Clonal expansion of T cells: A cytotoxic T-cell response in vivo that involves precursor cell proliferation

(Conjugate formation/T-cell proliferation/clonal selection)

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ABSTRACT The response of peritoneal exudate lymphocytes to allogeneic tumor cells was used to determine whether the in vivo generation of cytotoxic T cells (CTL) involved the proliferation of precursor cells. Ten days post-injection, both cytotoxic activity and the formation of conjugates between lymphocytes and target cells were shown to be specific for the immunizing tumor alloantigens and to be effected by Ly-2+ cells. A cell-sorting-based procedure was developed to isolate specific conjugates between red-fluorescence-tagged CTL and blue-fluorescence-tagged tumor target cells. When [3H]thymidine was administered during the response, almost all isolated conjugate-forming CTL were [3H]-labeled on autoradiography. Thus, the CTL were clearly products of dividing cells, a result that contradicts published data. Reassessment of a previously studied system, which suggested that CTL were not products of cell division, indicated that in that system many of the conjugate-forming cytotoxic cells studied were Ly-2+ and nonspecific, and thus perhaps not T cells. We conclude that the clonal selection model is applicable to at least one in vivo T-cell response.

Burnet's clonal selection theory (1) is a cornerstone of cellular immunology. However, the classic experiments validating the theory, and the closest approach to a formal proof (2), are essentially experiments on B cells. T cells can be shown to give a selective proliferative response to antigenic challenge in culture, and the same had been assumed to occur in vivo. This assumption has recently been challenged by Mitchison, Eichmann, and colleagues (3, 4). They have advanced the view that the unprimed animal already contains a large number of precursor T cells potentially reactive with a given antigen but suppressed by control systems. Induction of antigen serves to activate these precursors directly into effector function, without the need for much (or perhaps any) selective clonal expansion.

A study of the development of cytotoxic T lymphocytes (CTL) by Kimura and Wigzell (5) produced results supporting the extreme view that no clonal expansion was required in a vigorous in vivo T-cell response to alloimmunization. Mice were injected i.p. with irradiated allogeneic spleen cells. [3H]Thymidine was administered throughout the 6 days of the reaction. Peritoneal cells were then harvested at the peak of cytotoxic activity, conjugates were formed with tumor cells bearing the immunizing alloantigens, and the preparation was smeared and subjected to autoradiography. Very few of the conjugate-forming cytotoxic cells were labeled with [3H]thymidine.

It could be argued that responses to alloantigens are exceptional, the frequency of responsive precursors being so high initially that no expansion is required. Nevertheless, the result was extremely clear-cut and constituted one of the strongest arguments against clonal expansion of T cells in response to antigen in vivo. In view of its importance, we attempted to confirm this basic experiment. Our results lead to an opposite conclusion, supporting the concept of clonal expansion.

MATERIALS AND METHODS

Mice. Inbred specific-pathogen-free 8- to 10-week-old male mice of the strains C57BL/6J Wehi (H-2Kb), CBA/CaH Wehi (H-2d) and BALB/c (H-2b) were bred at the Hall Institute.

Tumor Cells. P815, an H-2Kb mastocytoma, and EL4, an H-2Kd T lymphoma, were maintained by i.p. transfer every 7 days in BALB/c (H-2b) and C57BL/6 mice, respectively. YAC-1, an H-2Kd lymphoma, maintained in culture.

Immunoassays. Mice were injected i.p. with 5 x 10⁶ x-irradiated (2000 rad; 1 rad = 0.01 Gy) allogeneic spleen cells, and the peritoneal exudate lymphocytes (PEL) were harvested at day 6. Alternatively, mice were injected i.p. with 2.5 x 10⁷ nonirradiated viable allogeneic tumor cells, and the PEL were harvested at day 10.

[3H]Thymidine Administration. [3H]Thymidine, 25 μCi in 0.5 ml of saline (1 Ci = 37 GBq), was injected i.p. each 24 hr over the periods stated. Unlabeled thymidine (10 mg in 0.5 ml of saline) was injected i.p. 4 hr prior to killing the mice.

PEL Preparation and Sorting. The peritoneal cavity was washed out with 5 ml of balanced salt solution. The PEL were suspended in balanced salt solution containing 10% fetal calf serum, then macrophages were depleted by incubation with carbonyl iron, followed by removal with a magnet, as detailed elsewhere (6). For separation of Ly-2+ and Ly-2- cells, the PEL were labeled with a directly fluoresceinated anti-Ly-2 monoclonal antibody and sorted on a FACS II instrument (Becton Dickinson) as detailed elsewhere.

