Immunologic Functions of Isolated Human Lymphocyte Subpopulations

IV. Stimulation of MLC and CML by Human T Cells

PAUL M. SONDEI, LEONARD CHESS, AND STUART F. SCHLOSSMAN

The Division of Tumor Immunology, The Sidney Farber Cancer Center—Department of Medicine, Harvard Medical School, 35 Binney Street, Boston, Massachusetts 02115

Received January 22, 1975

Surface immunoglobulin positive and immunoglobulin negative human lymphocyte populations were obtained by immunoabsorbent column chromatography. Both cell populations were effective as stimulating and target cells in allogeneic MLC and CML reactions. The immunoglobulin negative population was further depleted of both EAC rosette forming cells and nylon wool adherent cells. The resulting highly purified T cell population was also able to stimulate allogeneic cells in MLC, and induce the generation of specifically cytotoxic killer cells in CML.

The proliferative response observed in the mixed lymphocyte culture (MLC) has been shown to correlate with graft-versus-host disease, and rejection of allografts in vivo (1–5). This same in vitro interaction has been utilized to produce cytotoxic effector cells which can mediate specific killing in the in vitro cell mediated lympholysis (CML) assay, and lead to accelerated graft rejection in vivo upon adoptive transfer (6–9). Both the recognitive (proliferative) and effector (cytotoxic) phases have been shown in vitro to be mediated solely by T cells in both mouse and man (10–13). However, the cellular distribution of antigens recognized as "foreign" on stimulating and target cells remains unclear. To further resolve the nature of the stimulatory cell in MLC, a number of separation and depletion techniques have been utilized to obtain purified human or mouse T and B lymphocytes for in vitro studies. Conflicting results have been obtained by different laboratories, since it has been shown that T and B cells stimulate similarly (14, 15), that T cells stimulate better than B cells (16), and that B cells stimulate better than T cells (17, 18). The apparent contradictions found in these studies may in some way relate to differences in MLC methodology or cell purification techniques.

In the present studies we have utilized an anti-human Fab immunoabsorbent column to separate human lymphocytes into a surface immunoglobulin bearing (Ig+) cell population containing mostly B cells (> 97% Ig+ cells), and a surface immunoglobulin negative (Ig-) population which contains virtually no B lymphocytes (< 1% Ig+ cells) (19). Both populations were tested for their ability to stimulate allogeneic cells in MLC, induce the in vitro generation of cytotoxic lymphocytes, and serve as target cells in the CML assay. In all cases, the Ig- and Ig+ populations were stimulatory and behaved similarly.

Further purification of the Ig- population of cells was accomplished using EAC rosette sedimentation and nylon wool adherence to deplete EAC positive but
immunoglobulin negative cells and monocytes, respectively (20). The resulting purified population of T lymphocytes was also able to induce stimulation of allogeneic cells in MLC, and stimulate the generation of cytotoxic lymphocytes in vitro.

MATERIALS AND METHODS

Cell Separation Techniques

Fractionation of lymphocytes into nonimmunoglobulin bearing and immunoglobulin bearing populations. The method of quantitatively separating and then recovering B lymphocytes from human peripheral blood by means of a sephadex G-200 immunoabsorbent column to which purified rabbit anti-human Fab is covalently bound has been described in detail elsewhere (19, 21). In brief, ficoll-hypaque separated mononuclear cells from human peripheral blood are washed three times in media 199 + 5% fetal calf serum (FCS) and EDTA, and passed through the immunoabsorbent column. Cells bearing surface immunoglobulin (B cells) are retained on the column, and the cells passing directly through the column are less than 1% immunoglobulin bearing as shown by immunofluorescence with fluoresceinated rabbit anti-human Fab. The immunoglobulin bearing cells (Ig⁺) bound to the column are then recovered by competitive elution with a 1% human immunoglobulin solution. The immunoglobulin eluted population of cells was examined for surface immunoglobulins with fluoresceinated rabbit anti-human Fab and found to be greater than 97% surface immunoglobulin positive. As reported previously, the recovered Ig⁺ and Ig⁻ separated cell fractions comprise 98% of the cells initially put on the immunoabsorbent column (19).

