Idiotype positive T cells visualized by autoradiography and electron microscopy

(idiotypes/anti-alloantisera)

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ABSTRACT Anti-idiotypic antibodies were prepared in (CBA × C57BL/6)F1 hybrid mice by immunization either with CBA anti-C57BL/6 alloantisera or with purified CBA thymus-processed lymphoid cells (T cells). Iodinated anti-mouse Ig or triple sandwich ferritin-labeling techniques served to visualize the reaction between idiotype and antiidiotype. From 5 to 10% of purified CBA T cells appeared to carry receptors for C57BL/6 antigens. Heavily labeled cells had the morphology of small lymphocytes.

While postulated by most current immunological theories, the existence of specific receptors for antigen on thymus-processed lymphoid cells (T cells) has yet to be unequivocally demonstrated. Barring a completely different recognition process for T cells as opposed to thymus-independent lymphoid cells (B cells) or antibody, the minimum requirement for a T-cell receptor would seem to be possession of a variable region of similar design to that of immunoglobulins, whereas those parts of the receptor corresponding to immunoglobulin constant regions might conceivably be different. Hence, to reveal T-cell receptors, a reagent is needed that binds to specific features within variable regions.

Idiotypic determinants, antigens located in the variable region, provide such specific features (1). Evidence has been accumulating that the postulated T-cells share idiotypic determinants with alloantibody of like specificity (2–6). Antibodies prepared in semisynthetic animals against idiotypes of alloantibodies and against T cells react with T-cell idiotypes and can be used as probes for locating the T-cell receptor. Since these latter antibodies are ordinary immunoglobulins, they can easily be detected by conventional techniques using labeled anti-immunoglobulin from a different species. The major difficulty in this approach lies in the fact that some lymphoid cells, particularly B cells, normally carry immunoglobulin determinants on their surface. T cells, therefore, must first be purified from the bulk of B cells. In this paper we show that from 5 to 10% of mouse T cells react with anti-idiotypic antibody of proper specificity.

MATERIALS AND METHODS

Animals. Specific pathogen-free mice of the inbred strains CBA/H, C57BL/6, and F1 hybrids between these two strains were raised and maintained at the National Institute for Medical Research, Mill Hill, London. Young adults, either males or females, were used for each experiment.

Normal Spleen Cell Suspension. Mice were killed by cer-

vical dislocation and their spleens were teased with forceps in plastic petri dishes containing RPMI 1640 (Bio cult Laboratories, Paisley, Scotland) supplemented with 2.5 mg/ml of glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% fetal calf serum (Flow Laboratories Ltd, Ayrshire, England). The clumped cells were allowed to settle for 5 min at 4°. The suspension of dissociated cells was washed twice with the same medium.

Purification of Mouse T Cells. This was done as described (7). Briefly, mouse spleen cells were passed through a column of Degalan beads coated with normal mouse Ig and an excess of rabbit anti-mouse Ig serum. T cell populations prepared by this method showed less than 2% contamination by B cells, as measured with fluorescent rabbit anti-mouse Ig.

Induction of Alloantisera. Alloantisera of specificity CBA anti-C57BL/6 were induced in CBA mice by three injections of (CBA × C57BL/6)F1 spleen cells at monthly intervals. Each injection consisted of 5 × 10⁶ spleen cells given intraperitoneally. For the first injection the cells were mixed with an equal volume of Freund’s complete adjuvant. Animals were bled 10 days after the third injection and the sera were pooled, heat-inactivated (30 min, 56°), sterile filtered, and stored at –70°. Such sera contained cytotoxic alloantibodies of titers between 1:128 and 1:512, as measured in an assay described previously (8).

Induction of Anti-Idiotype Antisera. (A) By injection of alloantibodies: Crude Ig was precipitated from CBA anti-C57BL/6 alloantisera by dialysis against 18% and 15% Na₂SO₄. The final precipitate was mixed with complete Freund’s adjuvant. Each (CBA × C57BL/6)F1 host to be immunized received 0.2 ml of this mixture (corresponding to approximately 25 μg of protein) intraperitoneally. The animals received three more identical injections at 3-week intervals and were bled 10 days after the last injection. The sera of 25 mice were pooled, heat-inactivated, sterile filtered, and stored as described for alloantisera.

