Brief Definitive Reports

GENERAL METHOD FOR ISOLATION AND RECOVERY OF B CELLS BEARING SPECIFIC RECEPTORS*

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(Received for publication 10 January 1974)

Starting with the work of Wigzell and Andersson (1) a number of methods have been described for the isolation of cells bearing specific receptors. Depletion of a particular set of cells has been accomplished with a variety of immunoadsorbents. Recovery of cells is a more complex problem. As shown by Wofsy et al., cells with antihapten receptors can be recovered in a viable state by elution with free hapten at neutral pH (2). A more general method consists in adsorbing specific populations of cells onto Sephadex beads to which antigen is covalently conjugated. The Sephadex is then digested with dextranase to liberate the cells (3). Edelman and his collaborators adsorb cells onto nylon strings to which the antigen is covalently linked and recover the cells by plucking the strings. The mechanism may involve either the removal of receptors from cells under the mechanical stress or the breaking of noncovalent bonds linking the cell receptor to the antigen. The ingenious cell sorter devised by Herzenberg and his associates provides an effective general technique for depletion or recovery of a specific population of cells (5). The instrument is, however, quite expensive. Cells obtained by this method necessarily have some fluorescent antigen, or fluorescent antibody directed to a cell-surface antigen, on their surfaces; this does not appear to affect their utility for many biological experiments.

We report here a method, carried out at neutral pH, which may be generally applicable to the isolation of B cells bearing specific receptors. In principle, the method consists in coating polystyrene tubes (6) with specifically purified rabbit anti-*p*-azobenzoate antibody. This is followed by an antigen-azobenzoate conjugate; a few micrograms are bound. Cells with receptors for the antigen are then bound specifically to the tube, and are released upon addition of free hapten. Only a small fraction of the antigen-azobenzoate conjugate is released under conditions which suffice to permit recovery of nearly all of the cells.

* Supported by grant AI-06281 from the National Institutes of Health.

‡ Recipient of a Postdoctoral Fellowship of the National Kidney Foundation.

§ Supported by a Surgery Academic Training Grant from the National Institutes of Health (GM-1930).

Materials and Methods

Adsorption and Elution of Cells.—6-ml polystyrene tubes (12 × 75 mm, Falcon Plastics Co., Div. of BioQuest, Oxnard, Calif.) were used. To each tube was added 1 ml of specifically purified rabbit anti-*p*-azobenzoate antibody (7), at a concentration of 1.5 mg/ml in saline-borate buffer, pH 8, ionic strength 0.16. The tube was rotated in a horizontal position at 5–10 rpm for a minimum of 4 h at room temperature. Alternatively, the tube was filled with antibody solution and allowed to stand overnight at room temperature with gentle agitation. Experiments with radiolabeled antibody indicated that approximately 20 μg was irreversibly bound to each tube. Tubes were washed repeatedly with neutral buffer, exposed to a solution of bovine serum albumin (5 mg/ml in saline-borate buffer, pH 8) for 2 h at room temperature, then washed again.

The experiments reported here were carried out with keyhole limpet hemocyanin (KLH) (Calbiochem, San Diego, Calif.) as the antigen. A conjugate of this protein with *p*-azobenzoate groups was prepared by diazotization (8). Approximately 18 mol of hapten were conjugated per 10^5 g of protein.

Tubes coated with the antibenzoate antibody were filled with a solution of KLH-*p*-azobenzoate (1 mg/ml in saline-borate buffer). After gentle agitation for 2 h at room temperature the tubes were washed repeatedly. In preliminary experiments, using ^{125}I-labeled conjugate, 6–12 μg were bound per tube.

Subsequent steps were carried out at 4°C. Each tube was rinsed once with RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) and 15–25 × 10^6 lymphoid cells (see below) suspended in 0.6 ml of medium were added to each tube. The capped tubes were rotated in a horizontal position at 0.3–0.5 rpm for 40 min to adsorb cells with receptors specific for KLH. The suspension containing unbound cells was poured off and the tubes washed gently with three 2-ml portions of cold medium. 3 ml of medium containing 1 × 10^{-3} M *p*-nitrobenzoate was then added. The tubes were again rotated horizontally at 1–2 rpm for 30 min, then centrifuged at 200 *g* for 10 min to recover the adsorbed cells. The eluted cells, present in the pellet, were resuspended in medium.

