SEQUENTIAL RESPONSES OF MOUSE SPLEEN T CELLS IN MIXED LYMPHOCYTE CULTURE-INDUCED CYTOLYSIS*

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Lymphocytes responding to allogeneic cells in one-way mixed lymphocyte culture (MLC) undergo blast transformation and subsequent cell divisions, and generate killer cells. Both the proliferative response (MLC) and target cell lysis (CML) are immunologically specific phenomena (1–3). It has also been shown both in vivo (4, 5) as well as in vitro (6, 7) that blast cells primed by various antigens (4), including histocompatibility antigens (7), or stimulated by mitogens (6) have the capacity of returning to the resting state, “secondary lymphocytes.” Thus blast cells recovered from one-way MLC by 1 g velocity sedimentation revert to secondary lymphocytes when placed on a feeder layer syngeneic to responder cells (8). These secondary lymphocytes are T cells since they are sensitive to anti-φ serum plus complement and have the electrophoretic mobility of T cells (9). They display, however, certain properties strikingly dissimilar from nonprimed T cells: (a) They respond promptly to the original stimulator cells in secondary (2°) MLC but with a fairly limited vigor to other allogeneic stimulator cells carrying cross-reactive H-2 specificities; (b) They are killer cells to relevant allogeneic target cells in vitro; and (c) They are promptly and efficiently stimulated to maximal cytotoxicity by second exposure to the original histoincompatible cells (9–11). This communication reports that the same secondary T cells (and their progeny) can respond by sequential blast transformation, proliferation, and cytotoxicity repeatedly in successive MLC cultures.

Materials and Methods

Cultures. CBA/H-T6T6 spleen lymphocytes were used as responders. They were purified by lysing the erythrocytes with 0.83% NH₄Cl followed by adhesion on glass and by iron powder plus magnetic treatment (12). This procedure results in cells of which over 85% have lymphocyte morphology (12). Mitomycin-C (Calbiochem., Los Angeles, Calif.) blocked DBA/2 spleen cells (DBA/2in) were used as stimulators. Erythrocytes were lysed from the stimulator cell population as described above, but no further purification steps were employed in order to ascertain sufficient numbers of macrophages to support an optimal MLC response (9). The cultures were established in conditions in which only T cells respond (9, 13); in 11 x 110-mm round-bottom glass tubes

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(Münnerstadt Glasswahrenfabrik, Münnerstadt, Germany) with $1.5 \times 10^4$ responder cells and $3.0 \times 10^4$ stimulators/tube in 2 ml of Eagle's MEM supplemented with 5% fetal calf serum (12), and incubated at 37°C in a humified atmosphere of 5% CO$_2$ in air (12).

The MLC responses were quantitated after 3 h Colcemid-Ciba arrest by differential counts from May-Gruenwald-Giemsa stained cytocentrifuged (Shandon Scientific Co., London, England) cell smears and the actual cell numbers were assessed by adding a known number of glutaraldehyde-fixed chicken erythrocytes to the cultures before making the smears (12). This method, although laborious, enables the quantitation of the following parameters of the response: the number of blasts, the number of lymphocytes, total number of lymphoid cells, and the number of dividing cells (12).

Restimulations. Upon each restimulation $2.0 \times 10^4$ fresh stimulator cells were added to the culture tubes and the culture medium was replaced by fresh medium.

Estimation of the Number of Histocompatibility Antigen-Reactive Cells (H-ARC). $10^{-3}$ M hydroxyurea (HU) was added to duplicate cultures. HU at this concentration allows blast transformation but inhibits the cells from entering DNA synthesis (14). Since the HU-arrested blasts survive in vitro only for approximately 40 h, the peak number of blasts in HU-arrested cultures was used to estimate the number of H-ARC (10).

Cell-Mediated Target Cell Lysis (CML). The CML response was quantitated by the $^{51}$Cr (sodium chromate, The Radio Chemical Centre, Amersham, England) method in microconditions in Falcon Microtest II tissue culture trays (Falcon Plastics, Div. of Bioquest, Los Angeles, Calif.) by employing 10,000 target cells (P-815 of DBA/2 strain, a gift from Dr. K. T. Brunner, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), a rocking platform, and 6 h exposure time as described.

Results

The sequential blast and proliferative responses in $1^\circ \ldots 4^\circ$ MLC in one out of three experiments performed are described in Fig. 1. The peak of the blast and proliferative response took place in primary ($1^\circ$) MLC on the 7th culture day. Thereafter, the blast cells disappeared from these cultures, and on the 15th culture day no blast cells prevailed. Concomitantly with the disappearance of the blasts the number of lymphocytes increased, confirming that a certain number of blast cells reverted to lymphocytes during the second half of the $1^\circ$ MLC (10). The number of H-ARC, as estimated from the HU-arrested cultures in $1^\circ$ MLC was of the order of $60 \times 10^9$, i.e., approximately 3% of the input of the responder lymphocytes.

By second stimulation with DBA/2m the blast and proliferative response profiles were strikingly dissimilar. The response in $2^\circ$ MLC took place promptly and was already maximal on the 1st or 2nd culture day. The first prompt peak was sometimes followed by a second minor peak taking place on approximately the 6th day of culture. The number of H-ARC was $150 \times 10^9$ in $2^\circ$ MLC, i.e., approximately 98% of the responder lymphocytes remaining from the $1^\circ$ culture (Fig. 1). In $3^\circ$ and $4^\circ$ MLC the responses were equally prompt, and the number of H-ARC was 110% and 97% of the input of responder cells, respectively.