EAC rosette sedimentation. The Ig⁻ cell population described above contains at least three different cell populations: T cells which are E rosette positive, a population of cells forming EAC rosettes, and a latex ingesting monocyte population (26, 27). The EAC positive cells which may bear both C3 and Fc receptors can be separated from T cells by forming rosettes with sheep erythrocytes pretreated with rabbit anti-sheep RBC antibody and complement components 1, 4, 2, 3 (EAC cells, Cordis Labs, Miami, Fla.) by the method of Mendes (23). In brief, 0.5% EAC cells and 10⁶ washed Ig⁻ cells are incubated together for 30 min at 37°C and then spun at 200g for 5 min. The cell button was gently resuspended and sedimented over ficoll-hypaque. The mononuclear cells recovered at the interface were largely depleted of EAC rosette forming cells (EAC-RFC⁺).

Depletion of adherent cells. The EAC-RFC depleted Ig⁻ cell population was incubated on a media 199 + 5% FCS washed nylon wool column for 30 min at 37°C, to remove adherent cells (20); the nonadherent cell population was eluted with media 199 containing 5% FCS.

Proliferative studies. Standard one-way mixed lymphocyte cultures (MLC) were established by the method of Hartzman (24) in flat bottomed microtiter plates (Falcon Plastics, Oxnard, Calif.) using triplicate wells, each containing 0.2 x 10⁶ responding cells and 0.2 x 10⁶ stimulating cells which had been pretreated with 50 μg/ml of mitomycin-C for 30 min at 37°C and then washed three times. All cultures were established in “final medium” (Media containing 1% penicillin-streptomycin, 200 mM L-glutamine, 25 mM Hepes buffer, and 0.5% NaHCO₃; Microbiological Associates) supplemented with 20% heat inactivated human AB serum. After 5 days the cells were pulsed with 0.2 μCi of [³H]thymidine (sp act 1.9 Ci/m mole, Schwarz-Mann, Orangeburg, NJ) and harvested 16 hr later with a
MASH II apparatus (Microbiological Associates, Bethesda, Maryland). Radioactivity was measured in a Packard liquid scintillation counter, and the results expressed as counts per minute ± standard error of the mean.

Cell-Mediated Lympholysis

In vitro allogeneic sensitization. 7.5 × 10⁶ Responding cells + 7.5 × 10⁶ mitomycin-C treated stimulating cells were cultured upright in 5.0 ml final medium in a 30 ml tissue culture flask (Falcon Plastics) in a 5% CO₂, 37°C humid atmosphere. Cultures were fed 2.5 ml of final medium after 48 and 96 hr. On day 6 of culture the in vitro sensitized killer cells were harvested, purified by Ficoll–Hypaque centrifugation, washed and counted.

Target cell preparation. 3 × 10⁶ Lymphocytes obtained on the day of sensitization culture establishment were aliquoted in 16 × 150 mm capped tubes in 1.0 ml of final medium. After 2 days, 2 μg of PHA were added to each tube (PHA, Burroughs-Wellcome). On day 6 the tubes were pooled and 5–10 × 10⁶ cells were resuspended in 0.2 ml of medium and incubated at 37°C with 0.1–0.25 mCi of ⁵¹Cr (sp act 200 mCi/mg, New England Nuclear). After 1 hr dead cells and labeled debris were removed by ficoll–hypaque centrifugation at 4°C. Labeled target cells were removed from the interphase, washed twice, and resuspended at 10⁶ cells/ml in iced final medium.

⁵¹Cr release assay. 0.2 ml of media or putative killer cell suspension and 0.1 ml of target cells were placed in 10 × 75 mm glass test tubes, centrifuged at 150g for 5 min and incubated at 37°C in a 5% CO₂, 95% air humid atmosphere. After 4 hr, the cells were resuspended with 1.7 ml of iced medium and centrifuged at 250g for 10 min. One milliliter of supernatant was collected and counted for ⁵¹Cr content in a gamma counter (Searle, Inc., Des Plains, Ill.). Results are expressed as the average ⁵¹Cr cpm ± SD from triplicate tubes. To compare results from different targets, results are also expressed as percent cytotoxicity:

\[
\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Spontaneous Release}}{\text{Freeze–Thaw} - \text{Spontaneous Release}} \times 100,
\]

| TABLE I |
|-----------------|-----------------|-----------------|
| Responding cells | Stimulating cells | [³H]TdR (cpm) |
| R₁₄⁺ | Medium | 67 ± 54 |
| S₁₄⁻ | S₁₀⁻ | 13,272 ± 2492 |
| R₁₄⁺ | S₁₄⁺ | 13,138 ± 736 |
| R₁₄⁺ | S₁₀⁺ | 12,662 ± 1213 |
| S₁₀⁻ | S₁₄⁻ | 1976 ± 1253 |