Immunization of Mice.—A/J mice were inoculated intraperitoneally with 0.5 mg of KLH in 0.2 ml of Freund's complete adjuvant. Spleens were removed 2–3 wk after the injection. Single-cell suspensions were prepared by teasing the cells through a 100-mesh stainless steel screen in the presence of medium. After washing once with medium, the cells were passed through a column containing 0.1 g of cotton wool per 10^8 cells, then washed three times with medium. More than 95% of the leukocytes present had the morphology of small lymphocytes.

RESULTS

Fig. 1 shows photomicrographs of sections of the polystyrene tubes after exposure to cell suspensions under various conditions. Each tube was previously coated with antibenzoate antibody and then with KLH-*p*-azobenzoate. In Fig. 1 *a* the tube had been exposed to 2 × 10^7 spleen cells (filtered through cotton wool) from a mouse immunized with KLH. The surface is densely coated with lymphocytes. Fig. 1 *b* shows the effect of subsequent exposure for 30 min of such a tube of 1 × 10^{-3} M *p*-nitrobenzoate in medium at 4°C., followed by centrifugation at 200 *g* for 10 minutes. This resulted in essentially complete removal of the bound cells. Exposure to medium alone, followed by centrifugation had little obvious effect (Fig. 1 *c*).[1] Fig. 1 *d* shows the results of

[1] Other experiments indicated that centrifugation in the absence of hapten releases 10%–30% of the bound cells. This step is generally omitted to minimize the time required for the procedure.

