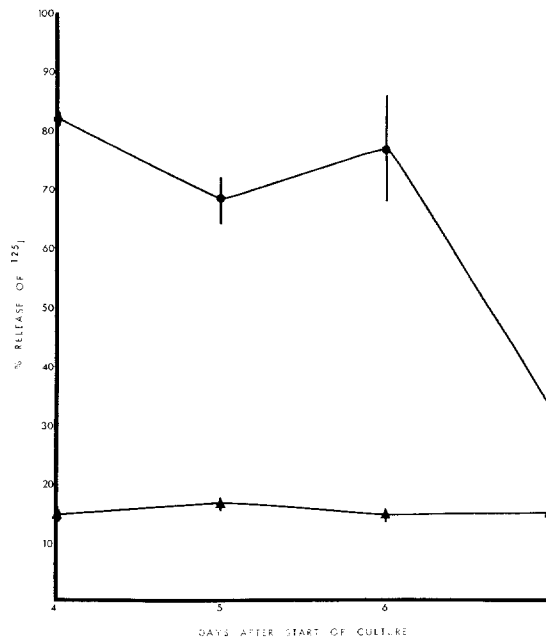


FIG. 1. Percent release of  $^{125}\text{I} \pm \text{SEM}$  from  $[^{125}\text{I}]\text{UdR}$ -labeled YAC cells added between 4 and 7 days after start of MLR cultures. ●, CBA + (A x CBA) $F_1$  cells; ▲, A x (A x CBA) $F_1$  cells. Percent release of  $^{125}\text{I}$  from nonmixed cultures after 4 days was  $15.1\% \pm 0.9$ .

cytotoxic effect being smaller at earlier times (data not shown). The decline in cytotoxicity on day 7 probably reflects the diminished number of viable cells remaining at that time.

In these experiments, the cytotoxic effect observed was specific. Thus, the amount of isotope released in nonmixed cultures was the same as that seen in mixed cultures where the responding lymphocytes and the target cell were of the same H-2 genotype. At the effector to target cell ratios employed in these experiments (approx. 25:1), responding lymphocytes that were specifically sensitized against target cell antigens caused near maximal release of isotope from the labeled YAC cells. Thus, after 4 days of culture, the percent isotope released from YAC cells that were added to nonmixed cultures was 15.1%. Similar release occurred when YAC was added to a MLR between A/Sn and (A × CBA)F<sub>1</sub> (Fig. 1). In this case, the responding cells and the target cells were of the same H-2 genotype (*H-2<sup>a</sup>*), and the responding A lymphocytes would not be expected to react against the *H-2<sup>a</sup>* target cells. The specific MLR directed against the target cell H-2 antigens [CBA lymphocytes mixed with (A × CBA)F<sub>1</sub> cells] resulted in 83% release of isotope from the labeled *H-2<sup>a</sup>* tumor target cells.

*Characteristics of the Effector Cells Mediating the Cytotoxic Effect.*—To test whether adherent cells were responsible for the cytotoxic effect in this system, MLR were incubated for 5 days and thereafter nonadherent cells were obtained by pouring the cell suspensions in the plastic Petri dishes directly into new dishes. The cytotoxic effect of these nonadherent lymphocytes against YAC targets was compared with groups where no cell separation was performed by adding labeled YAC cells and determining the isotope release 16 h later.

It was found that the cytotoxic effect of the nonadherent cell populations was similar to that found in the unseparated groups (Table I), indicating that adherent cells were not required for the expression of cytotoxicity of MLR-activated effector lymphocytes, in agreement with the findings of Häyry et al. (5).