* 0.2 × 10⁶ Responding cells + 0.2 × 10⁶ mitomycin-C treated stimulating cells were cultured in 0.25 ml microplates for 136 hr. [³H]TdR was added for the last 16 hr, and cells were harvested onto glass fiber filters for scintillation counting. Results are expressed as the mean [³H]TdR cpm of triplicate cultures ± SEM. All lymphocytes used were from unrelated human donors R and S. Subscripts indicate unfractionated lymphocytes (UNF), immunoabsorbent purified surface immunoglobulin negative lymphocytes (Ig⁻), or immunoabsorbent eluted immunoglobulin bearing lymphocytes (Ig⁺).
FIG. 1. CML on Ig- and Ig+ target cells. Four-hour $^{51}$Cr release from 10$^4$ Ig$^+$ and Ig$^-$ target cells using either 30 $\times$ 10$^4$ (30/liter) or 5 $\times$ 10$^4$ (5/liter) killer cells sensitized in vitro. Killer cells were sensitized with either allogeneic Ig$^+$ or Ig$^-$ mitomycin-C treated stimulating cells. % Cytotoxicity values $\pm$ S are based on the following freeze-thaw (F.T.) and Spontaneous Release (S.R.) values ($^{51}$Cr cpm of triplicate tubes $\pm$ SD): Ig$^+$ targets, F.T. = 750 $\pm$ 38, S.R. = 169 $\pm$ 7; Ig$^-$ targets, F.T. = 618 $\pm$ 64, S.R. = 110 $\pm$ 9.

where spontaneous release equals the $^{51}$Cr counts obtained from targets incubated for the 4-hr assay period in medium alone; freeze-thaw equals the $^{51}$Cr released from targets after repetitive freeze-thawings; and the experimental value equals the $^{51}$Cr released from targets incubated with killer cells.

RESULTS

Isolated immunoglobulin positive and negative lymphocytes were treated with mitomycin-C and tested for their ability to stimulate proliferation of either unfractionated or Ig$^-$ allogeneic lymphocytes in MLC. Both Ig$^+$ and Ig$^-$ populations, as well as unfractionated lymphocytes, stimulate allogeneic responding cells to the same degree (Table 1). In addition, Ig$^+$ and Ig$^-$ cells were tested for their ability
TABLE 2
CML FOLLOWING SENSITIZATION IN VITRO WITH PURIFIED HUMAN LYMPHOCYTE^a

<table>
<thead>
<tr>
<th>Responding cells</th>
<th>Stimulating cells</th>
<th>^aCr cpm</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_Ig^-</td>
<td>C_Ig^-</td>
<td>51 ± 9</td>
<td>-0.1 ± 2.0</td>
</tr>
<tr>
<td>D_UNF</td>
<td>D_UNF</td>
<td>50 ± 9</td>
<td>-0.5 ± 2.0</td>
</tr>
<tr>
<td>D_UNF</td>
<td>C_UNF</td>
<td>230 ± 11</td>
<td>62.6 ± 2.3</td>
</tr>
<tr>
<td>D_UNF</td>
<td>C_Ig^-</td>
<td>215 ± 9</td>
<td>57.3 ± 2.1</td>
</tr>
<tr>
<td>D_UNF</td>
<td>C_T</td>
<td>145 ± 8</td>
<td>33.0 ± 2.0</td>
</tr>
</tbody>
</table>

^a Subpopulations. Four-hour ^aCr release from 10^4 target cells using 20 X 10^4 in vitro sensitized killer cells. All targets are from individual C. Responding and stimulating cells obtained by several purification methods (see text) are from unrelated individuals C and D. Subscripts indicate purified T lymphocytes (T); (UNF), (Ig^-) and (Ig^+) are described in the legend to Table 1. % Cytotoxicity values are based on the following freeze-thaw and spontaneous release values (mean ^aCr cpm of triplicates ± SD); freeze-thaw = 337 ± 9; spontaneous release = 51 ± 8.