The CML (lytic) response, as quantitated on a cell-to-cell basis, was hardly detectable in $1^\circ$ MLC on the 4th culture day, clearly visible on the 6th culture day, whereafter the capacity of the MLC-primed cells to lyse relevant allogeneic target cells declined. In $2^\circ$ MLC, target cell lysis was prominent already on the 3rd day and maximal on the 4th-7th culture day, In $3^\circ$ and $4^\circ$ MLC the CML responses were even more prompt and were maximal already on the 3rd day of culture. In $4^\circ$ MLC significant target cell lysis in the short-term 6-h assay was
FIG. 1. Blast and proliferative responses in 1°... 4° MLC of CBA/H-T6T6 plus DBA/2m. All cultures were initiated at day 0 with 1,200 culture tubes. Duplicate to triplicate tubes were removed from the pool on each indicated day and used for quantitation of the various responses. On day 8 all cultures were refed by replacing half of the culture medium with fresh medium. On days 15, 22, and 28 fresh DBA/2m stimulator cells were added to the cultures and the culture medium was changed. Closed circle and solid line: blasts per culture; open circle and solid line: lymphocytes per culture; broken line: cells in mitosis per culture after 3 h Colcemid-Ciba arrest. Shaded areas indicate blast responses in cultures containing 10⁻⁸M HU. Karyotype analysis (19) was made from parallel cultures on days indicated by asterisks: out of 211 metaphase plates analyzed 208 carried the T6T6 chromosome markers of the responder cell type. Background responses in nonmixed controls, maximally 3 blasts/culture x 10³.

**TABLE I**

Effect of Subsequent Stimulation in the MLC on the CML

| No MLC* | Days in culture | Days after last stimulation | Specific ¹⁰⁶Cr release† |
|---------|-----------------|----------------------------|------------------------|
|         |                 | 16:1 | 8:1 | 4:1 | 2:1 | 1:1 | 0.5:1 | 0.25:1 | 0.125:1 |
| 1°      | 4               | 5    | 2   | 2   | 1   |
|         | 7               | 59   | 22  | 9   | 5   |
|         | 14              | 26   | 12  | 5   | 1   |
| 2°      | 18              | 14   | 6   | 3   | 4   | 1   |
|         | 19              | 62   | 50  | 23  | 12  | 3   | 3   |
|         | 22              | 78   | 53  | 43  | 25  | 14  | 6   |
| 3°      | 24              | 50   | 29  | 15  | 7   | 5   | 2   |
|         | 25              | 42   | 28  | 15  | 6   | 3   | 1   |
|         | 28              | 40   | 26  | 14  | 8   | 4   | 3   |
| 4°      | 31              | 76   | 51  | 27  | 13  | 8   | 5   |
|         | 32              | 42   | 18  | 11  | 5   | 2   | 2   | 0   |
|         | 35              | 26   | 16  | 7   | 4   | 2   | 1   |

* Primary culture: CBA + DBA/2m. Subsequent stimulations: + DBA/2m.
† Target cell in CML: P-815 of DBA/2, 6 h exposure time, ratios refer to various effector-target cell ratios.
observed in effector-target cell ratios as low as 0.125:1, compared to 1:1 which is the lowest ratio giving significant lysis in 1° MLC (Table 1).

Discussion

The interpretation of these results must take into consideration the following points: The cultures were performed in conditions where only T cells are responsive (9, 13). The culture conditions employed favor the survival of responding cells (10, 12). The stimulator cells in these conditions die fast and are practically nonexistent from the 4th culture day onwards (12); neither do they participate in the proliferative response to any measurable extent (see legend of Fig. 1). Thus it seems evident that the data presented represent CBA T-cell responses to DBA/2.

The number of H-ARC in 1° MLC—as quantitated by the HU-block method—is of the order of 3%, which is roughly in agreement with other estimates on the number of H-ARC in the MLC response (1). The 1° MLC procedure results in a manyfold concentration of the H-ARC, as approximately 98% of the lymphocytes remaining from the primary culture are H-ARC by second stimulation. In 3° and 4° MLC the number of H-ARC still further approximates 100%, favoring the interpretation that the same population of cells, already enriched by the primary culture, performs the subsequent MLC responses. It is also evident that the cells which respond by proliferation in these cultures, respond to histoincompatible cells rather than to some antigenic components in the culture medium, since changing of the medium (without adding new stimulator cells) on days 8, 14, and 21 of 1° MLC does not induce any blast or proliferative response (10).

Indirect evidence based on synergy in the graft-vs.-host (15) and MLC-CML (16–18) reactions performed with lymphoid cells of various anatomical origin has prompted the hypothesis that two or more “subsets” of T cells cooperate in the T autonomous phase of the allograft response. Furthermore, it has been suggested that one of these subsets would primarily be involved in the proliferative phase of the response, whereas the other would be the progenitor for killer cells (15–17). In our experiments both the proliferative and the cytotoxic responses take place concomitantly and the result neither proves nor disproves the possibility of two interacting T-cell subsets.

Summary

T cells triggered to blast transformation and proliferation by histoincompatible cells have the capacity of reverting “back” to lymphocytes. These “secondary” lymphocytes and their progeny cells are able to respond repeatedly to the same allogeneic stimulus in vitro.

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