There appeared to be a requirement for close contact between effector and target cell for expression of cytotoxicity, since supernatants from MLR cultures were not cytotoxic to target cells by themselves. After 5 days of MLR, cultures

TABLE I  
Percent Release of <sup>125</sup>I from [<sup>125</sup>I]UdR-Labeled YAC Cells in the Presence of day 5 MLR Cultures in Which Adherent Cells Had Been Removed before Testing

| Parental cell | F <sub>1</sub> hybrid cell |                              | % release <sup>125</sup> I ± SEM |
|---------------|----------------------------|------------------------------|----------------------------------|
| —             | (A × CBA)                  | Unseparated (whole) cultures | 17.9 ± 0.7                       |
| A             | (A × CBA)                  | “ “ “                        | 13.6 ± 1.6                       |
| CBA           | (A × CBA)                  | “ “ “                        | 88.9 ± 3.7                       |
| —             | (A × CBA)                  | Nonadherent cells only       | 13.7 ± 1.7                       |
| CBA           | (A × CBA)                  | Nonadherent cells only       | 80.7 ± 1.8                       |

were tested for their cytotoxic effect against YAC targets and compared in this respect with cell-free supernatants obtained from parallel cultures. It was found that the lymphocytes obtained in the MLR were strongly cytotoxic to YAC cells, whereas the supernatant from parallel cultures had no effect (Fig. 2).

It has been reported that thymus-derived lymphocytes are competent to release substances upon confrontation with histoincompatible cells that can activate macrophages into cytotoxicity (18). Furthermore, antibody can induce

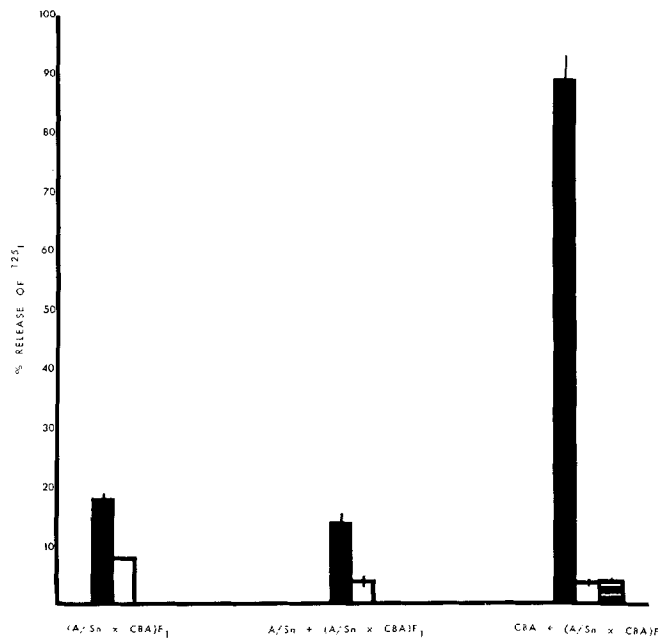


FIG. 2. Percent release of  $^{125}\text{I}$   $\pm$  SEM from [ $^{125}\text{I}$ ]UdR-labeled YAC cells. After 5 days of MLR, whole cultures were tested for their cytotoxic effect (solid bars). Supernatants from parallel cultures (open bars), or supernatants and  $10^7$  new nonimmune CBA spleen cells (horizontal-lined bars) were also tested against YAC.

nonsensitized bone marrow-derived (B) cells into cytotoxicity against YAC.<sup>2</sup> Thus, supernatants were removed from the active MLR, as described above, and tested against YAC cells in the presence of fresh normal CBA spleen cells ( $10^7$ /dish) to study whether antibody or other substances released into the supernatants would induce cytotoxicity in nonsensitized spleen cells.

As shown in Fig. 2 there was no cytotoxicity induced when fresh spleen cells were added to the supernatants from MLR cultures that contained cytotoxic

<sup>2</sup> Britton, S., and J. Forman. 1973. Some characteristics of the antibody-induced cell-mediated cytotoxicity against leukemia cells. *Transplantation*. In press.

effector cells. This is consistent with thymus-derived cells as the actual killer cells in *in vitro* MLR.

*Specificity of the Cytotoxic Effect Induced by In Vitro- and In Vivo-Sensitized Cells. Effect on Innocent Bystander Targets.*—Nonspecific killing of cells can occur during the homograft reaction (9–12). Such innocent bystander effects are thought to occur when there is close contact between the specific target undergoing destruction and innocent third-party cells. So far we have demonstrated that killing of self did not occur in these MLR reactions [A lymphocytes activated against (A × CBA)F<sub>1</sub> cells and tested against strain A target cells], but it is conceivable that this may occur if specific (but unlabeled) activating cells (CBA) were also present during the effector stage together with labeled YAC target cells.