Conjugate Formation, Scoring, and Sorting. This was a development of earlier procedures (6, 8, 9) and used red-fluorescence (rhodamine)-tagged effector cells and blue-fluorescence (monobromobimane)-tagged target cells. Tumor target cells were washed in balanced salt solution and suspended at 10⁷ cells per ml. Monobromobimane (Thiolyte, Calbiochem) (1 μl of a 50 mM solution) was added. After 10 min at room temperature, the suspension was layered over fetal calf serum and centrifuged, then the cells were washed twice before suspension in balanced salt solution/10% fetal calf serum at 10⁷ cells per ml. PEL effector cells were washed in balanced salt solution and suspended at 10⁷ cells per ml. Rhodamine isothiocyanate (Research Organics, Cleveland, OH) (5 μl of 100 μg/ml) was added. After 10 min at room temperature, the cells were washed in balanced salt solution and then in balanced salt solution/10% fetal calf serum. The cells were again incubated for 10 min at 37°C to remove easily

Abbreviations: CTL, cytoxic T lymphocyte(s); PEL, peritoneal exudate lymphocyte(s); NK cell, natural killer cell.
shed fluorescent material, washed, then finally suspended at 2 \times 10^5 cells per ml in balanced salt solution/10% fetal calf serum. To form conjugates (complexes between CTL and target cells), 100 \mu l of the effector PEL suspension and 100 \mu l of the tumor target suspension were mixed in 96-well flat-bottomed microtiter trays, and the cells were spun down (200 \times g, 30 sec) and then incubated 10 min at room temperature. The cells were resuspended by using a standard shear force of 20 aspirations in and out the disposable tip at 150 \mu l. For microscope scoring, the suspension was diluted 1:4 and examined using low power phase-contrast and epi-illumination for fluorescence at the same time. The tumor targets appeared blue, and the PEL effectors were uncolored. For flow cytometric analysis and sorting, a modified FACS II instrument (Becton Dickinson) with a second separately aimed laser and time-delay electronics was used as described elsewhere (9). Red rhodamine fluorescence was excited by laser 1 emitting at 514.5 nm and development, the slides were labeled with ultraviolet lines at 351 nm and 364 nm and filtered by a 450-nm-wide band pass filter. For sorting target cells, 100 \mu l of the PEL suspension and 100 \mu l of the tumor target suspension were mixed in 96-well medium/50% fetal calf serum. Spontaneous release averaged 7% for P815 and 5% for EL4. All results are means of five or six separate assays. Errors were uncolored. For flow cytometric analysis and sorting, a modified FACS II instrument (Becton Dickinson) with a second separately aimed laser and time-delay electronics was used as described elsewhere (9). Red rhodamine fluorescence was excited by laser 1 emitting at 514.5 nm and was filtered by a 580 nm/FT dichroic mirror and a 570-nm colored glass filter; forward light scatter was also measured with this laser. Blue bimane fluorescence was excited by laser 2 with ultraviolet lines at 351 nm and 364 nm and filtered by a 450-nm-wide band pass filter. For sorting conjugates, tight gates were set around the entities showing both the red fluorescence of the PEL and the blue fluorescence of the targets, with low-angle light scatter set to exclude entities smaller than the target cells.

Autoradiography. Concentrated cell suspensions in fetal calf serum were smeared on gelatin-coated slides, air-dried, and fixed in methanol/acetic acid. The dry slides were dipped in Kodak NTB/2 emulsion, and exposed for 14 days. After development, the slides were Giemsa stained.

RESULTS

The Nonspecific Peritoneal Response to Irradiated Allogeneic Spleen Cells. To mimic the conditions of the key Kimura and Wigzell (5) experiment, C57BL/6 mice were injected i.p. with irradiated BALB/c spleen cells, and the PEL were harvested at day 6, the peak of the response. Excellent lysis was obtained of tumor cells bearing the sensitizing alloantigen (P815), but surprisingly equally good lysis was obtained of unrelated and even syngeneic tumor cells (EL4) (Fig. 1). In addition, the natural killer (NK) target YAC-1 was lysed (data not shown). Similar nonspecificity was obtained with other strain combinations (Fig. 1) except for BALB/c, which when sensitized with C57BL/6 showed partial specificity but much poorer lysis overall. To determine whether the PEL causing lysis were of the CTL lineage, C57BL/6 anti-BALB/c PEL were labeled with anti-Ly-2 antibody and sorted. Both Ly-2^- and Ly-2^+ cells killed the P815 tumor cells bearing the sensitizing alloantigen (Fig. 2), but only Ly-2^- cells killed the unrelated tumor targets EL4 (Fig. 2) and YAC-1. We concluded that PEL contained some allospecific Ly-2^- CTL, but also contained many nonspecific Ly-2^- cytotoxic cells, perhaps of the NK type.