The stimulatory capacity of mitomycin-C treated Ig^- cells to induce both a proliferative and cytotoxic response indicates that this in vitro stimulatory ability is not solely a property of surface immunoglobulin positive B cells. The Ig^- cell population is comprised of T cells, monocytes, and an EAC rosette positive cell population (Null cells), each of which could provide the allogeneic stimulus. To ascertain whether T cells were stimulatory on their own, required effective depletion of both monocytes and EAC positive cells from the Ig^- population.

TABLE 3
MLC STIMULATION BY PURIFIED HUMAN T LYMPHOCYTES^a

<table>
<thead>
<tr>
<th>Responding cells</th>
<th>Stimulating cells</th>
<th>[^3H]TdR^a (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_Ig^-</td>
<td>X_UNF</td>
<td>2189 ± 558</td>
</tr>
<tr>
<td>X_Ig^-</td>
<td>X_T</td>
<td>2514 ± 650</td>
</tr>
<tr>
<td>X_Ig^-</td>
<td>Y_T</td>
<td>26,472 ± 588</td>
</tr>
</tbody>
</table>

^a Standard microplate MLC reactions were performed with various purified lymphocyte populations from unrelated individuals X and Y. Each microwell contained 0.02 X 10^4 responding cells ± 0.02 X 10^4 mitomycin-C treated stimulating cells. Results are expressed as the mean [^3H]HDT cpm of triplicate cultures ± SEM.
**TABLE 4**

**MLC Stimulation by Purified Human T Lymphocytes**

<table>
<thead>
<tr>
<th>Responding cells</th>
<th>Stimulating cells</th>
<th>$[^3H]TdT^a$ (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_T$</td>
<td>$X_{UNF}$</td>
<td>2878 ± 321</td>
</tr>
<tr>
<td>$X_T$</td>
<td>$Y_T$</td>
<td>4380 ± 2196</td>
</tr>
<tr>
<td>$X_T$</td>
<td>$Y_T + X_{Ig}^b$</td>
<td>23,452 ± 3008</td>
</tr>
</tbody>
</table>

*Standard microplate MLC reactions were performed with various purified lymphocyte populations from unrelated individuals X and Y. Each microwell contained 0.02 × $10^8$ responding cells + 0.02 × $10^8$ mitomycin-C treated stimulation cells. The response of 0.2 × $10^6$ $X_T$ cells is reconstituted by adding 0.1 × $10^6$ mitomycin-C treated $X_{Ig}^b$ cells as a source of nonresponsive, non-antigenic adherent cells. Results are expressed as the mean $[^3H]TdT$ cpm of triplicate cultures ± SEM.

Ig$^-$ cell fractions obtained from sephadex anti-Fab immunoabsorbent columns were depleted of EAC positive by EAC rosette sedimentation. As described previously (20), this technique decreased the percent of EAC positive cells from 26 ± 3 to 3 ± 2. These EAC-RFC depleted populations which still contained 4% latex ingesting cells were then depleted of monocytes by adherence to nylon wool columns. The resultant purified T cell populations (0% latex ingesting cells, 96% E rosette positive) were treated with mitomycin-C, and tested for their ability to stimulate in standard MLC and to generate cytotoxic lymphocytes in CML sensitization cultures. These purified T cells were efficient in generating cytotoxic lymphocytes (Table 2) as well as stimulating a one way MLC (Table 3).

Further studies were undertaken to demonstrate that the observed T cell stimulation was not caused by residual monocytes. Since good proliferation has been induced by a small population of adherent cells (25, 26), the morphological absence of latex ingesting cells by itself cannot exclude a functionally significant monocyte contamination. However, the absence of functional macrophages from the pure T stimulatory cells is supported further by the finding that ($Y_T$) cells do not stimulate responding cells ($X_T$) which were monocyte depleted (Table 4). More important, this same monocyte depleted mixture gave a good proliferative response when a source of mitomycin-C treated nonresponsive adherent cells from the responder (X Ig$^-$) was added to reconstitute the depleted monocytes. The number of adherent cells needed to reconstitute a monocyte depleted MLC has been shown to be less than that required to stimulate allogeneic cells (26); therefore, the ability of purified T cells to effectively stimulate only when responder adherent cells are present supports the view that stimulation was induced by the T cells, and not by contaminating monocytes.