(B) By injection of T cells: (CBA × C57BL/6)F1 hosts were injected four times with 5 × 10⁷ CBA T lymphocytes intraperitoneally at 2-week intervals, the first injection containing an equal volume of Freund’s complete adjuvant. Animals were bled 10 days after the last injection, and the serum pools of 25 mice were handled as above.

F₁ antisera provoked by either method had the capacity to kill, with the aid of complement, cells reactive in specific mixed leukocyte responses in vitro or in specific graft-versus-host reactions in vivo (H. Binz and B. Askonas, in preparation).
Absorption of F₁ Antiserum with Erythrocytes. Before use, all F₁ antisera or normal control sera were absorbed with erythrocytes from (CBA × C57BL/6)F₁ mice. One milliliter of F₁ serum was absorbed twice with 0.5 ml of packed, twice-washed erythrocytes for 30 min at 4°C. Cells were removed by centrifugation for 10 min at 5000 rpm.

Iodination of Rabbit Anti-Mouse Ig. Rabbit antisera against mouse IgG was a gift from Dr. B. Askonas. The IgG fraction of this serum was prepared by standard procedures and was iodinated with ¹²⁵I (Radiochemical Centre, Amersham, England) to a specific activity of 2 mCi/mg (9).

Ferritin-Labeling of Guinea Pig Anti-Rabbit Ig. The IgG fraction of serum from guinea pigs immunized with rabbit IgG was coupled with ferritin as described (10).

Reaction of Parental T Cells with F₁ Sera. (A) For autoradiography: 0.1 ml of T cell suspension (5 × 10⁸ cells per ml) and 0.1 ml of undiluted, absorbed (see above) F₁ serum were mixed and allowed to stand for 1 hr at 4°C. The cells were then washed five times with medium containing 5% fetal calf serum. The final pellet was resuspended in 0.1 ml of iodinated rabbit anti-mouse Ig (5 × 10⁷ cpm/ml). After further incubation for 1 hr at 4°C, the cells were washed five times with medium and finally resuspended in 2 drops of 100% fetal calf serum. Smears of such cells on cleaned slides (Clay-Adams Inc., New York, N.Y.), dried at room temperature and fixed for 2 hr in 1% osmium tetroxide (11). After fixation, the cells were washed in serum-free medium, dehydrated, and embedded in Epon.

As a check on the specificity of the reaction, the radioactivity of cell suspensions prepared as above was determined in a gamma spectrometer. Significant uptake of radioactivity occurred in experiments of parental T cells with matching anti-idiotypic serum (H. Binz, unpublished).

(B) For immunoferritin labeling: Reactions of parental T cells with F₁ sera were as above. For the second sandwich layer, undiluted, unlabeled rabbit anti-mouse IgG was used. This was followed by a third incubation (1 hr at 4°C) with ferritin-labeled guinea pig anti-rabbit IgG. After five final washes the cells were resuspended in serum-free medium and processed for electron microscopy as above. The thin sections were stained with uranyl acetate and lead citrate.

Autoradiographs. (A) Light microscopy: Fixed and dried

<table>
<thead>
<tr>
<th>Purified T cells from strain</th>
<th>First serum sandwich layer*</th>
<th>No. of grains per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td>CBA</td>
<td>F₁, anti-(CBA anti-C57)†</td>
<td>925 (88)§</td>
</tr>
<tr>
<td>CBA</td>
<td>F₁, anti-CBA T</td>
<td>970 (88)§</td>
</tr>
<tr>
<td>CBA</td>
<td>F₁, normal serum</td>
<td>654 (94)§</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>F₁, anti-(CBA anti-C57)</td>
<td>706 (94)§</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>F₁, anti-CBA T</td>
<td>894 (94)§</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>F₁, normal serum</td>
<td>752 (94)§</td>
</tr>
<tr>
<td>(CBA × C57BL/6)F₁</td>
<td>F₁, anti-(CBA anti-C57)</td>
<td>803 (94)§</td>
</tr>
<tr>
<td>(CBA × C57BL/6)F₁</td>
<td>F₁, anti-CBA T</td>
<td>912 (94)§</td>
</tr>
<tr>
<td>(CBA × C57BL/6)F₁</td>
<td>F₁, normal serum</td>
<td>926 (94)§</td>
</tr>
</tbody>
</table>

* Cells were first treated with the indicated serum and were then reacted with iodinated rabbit anti-mouse Ig as the second sandwich layer (see Materials and Methods).
† Serum raised in (CBA × C57BL/6)F₁ animals by the injection of CBA anti-C57BL/6 alloantibody.
‡ Serum raised in (CBA × C57BL/6)F₁ animals by the injection of purified CBA T cells.
§ Figures within parentheses denote percentage of cells out of total.