To test this, the following experiment was performed: a MLR between A and (A × CBA)F<sub>1</sub> cells was allowed to proceed for 5 days. Thereafter, 10<sup>5</sup>-labeled YAC cells were added to these cultures. Since YAC and the A effector cells are of the same H-2 genotype, no cytotoxicity was expected to occur and was not observed (Table II). To test for innocent bystander killing, we added labeled YAC targets to parallel cultures together with an equal number of unlabeled specific targets (CBA spleen cells). We found that the release of <sup>125</sup>I from the labeled YAC cells was not higher in these groups, and in the same range as non-mixed control cultures. Thus, the presence of activating lymphocytes even during the effector stage did not cause a cytotoxic effect on target cells having the same H-2 genotype as the activated lymphocytes.

Similar experiments were carried out with lymphocytes from animals immunized *in vivo*. CBA and A mice were inoculated *i.p.* with 10<sup>7</sup> (A × CBA)F<sub>1</sub> spleen cells. 7 days later, their spleens were removed and tested *in vitro* for their cytotoxic effect against YAC cells. It was found (Table III) that primed CBA spleen cells displayed a cytotoxic effect. Furthermore, when 10<sup>5</sup> CBA spleen cells (specific targets) were added to the group containing the immunized A spleen cells together with an equal number of labeled YAC cells, there was no

TABLE II  
*Percent Release of <sup>125</sup>I from [<sup>125</sup>I]UdR-Labeled YAC Cells in the Presence of day 5 MLR Cultures. Effect of Reintroducing Unlabeled Specific Target Cells when Effector and Labeled Targets Are of the Same H-2 Genotype*

| Parental cell | F <sub>1</sub> hybrid cell | Specific target added at the time of the cytotoxic assay | % release of <sup>125</sup> I ± SEM |
|---------------|----------------------------|--|-------------------------------------|
| CBA           | (A × CBA)                  | —  | 88.9 ± 3.7                          |
| A             | (A × CBA)                  | —  | 13.6 ± 1.6                          |
| A             | (A × CBA)                  | Yes*   | 14.5 ± 2.2                          |
| —             | (A × CBA)                  | —  | 17.9 ± 0.7                          |

\* 10<sup>5</sup> unlabeled CBA cells were added together with 10<sup>5</sup> labeled YAC cells during the cytotoxic assay.

TABLE III  
*Release of  $^{125}\text{I}$  from [ $^{125}\text{I}$ ]UdR-Labeled YAC Cells in the Presence of In Vivo-Sensitized Spleen Cells. Effect of Reintroducing Unlabeled Specific Target Cells when Effector and Labeled Targets Are of the Same H-2 Genotype*

| Host spleen cells | Donor inoculum         | Effector to target cell ratio | Specific target added at the time of the cytotoxic assay | % release of $^{125}\text{I} \pm \text{SEM}$ |
|-------------------|------------------------|-------------------------------|--|--|
| CBA               | (A $\times$ CBA) $F_1$ | 100:1                         | —  | 40.3 $\pm$ 0.9                               |
| CBA               | (A $\times$ CBA) $F_1$ | 50:1                          | —  | 24.2 $\pm$ 1.9                               |
| CBA               | (A $\times$ CBA) $F_1$ | 10:1                          | —  | 17.3 $\pm$ 0.9                               |
| A                 | (A $\times$ CBA) $F_1$ | 100:1                         | —  | 8.6 $\pm$ 0.3                                |
| A                 | (A $\times$ CBA) $F_1$ | 100:1                         | Yes*   | 9.6 $\pm$ 0.5                                |
| CBA               | —                      | 100:1                         | —  | 19.7 $\pm$ 0.7                               |
| A                 | —                      | 100:1                         | —  | 11.6 $\pm$ 0.8                               |

\*  $10^5$  unlabeled CBA cells were added together with  $10^5$  labeled YAC cells during the cytotoxic assay.

increase in the amount of isotope release. Thus, in this suspension assay system, there is no evidence for innocent bystander killing.