The Specific Peritoneal Response to Viable Allogeneic Tumor Cells. We therefore sought a situation where the cytotoxic response was clearly due to specific CTL. Previous studies by us (6, 8) and by Kimura and Wigzell (5) suggested i.p. injection of viable allogeneic tumor cells should give a high level of specific CTL in the peritoneal cavity just at the point when the tumor cells are all eliminated (day 10). As shown in Fig. 3, the PEL from C57BL/6 mice injected with P815, or from BALB/c mice injected with EL4, gave excellent lysis specific for the sensitizing tumor; in addition, only marginal lysis of YAC-1 was detected and only at high effector to target cell ratios. The specificity was further demonstrated by cold-target inhibition studies of the lysis of P815 by C57BL/6 anti-P815 PEL. P815 blocked the lysis; EL4 and TIKAUT (a T lymphoma of H-2k origin) did not. B-cell blasts from lipopolysaccharide-stimulated BALB/c mice blocked lysis; those from CBA or C57BL/6 mice did not (data not shown). In addition, conjugate formation between PEL and tumor targets showed specificity for the sensitizing tumor (Table 1). It has been shown (10) that 80–90% of such conjugate-forming cells are active CTL.

The Specific Cytotoxic Cells are Ly-2^- cells. PEL from C57BL/6 mice sensitized with P815, and from other mouse strain/tumor combinations, were sorted into Ly-2^- and Ly-2^+ fractions and each was tested for cytotoxicity. Over several experiments, a mean of 60% (±12%) of the PEL were Ly-2^-.

In all cases, virtually all activity was due to Ly-2^- cells (Fig. 4). Likewise, the ability to form conjugates with the sensi-
tumor cells conjugated 29%, conjugated populations give us to conclude that the cytotoxic cells developed were predominantly specific Ly-2⁺ CTL, in contrast to those obtained with the irradiated spleen cell sensitization procedure.

A Cell Sorting Procedure for Isolation of CTL-Target Cell Conjugates. In our experience, a high proportion of the conjugates originally detected in free suspension are dissociated or lost when smears are made for autoradiography. In addition, on smears it is hard to distinguish true conjugates from accidental contacts. Accordingly, we developed a procedure where conjugate-forming PEL were positively isolated by cell sorting prior to preparing smears. Target cells were tagged with blue-fluorescent bimane, effector cells were tagged with red-fluorescent rhodamine, and double-positive red- and blue-fluorescent conjugates were isolated by cell sorting with a two-laser instrument. This procedure gave clearer sorting than an earlier technique using other fluorescent tags (9), where autofluorescence and transfer of label complicated longer runs. As shown in Fig. 5, the “double-positive” regions corresponding to conjugates could readily be distinguished. Specificity for the sensitizing tumor was confirmed in “criss-cross” experiments (Fig. 5). Flow cytometric analysis gave a similar level of conjugates and a similar specificity pattern to analysis by microscope examination (Table 1).

Are the Specific Conjugate-Forming CTL Products of Cell Division? Having established a sensitizing protocol giving specific Ly-2⁺ CTL and a sorting procedure to isolate specific conjugates, we repeated the key experiment. [³H]Thymidine was injected daily during various stages of the 10-day response of C57BL/6 mice to P815 tumor cells. At day 10, the PEL were harvested and tagged with rhodamine, conjugates were formed with bimane-tagged P815, and the conjugate fraction was sorted. The sorted cells were smeared and subjected to autoradiography. After staining, the PEL were readily distinguished from the P815 tumor targets on the autoradiographs. Some PEL and tumor targets remained as conjugates (Fig. 6); most had dissociated. The results, summarized in Table 2, are based on counts of all PEL in the

Table 1. Comparison of flow cytometric analysis and microscopic examination for scoring conjugates

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Tumor immunogen</th>
<th>Conjugate counting procedure</th>
<th>% tumor cells as conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>EL4</td>
<td>Microscope</td>
<td>24 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flow cytometry</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>P815</td>
<td>Microscope</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flow cytometry</td>
<td>7 ± 3</td>
</tr>
</tbody>
</table>

Results are the means ± SEM of 10 experiments for microscope counts and 3 experiments for flow cytometry.

Fig. 3. The cytotoxic specificity of PEL obtained 10 days after i.p. injection with allogeneic tumor cells. Only the sensitizing tumor cells were lysed, in contrast to the data shown in Fig. 1.