**RESULTS**

Light microscopic autoradiographs

Most cells showed no silver grains or a few grains corresponding to the general background scatter. Approximately 5% of cells had low numbers of grains (5–10), and very few had between 10 and 90 grains (Table 1). Some cells, however, appeared rather heavily labeled, with grain counts well above 30 as estimated visually (photographs, like Fig. 1, show fewer grains in sharp focus).

T-cell suspensions in which the first sandwich layer consisted of normal F₁ serum (Table 1, lines 3, 6, and 9) had fewer than 2% of such heavily labeled cells. These presumably represented contaminating B cells. Similar figures were obtained when B-cell contamination was estimated by surface immunofluorescence.

When the first sandwich layer consisted of anti-idiotypic serum, the number of heavily labeled cells remained unchanged in those T-cell populations which, for genetic reasons, could not be expected to carry the relevant idiotype: C57BL/6 and (CBA × C57BL/6)F₁ (Table 1, lines 4, 5, 7, and 8). However, the same anti-idiotypic sera, when applied to T cells from CBA mice, significantly increased the numbers of heavily labeled cells (Table 1, lines 1 and 2). In the experiment shown on Table 1 both anti-idiotypic sera labeled 7% of CBA cells heavily. In other experiments of similar design, 5–10% of such cells were consistently observed.
Figs. 1–3. CBA T-cells reacted with (anti-CBA anti-C57) serum (anti-idiotypic serum) and 125I-labeled rabbit anti-mouse IgG. Fig. 1. Light microscopic autoradiography of whole cells. The selected field shows one reactive cell covered with numerous grains (see Table 1). Fig. 2. Autoradiography after 10 weeks of exposure. The localization of 8 grains on the periphery of the cell indicates specific surface binding. X12600. Fig. 3. Autoradiography of a thin section with a population of cells among which one was reactive with the anti-idiotypic serum, as indicated by the presence of 4 grains after 4 weeks of exposure. X6300.

Electron microscopic autoradiographs

Careful scanning of CBA cell suspensions with anti-idiotypic serum as the first sandwich layer revealed occasional cells with evidence of radioactive decay at their surface (Figs. 2 and 3). The number of grains associated with cells, after 4 weeks of exposure, never exceeded 5 (Fig. 3), whereas after 10 weeks, cells associated with 5–10 grains were typically present (Fig. 2). Numerous unlabeled cells in the immediate vicinity of labeled cells attested to the specificity of the reaction. In preparations with C57BL/6 cells or (CBA × C57BL/6)F1 cells, a prolonged search was able to detect only one labeled cell, presumably a B cell contaminant. In spite of intense efforts, no labeled CBA cell could be seen in the preparation with normal F1 serum as the first sandwich layer.

Ferritin-labeled electron microscopy

With a similar amount of effort ferritin-labeled cells could be detected in CBA cell suspensions having anti-idiotypic serum as the first, rabbit anti-mouse Ig as the second, and ferritin-tagged guinea pig anti-rabbit Ig as the third sandwich layer. In these cells, fairly large surface areas were occupied by the immunologic reactants, suggesting a large number of specific receptors on individual cells. Again, adjacent cells were entirely free of label (Figs. 4 and 5). At higher magnification the structural composition of the triple sandwich could be readily interpreted. The surface mem-

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brane of the lymphocyte was covered with an electron-opaque layer representing the anti-idiotypic anti-globulin complex, covered with clusters of ferritin of the guinea pig anti-rabbit IgG conjugate (Fig. 6).

DISCUSSION

Our experiments showed that a certain proportion of CBA T cells carried antigenic determinants reacting specifically with antibodies induced in (CBA x C57BL/6)F1 animals either by CBA anti-C57BL/6 serum or by CBA T cells. The reasons for believing that these determinants formed part of the T-cell receptor for alloantigens were the following: Similar antibodies specifically blocked recognition of alloantigens by T cells in vitro (2, 3), impeded the mixed leukocyte reaction (H. Binz and B. Askonas, in preparation), and inhibited graft-versus-host reactions in vitro (4), three phenomena thought to depend on functionally intact T cells.