*Effect of Agglutinating Activated Effector Lymphocytes to Innocent Target Cells.*—It has been suggested that the mechanism by which activated thymus-derived lymphocytes kill target cells is basically nonspecific and that the only requirement for cytotoxicity to occur is the close contact is established between effector and target cell (19). Although innocent bystander effects have been observed (9–12), it is not clear whether killing in these cases was the result of release of short-lived toxic products from the specifically damaged targets. It is conceivable that such toxic products could be responsible for killing of the innocent bystanders being in close proximity, rather than a direct action of the activated killer lymphocytes on these target cells. In order to resolve this, we used Con A and PHA, which have been shown to cause nonspecific agglutination of cells (20), to bring about close contact between effector and target cells. Although these agents are also mitogens for T cells and have been shown to induce cytotoxicity in this cell population (13), they were added to the MLR at the same time as labeled target cells were admixed and cytotoxicity was assayed 6 h later, i.e., at a time when mitogen-induced cytotoxicity could not be detected in this system.

MLR cultures were allowed to proceed for 5 days and thereafter labeled YAC cells were added. At the same time, various concentrations of Con A or 1  $\mu\text{g}$  of PHA was also added to the Petri dishes and the resultant cytotoxicity was measured. Controls consisted of nonmixed lymphocytes treated with Con A and PHA as described for the MLR cultures.

During the time period (6 h) of the effector stage, Con A by itself had no effect on the viability of the YAC cells, nor did Con A induce cytotoxicity in unmixed cultures at any of the concentrations tested (Fig. 3). In the MLR

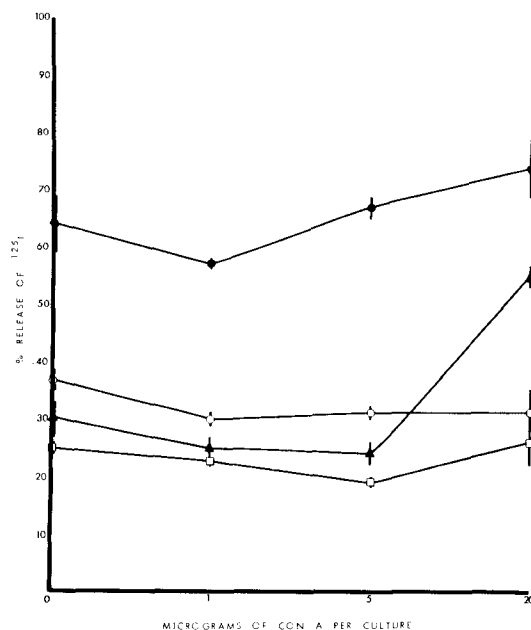


FIG. 3. Percent release of  $^{125}\text{I} \pm \text{SEM}$  from [ $^{125}\text{I}$ ]UdR-labeled YAC cells. YAC cells were added to day 5 MLR cultures with or without varying concentrations of Con A and isotope release was measured 6 h later. □, YAC cells alone; ○, (A × C57Bl)F<sub>1</sub>; ●, C57Bl + (A × C57Bl)F<sub>1</sub>; ▲, A + (A × C57Bl)F<sub>1</sub>.

[C57Bl mixed with (A × C57Bl)F<sub>1</sub>] where specific killing of YAC was observed, the presence of different concentrations of Con A did not significantly alter the specific isotope release. There was no cytotoxicity observed in the MLR containing A cells mixed with (A × C57Bl)F<sub>1</sub>, since the effector and target cells shared the same H-2 genotype. However, when Con A was added to the latter cultures at a concentration of 20 µg/ml, there was a pronounced cytotoxicity observed. Similar results were observed when 1 µg/ml of PHA was added to similar cultures in an analogous way (Table IV).

Both Con A and PHA induced mixed agglutination of lymphocytes and target cells. This is illustrated in Fig. 4, where we found nonspecific large aggregates of the two cell types in the MLR in the presence of 20 µg/ml of Con A.

These findings indicate that direct killing of H-2-compatible target cells can occur in specifically sensitized lymphocyte populations without the need of innocent bystander effects. These results are consistent with the prediction that killing by effector lymphocytes having specific receptors for alloantigens is basically a nonspecific process, specificity being determined by the activated effector cell having specific receptors for foreign antigens present on the target cell.