Fig. 4. The phenotype of PEL causing specific cytotoxicity. PEL harvested 10 days after injection of C57BL/6 mice with viable P815 tumor cells were labeled with fluorescent anti-Ly-2 and sorted into Ly-2⁺ and Ly-2⁻ fractions. These were tested on ¹¹¹In-labeled P815 target cells. Unseparated PEL gave the same cytolytic activity before and after labeling, so the results are pooled.

Fig. 5. Analysis and sorting of CTL-target cell conjugates by flow cytometry. PEL harvested 10 days after i.p. injection with allogeneic tumor cells were labeled with red fluorescent rhodamine, and the tumor target cells were labeled with blue fluorescent bimane. After interaction, the red- and blue-fluorescent conjugates formed where specific CTL were present could be analyzed and sorted from the free effectors and targets. The figures are contour plots all from one experiment where in each case the levels were 3, 10, and 30 cells per channel for 25,000 cells analyzed.
DISCUSSION

The specific Ly-2+ conjugate-forming CTL found in the mouse peritoneal cavity 10 days after injection of allogeneic tumor cells are the product of dividing cells, as judged by [3H]thymidine incorporation. This is in complete contrast with the results of an earlier study on the conjugate-forming cells found 6 days after injection of allogeneic spleen cells (5). Our results now suggest that many of the cytotoxic cells produced following the earlier protocol were nonspecific Ly-2- cells, perhaps of the NK type; in the earlier study, no specificity or phenotype studies were reported on this crucial group, although other sensitization protocols not used in the key experiments were examined. Nevertheless, we are surprised that so few labeled conjugate-forming cells were detected in these experiments since some should have been CTL. Other technical problems that might have reduced the labeling index are (i) the selective dissociation of CTL-target conjugates, (ii) the counting of false conjugates, and (iii) the relatively low overall labeling of lymphoid cells. However, it is also possible that the CTL found after different immunization protocols are produced in fundamentally different ways.

Our results provide one clear example where Ly-2+ T cells behave in vivo very much as in culture, with extensive clonal expansion associated with CTL generation. This is encouraging for those using clonal selection as the central model for T-cell responses and for those who trust that interleukin 2 and its receptor have an in vivo role. Nevertheless, we note that our protocol gives a prolonged and exceptionally intense stimulation, and the situation may be very different with more modest and transient T-cell responses. These would also be more difficult to study. Also, we note that the extent of clonal proliferation in vivo for T cells may still be less than that of B cells, as Mitchison and Pettersson have suggested (3). Our results do at least eliminate the extreme model, which proposes a complete absence of antigen-induced expansion of T cells in vivo.

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Table 2. Autoradiographic assessment of conjugate-forming PEL

<table>
<thead>
<tr>
<th>% [3H]thymidine-labeled cells</th>
<th>Days</th>
<th>Days</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–9</td>
<td>4–9</td>
<td>6–9</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>46 ± 7</td>
<td>28 ± 9</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Lymph node cells</td>
<td>41 ± 4</td>
<td>20 ± 6</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>All PEL</td>
<td>75 ± 7</td>
<td>71 ± 3</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>PEL in conjugate fraction</td>
<td>90 ± 2</td>
<td>91 ± 1</td>
<td>82 ± 1</td>
</tr>
</tbody>
</table>

Autoradiographic counts of labeled lymphoid cells from C57BL/6 mice injected i.p. with P815. [3H]Thymidine was administered each 24 hr over the time period stated. PEL were harvested at day 10, freed of macrophages, and reacted with P815 to form conjugates, which were then isolated by cell sorting. Spleen and lymph node cells from the same animals were also studied to provide a basis for comparison. Results are the means ± SEM of three experiments in each case, with 300 lymphoid cells scored in each group of each experiment.

Fig. 6. Autoradiograph of sorted conjugates. The unlabeled P815 target cells are readily distinguished from the labeled PEL.

conjuate fraction, whether still as conjugates or not. It is obvious that when [3H]thymidine was administered throughout the response, most of the unselected PEL were labeled. This result is not surprising, since these cells were induced by the sensitization procedure. The PEL from the sorted conjugate fraction showed an even higher labeling index. Limiting the [3H]thymidine exposure to the last 3 days of the response still gave a majority of conjugate-forming CTL labeled, as would be expected of a continuously expanding population. A separate count of the small proportion of PEL in the sorted conjugate fraction still in the form of conjugates on the smears showed that the majority of these (97%) were labeled, as shown in Fig. 6.