The T-cell receptor has been considered "elusive" (14) mainly because it failed to react with conventional anti-immunoglobulin reagents. This has led to the speculation that T cells might depend on a recognition system fundamentally different from immunoglobulin. However, when it is realized that conventional anti-immunoglobulin reagents all address themselves to parts of the molecules that are actually irrelevant in antigen recognition, such extreme views become less plausible. The present communication shows that T cells react perfectly well with antibodies directed at variable region determinants of alloantibodies. The antigens concerned, usually called idiotypes, are coded for by the
same v genes that define the antibody combining site (1). In contrast to other systems, where antibodies of the same specificity can have differing idiotypes (15), and where the same idiotype can belong to antibodies of differing specificity (16), in alloantibody a given combining specificity and its corresponding idiotype seem to be strongly correlated (17). Hence, the finding that mouse T cells carry idiotypes similar to those of alloantibodies of like specificity most probably means that they carry, as an essential part of their receptor, a similar or identical v gene product.

Since B cells normally have immunoglobulin molecules on their surface, and since our procedure made use of labeled anti-immunoglobulin for tagging, it was essential to start with a T-cell suspension largely devoid of B cells. This was achieved by an immunosorobent column retaining most of the B cells (7). Light microscopic autoradiography and surface immunofluorescence showed between 1 and 2% of cells to be tagged by anti-immunoglobulin reagents in the absence of the first (anti-idiotype) sandwich layer. This number, then, represented contamination by B cells and possibly by other cells carrying constant region immunoglobulin determinants on their surface. When the correct anti-idiotype first sandwich layer had been applied, the same labeled anti-immunoglobulin revealed significantly more tagged cells, between 5 and 10%.

An incorrect first sandwich layer reduced the number of positive cells to control levels. CBA cells first treated with anti-(CBA anti-C57BL/6) serum showed 7% labeled cells; the same cell preparation treated with normal F1 serum had only 1.5% labeled cells (Table 1). C57BL/6 and F1 hybrid cells, whether treated with normal F1 serum or with anti-idiotype serum, never showed more than 1–2% heavily labeled cells.

The possibility that the structures revealed were cytophilically adsorbed antibodies seems remote, since such immunoglobulin molecules should have reacted with antibodies directed at constant region determinants. Moreover, rat cells treated with trypsin and allowed to recover under tissue culture condition regenerated the same number of idiotype-carrying cells (H. Binz and H. Wigzell, unpublished).

The electron micrographs served two purposes. First, they added another type of label, ferritin, to that used in autoradiography. Second, they allowed a check on cellular morphology. On the other hand, the electron micrographs were more difficult to evaluate quantitatively. They did show, however, that labeled cells were fairly numerous in those reaction mixtures using the correct anti-idiotype and were quite rare in all control mixtures. For instance, many other examples of specifically labeled cells as shown in Figs. 2–6 could be found, whereas the contaminating positive cells in controls which were detectable in light microscopic preparations largely escaped observation in electron microscopic thin sections even after careful scanning of many grids.

The specifically labeled cells had features typical of small lymphocytes (Figs. 2–5). We were, however, unable to decide according to published morphological criteria (18, 19) whether they were to be reckoned as T or as B cells. Their T-cell nature was surmised from the other data presented.

Regarding the density of receptors on positive cells, the impression of scarcity created by the electron microscopic autoradiographs (Figs. 2 and 3) is certainly misleading. Actually, when the number of decays seen in thin section was extrapolated to the whole cell surface, a figure close to 500 grains per positive cell was obtained. A more realistic picture was provided by the ferritin labeling experiments, which revealed reactive patches extending over a considerable proportion of the cell surface (Figs. 4–6).

As documented before, anti-idiotype antibodies of the types used in the present experiments react specifically with recognition structures for the corresponding alloantigens (2–6, 8, 9, 17). The alloantigenic differences between the two mouse strains CBA and C57BL/6 involve a number of specificities, so that we cannot claim that each labeled cell represented the same specific idiotype. Nevertheless, we can say that roughly 7% of CBA T cells were collectively committed to recognizing the foreignness of C57BL/6 alloantigens. This may seem a surprisingly high figure, but is entirely in accord with other, fully independent estimates (20–22).

Antisera used in this work were produced at the National Institute for Medical Research, Mill Hill, London, by H.B. during his stay there. We thank Dr. B. Askonas for numerous discussions and Dr. K. Binz for help. H.B. is recipient of a Swiss National Foundation fellowship. The study was supported by the Swiss National Foundation, contract NCI-COB-33959, and the Swedish Cancer Society.