TABLE IV  
*Effect of the Addition of PHA on the Percent Release of  $^{125}\text{I}$  from [ $^{125}\text{I}$ ]UdR-Labeled YAC Cells in the Presence of day 5 MLR Cultures*

| Parental cells | F <sub>1</sub> hybrid cells | Presence of mitogen | % release of $^{25}\text{I} \pm \text{SEM}$ |
|----------------|-----------------------------|---------------------|---|
| —              | —                           | —                   | 3.9 $\pm$ 0.4*                              |
| —              | —                           | 1 $\mu\text{g}$ PHA | 4.1 $\pm$ 0.3*                              |
| —              | (A $\times$ C57Bl)          | —                   | 23.6 $\pm$ 4.6                              |
| —              | (A $\times$ C57Bl)          | 1 $\mu\text{g}$ PHA | 20.9 $\pm$ 4.0                              |
| C57Bl          | (A $\times$ C57Bl)          | —                   | 75.1 $\pm$ 4.6                              |
| C57Bl          | (A $\times$ C57Bl)          | 1 $\mu\text{g}$ PHA | 68.2 $\pm$ 2.5                              |
| A              | (A $\times$ C57Bl)          | —                   | 14.6 $\pm$ 1.5                              |
| A              | (A $\times$ C57Bl)          | 1 $\mu\text{g}$ PHA | 44.5 $\pm$ 2.1                              |

Cultures were run for 5 days. At that time,  $10^5$  [ $^{125}\text{I}$ ]UdR-labeled YAC cells were added and isotope release was determined after 6 h. Some cultures also received 1  $\mu\text{g}$  of PHA together with the labeled tumor cells.

\* Represents effect of mitogen on tumor cells alone.

#### DISCUSSION

The homograft reaction is generally believed to be a specific immunological phenomenon causing destruction of allogeneic grafted tissue by immunological effector mechanisms in the host, while the hosts and third-party cells (not antigenically foreign to the host) are not affected. Thymus-derived lymphocytes have been demonstrated to have receptors for alloantigens (21, 22). Specifically sensitized cells are cytotoxic to the corresponding target cells and can be specifically absorbed on cell monolayers, indicating that the sensitized cells also contain specific receptors (23, 24). This is believed to be the basis for the specificity of the T cell killing mechanism.

On the other hand, nonsensitized lymphocytes (and perhaps educated T cells [25]) that have receptors for the Fc portion of immunoglobulin (26) can also be transformed into killer cells by binding to antibody that is bound to target cells (27). While such cells do not necessarily contain specific receptors for the target cell in question, the specificity of the reaction is a result of the antibody itself, which can only bind to target cell antigens that are identical or similar to those on the sensitizing cell population.

Cells prestimulated by pokeweed mitogen or PHA and then washed free of these mitogens are cytotoxic for cells within the species but not across species (28).<sup>3</sup> However, if the stimulated cells are tested against target cells in the presence of PHA, where close contact between effector and target is achieved by the agglutinating properties of the mitogen, such cells do kill across the species barrier. Thus, even in the case of mitogenically activated, presumably

<sup>3</sup> Stejskal, V., G. Holm, and P. Perlmann. 1973. Pokeweed mitogen induces lymphocyte cytotoxicity in vitro. Manuscript in preparation.