**DISCUSSION**

Recent studies of both murine and human lymphocytes have suggested that the major antigenic stimulus recognized in MLC is present predominantly on B cells (17, 18, 36). In other studies, however, including our own, effective MLC stimulation was obtained with a population depleted of B cells (13, 15, 16, 22, 27–29, 37, 38). To further investigate the possible stimulatory capacity of T cells, we have in the present studies used a combination of separation techniques to...
deplete B cells, EAC positive cells, and monocytes from ficoll-hypaque purified human peripheral blood mononuclear cells. These procedures yielded a highly purified T cell population that was stimulatory in MLC and capable of inducing allogeneic cells to become specifically cytotoxic in CML. That this stimulation was actually a response to T cells, was demonstrated by exclusion of B cells, EAC positive cells, and monocyte contamination using both morphological and functional criteria.

As indicated previously, B cells which bear surface immunoglobulin can be quantitatively separated from human peripheral blood lymphocytes by an immunosorbent column (19). The cells passing through this column were virtually free of Ig⁺ cells as detected by fluorescence microscopy with a fluoresceinated rabbit anti-human Fab antiserum (19). Recent studies with the more sensitive Fluorescence Activated Cell Sorter (FACS) and with a ¹²⁵I labeled pepsin digested rabbit anti-human Fab have also failed to detect Ig⁺ cells in this Ig⁻ population (30).

This Ig⁻ population, devoid of B cells, was depleted of EAC positive cells by EAC rosette sedimentation (23). This method is effective in specifically eliminating the EAC rosette positive cells from Ig⁻ populations (22) as measured by EAC rosette formation and several functional assays. In this regard, EAC positive and Ig⁻ cells but not T cells are effective in mediating Antibody Dependent Cellular Cytotoxicity (ADCC); following EAC rosette depletion the nonrosetting cells are no longer capable of mediating ADCC (20).

The resultant T lymphocyte preparation was depleted of residual monocytes by incubation on a nylon wool column. To demonstrate that monocytic contamination of the nylon wool purified T cells could not have accounted for the observed MLC stimulation we took advantage of the fact that human MLC responses require a small number of monocytes to "support" proliferation (25). Studies by Alter and Bach have suggested that the number of monocytes needed to stimulate in MLC is substantially greater than that needed to "support" MLC proliferation (26). In this regard, we have shown that a purified T cell population depleted of nylon wool adherent cells does not contain a sufficient number of contaminating monocytes to "support" MLC activity and therefore should not contain a significant number of stimulatory monocytes. Taken together, the above experiments strongly suggest that mitomycin-C treated human T cells alone are capable of effectively stimulating in MLC.

The fact that T cells do stimulate allogeneic cells to proliferate is of considerable interest with respect to the genetic requirements of MLC activation. MLC stimulation has been shown in man (31) and other species to depend on the presentation of foreign "LD antigens" (32, 33) coded for genetically within the Major Histocompatibility Complex (MHC), yet genetically separable from the major serologically defined (SD) antigens (34). In addition to inducing a proliferative response in MLC, LD antigen recognition leads to a T cell collaboration that is required for the generation of cytotoxic lymphocytes (35). Proliferation alone, as induced by nonspecific plant mitogens, does not produce this collaborative interaction, suggesting that LD recognition may induce two separate responses: proliferation and collaboration (35). We would thus emphasize that the purified T cells examined in the present studies in addition to stimulating in MLC, effectively induced the differentiation of allogeneic effector cells to become specifically cytotoxic in CML. Therefore, the stimulation of both proliferation and collaboration by a
highly purified T cell population indicates that populations of T cells, as well as B cells, express LD antigens. Nevertheless the quantitative distribution of LD antigens on individual T and B cells remains unclear. Clonal expression of LD antigens could generate both stimulatory and nonstimulatory T and B cells. Unlike the immunoabsorbent column, many T cell purification methods are associated with large "nonspecific" cell losses, that could possibly separate the stimulatory T cells from the nonstimulatory ones. While the nonstimulatory T cells obtained by such a purification may be of great importance in studying in vitro cellular immune responses, they do not prove that all T cells are nonstimulatory or LD deficient. Because LD antigens are required but not necessarily sufficient for MLC stimulation, even the lack of MLC stimulation by these T cell subpopulations may not prove LD absence. However, the ability of a population of pure T cells to cause MLC stimulation and induce the generation of cytotoxic cells in CML does indicate the presence of LD gene activity. As such, this study demonstrates that some T cells as well as some B cells effectively express LD gene products.

REFERENCES

36. Simpson, E., Personal communication.