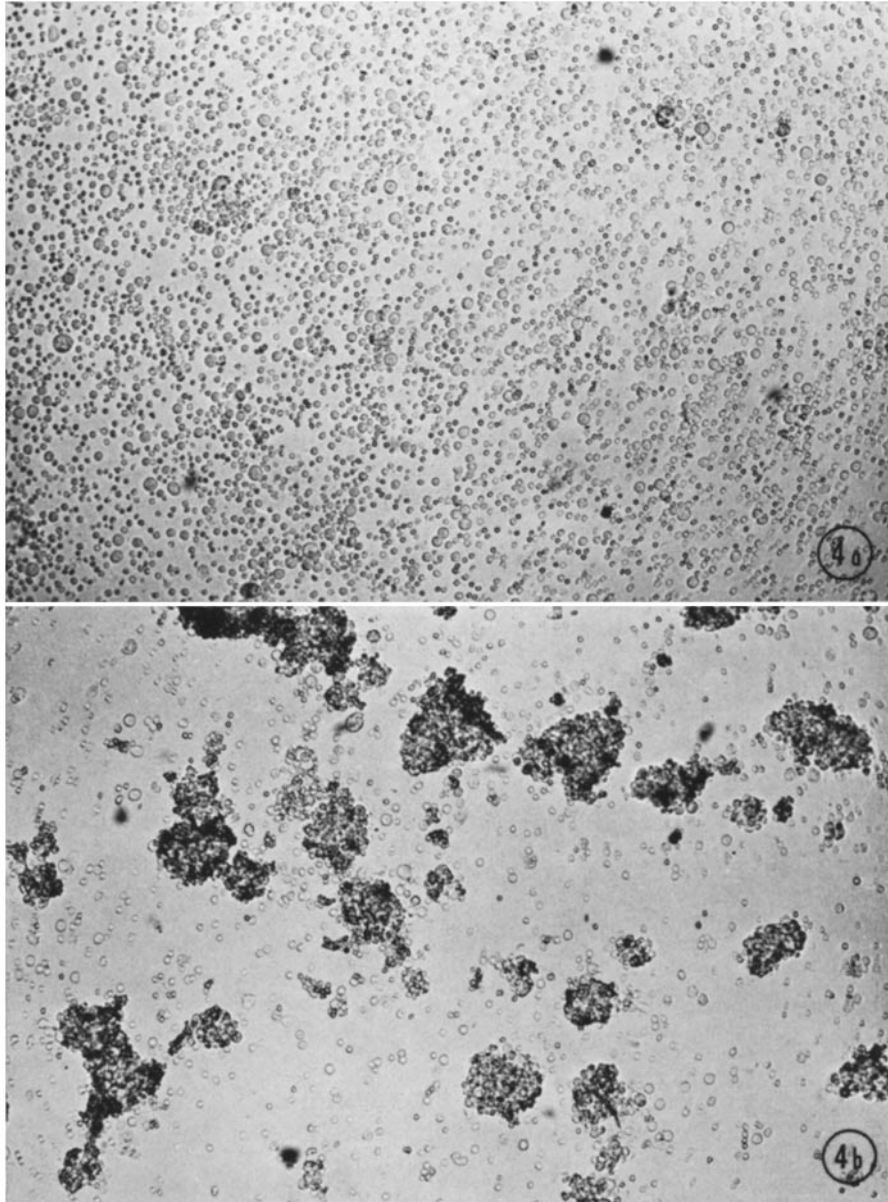


FIG. 4. Appearance of day 5 MLR cultures consisting of A + (A  $\times$  C57Bl)F<sub>1</sub> cells that had  $10^5$  YAC cells added 6 h previously (a), or 20  $\mu$ g of Con A added to the cultures together with the YAC cells (b).  $\times 150$ .

thymus-derived cells, there is some specificity in the killing process, although close contact will allow killing to occur across the species barrier.

Killing of targets syngeneic to the effector cells or targets against which the host is not presensitized (innocent bystander) is usually not observed, but can occur in certain situations, where there is very close contact between target and effector cell (9–12). In the experiments reported here both *in vivo*- and *in vitro*-stimulated lymphocytes failed to kill labeled target cells, when the effector lymphocytes were of the same H-2 genotype as the target cell, even though fresh specific target cells were added at the time of assay. Similar results have also been reported by others (5, 7, 8), although in other *in vitro* assay systems nonspecific effects have been seen (9, 10).

Klein and Klein (29) studied the fate of mixed inocula from a large series of histoincompatible tumor cells and in most cases they found that syngeneic tumors were not destroyed by the host in spite of the fact that they were injected along with a much larger number of allogeneic tumor cells. Contrariwise, Zbar et al. (11) have shown innocent bystander effects against one strain of guinea pig tumor cells, when the host was immune to a second antigenically non-cross-reacting tumor. This effect was seen at the site of delayed hypersensitivity skin reactions to the specific tumor where there was close contact between effector cells and specific and nonspecific targets. Similar results were also obtained in delayed hypersensitivity lesions if the tumor was injected intradermally with purified protein derivative into Freund's adjuvant-sensitized guinea pigs. Cohen and Feldmann (12) have shown that fibroblast targets to which effector cells were not sensitized released an increased amount of isotope when mixed on a monolayer with the specific targets.

In these types of experiments, the killing of the innocent bystander cells could be due to (a) the direct action of the killer cell on the innocent bystander itself, or (b) an indirect secondary effect from the release of short-lived toxic products from the specifically damaged cell that resulted in lysis of neighboring cells. These innocent bystander effects were most readily demonstrated when specific target cells were included in the reaction. Thus, it cannot be determined whether the specific targets served to activate the effector cell or merely played a passive role in bringing the effector cell in close contact with the syngeneic targets.

In the present experiments, we have shown that sensitized effector cells in MLR do kill H-2 syngeneic cells directly when agglutinating agents such as Con A or PHA are added to the test system during the cytotoxic assay. These mitogens did not by themselves stimulate such cells to become cytotoxic due to the short time of the incubation period (6 h). Nevertheless, without reintroducing specific targets, H-2<sup>a</sup> lymphocytes sensitized to H-2<sup>k</sup> target cells were cytotoxic to H-2<sup>a</sup> (YAC) targets in the presence of PHA or Con A, but not in their absence. This result indicates that the effector cells (probably thymus derived) are nonspecific in their killing mechanism and only need close contact

in order for killing to be expressed. While we cannot rule out the possibility that some antigen is bound to these effector cells from residual antigen in the cultures, it would appear that activated cells with specific alloantigenic receptors at this stage of development can kill (either specific or nonspecific targets) nonspecifically and need not require reactivation or an additional signal by interaction of its cell receptors with alloantigens on the target cell membrane. This data also indicates that killing is a direct consequence of the cytotoxic action of the effector cell on the syngeneic target rather than an indirect effect mediated by the release of short-lived toxic substances released from specific targets that secondarily destroy the bystander cells in close proximity.

While we have not directly examined the nature of the effector cells in this reaction, Häyry et al. (5) have separated effector cell populations stimulated in *in vitro* MLR in the mouse H-2 system and have found that thymus-derived cells are required and can express cytotoxicity by themselves. Our present results that demonstrate that removal of adherent cells from MLR-sensitized cultures does not alter the cytotoxicity of the effector cell population are in agreement with this finding.

We have also found that supernatants from MLR cultures containing active cytotoxic cells are not cytotoxic to YAC cells. In addition there was no cytotoxicity induced when fresh spleen cells were added to supernatants and target cells. This evidence argues against the participation of antibody-induced cytotoxic mechanisms to a significant degree in MLR cytotoxicity. Furthermore, IgG but not IgM is the antibody class required for inducing cytotoxicity, and IgG is usually not found in primary *in vitro* cultures (30), again arguing against antibody-induced mechanisms in these systems. Although it has been shown that macrophages can be activated from activated supernatants of spleen cells (18), the failure of these supernatants to induce cytotoxicity in normal spleen cells is consistent with findings by us and others that macrophages are not active in MLR cytotoxicity reactions.

#### SUMMARY

Generation of cytotoxic effector cells by a unidirectional mixed lymphocyte reaction (MLR) in the mouse H-2 system was studied using labeled YAC (*H-2<sup>a</sup>*) leukemia cells as targets. The responding effector cell displayed a specific cytotoxic effect against target cells of the same H-2 genotype as the stimulating cell population. Killing of syngeneic H-2 cells was not observed, even when the labeled target cells were "innocent bystanders" in cultures where specific target cells were reintroduced. Similar results were found with spleen cells taken from mice sensitized *in vivo* 7 days earlier. The effector cell was not an adherent cell and was not activated by supernatants from MLR. The supernatants were not cytotoxic by themselves. When concanavalin A or phytohemagglutinin was added to the cytotoxic test system, target and effector cells were agglutinated. Under these conditions, killing of *H-2<sup>a</sup>* target cells was observed in mixed

cultures where  $H-2^a$  lymphocytes were also the effector cells. These findings indicate that specifically activated, probably thymus-derived lymphocytes, can kill nonspecifically once they have been activated and providing there is close contact between effector and target cells. Thus, specificity of T cell killing appears to be restricted to recognition and subsequent binding to the targets, the actual effector phase being nonspecific.

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