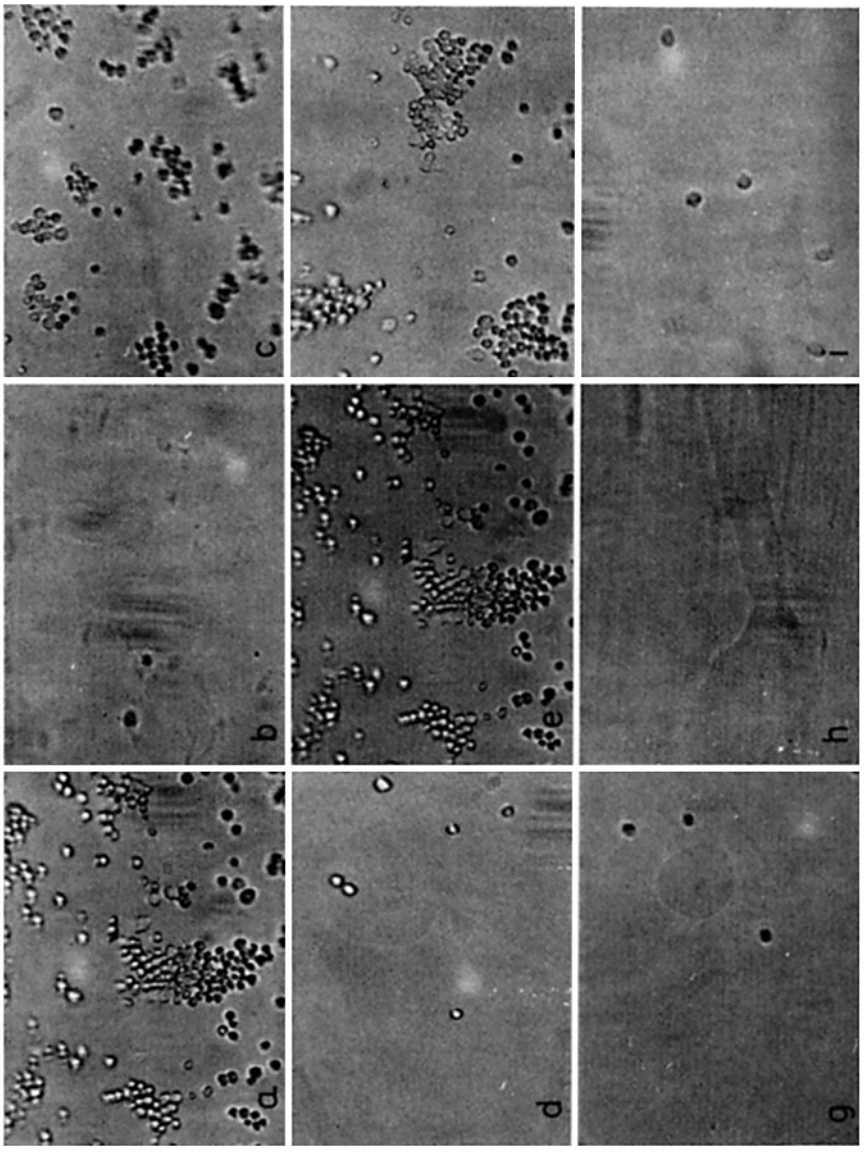

FIG. 1. Photographs of sections of polystyrene tubes coated with specifically purified anti-*p*-azobenzoate antibody and then with KLH-*p*-azobenzoate before exposure to 2×10^7 cells from A/J mice. (*a*) Exposed to KLH immune spleen cells; (*b*) treated as in (*a*) then exposed to 1×10^{-3} M *p*-nitrobenzoate and centrifuged at 200 *g* for 10 min; (*c*), as in (*a*), but exposed to medium lacking hapten, then centrifuged; (*d*), exposed to KLH immune cells in the presence of 50 μg/ml of KLH; (*e*) and (*f*), same as (*d*), except that bovine IgG or bovine serum albumin, rather than KLH, was present in the medium; (*g*), (*h*), (*i*), exposed to spleen, thymus or bone marrow cells, respectively, from nonimmunized A/J mice.

exposure of a coated tube to KLH immune cells suspended in medium containing 50 μg of KLH per ml. The antigen almost completely inhibited uptake of cells onto the tube. The presence of 50 μg/ml of unrelated proteins, bovine IgG or bovine serum albumin, had no apparent inhibitory effect (Figs. 1 *e* and *f*). Ovalbumin similarly was noninhibitory (not shown). Figs. 1 *g*, *h*, and *i* show the absence of cells on the surfaces of tubes exposed to spleen, thymus, or bone marrow cells, respectively, from nonimmunized mice.

Another test of specificity of uptake consisted in exposure of the tube to mouse anti-KLH antiserum before introduction of the immune cell suspension. This pretreatment completely blocked the uptake of cells.

Quantitative data on recovery of cells under the various conditions described above are presented in Table I. The results in the table are typical of those of many similar experiments. About 1% of the spleen lymphocytes, from mice primed with a single injection of KLH, were recovered. (It is important for maximum uptake to use a concentrated cell suspension [$>1 \times 10^7$ cells/ml]). There was essentially no uptake of KLH immune cells by tubes coated only with bovine serum albumin, or coated with normal rabbit IgG followed by KLH-*p*-azobenzoate.

In all experiments the viability of cells was tested by determining their capacity to exclude trypan blue. More than 80%, and usually more than 90%, of the cells were viable by this criterion. There was no decrease in cell viability after 30 min exposure at 4°C to hapten at a concentration (0.1 M) 100 times greater than that used for elution of cells from the tube.

Nearly all cells eluted were B cells. An anti-theta reagent (AKR anti-C3H),[2] which in the presence of guinea pig complement killed 62% of lymph node cells from an A/J mouse, had no discernible effect on cells eluted from a KLH-coated tube; the percentage of viable cells remained unchanged within experimental error.

The nature of the receptors on the various cell populations was investigated with fluoresceinated reagents (9). Cells were first exposed at 0–4°C to KLH at a concentration of 1 mg/ml in RPMI-1640. After washing with medium, they were treated at the same temperature with an IgG fraction of mouse anti-KLH antiserum to which fluorescein had been conjugated. In a typical experiment 1–3% of the KLH immune cell population (passed through cotton wool) and 35–55% of the lymphocyte population recovered from a tube was fluorescent. The values were obtained by counting a minimum of 300 cells having the morphology of small lymphocytes.

In other experiments, ^{125}I-labeled KLH-*p*-azobenzoate was used in the procedure. Cells recovered from the tube were washed six times and their radioactivity was determined. They were then exposed to 50 μg/ml of the same labeled preparation, washed and counted again. There was a 14–19-fold in-

[2] Generously provided by Dr. Ethel Jacobson.

TABLE I

*Numbers of Cells Eluted by 1 × 10^{-3} M p-Nitrobenzoate from Polystyrene Tubes Coated with Antibenzoate Antibody and KLH-p-Azobenzoate**

Cell population	Inhibitor (50 μg/ml)	Number of cells eluted per tube
KLH immune, spleen	—	286,000
KLH immune, spleen	KLH	<4,000
KLH immune, spleen	Bovine IgG	334,000
KLH immune, spleen	Bovine serum albumin	316,000
KLH immune, spleen	Ovalbumin	260,000
Nonimmune spleen	—	<4,000
Nonimmune thymus	—	<4,000
Nonimmune bone marrow	—	<4,000
KLH immune, thymus	—	<4,000

* All tubes were exposed to a suspension containing 2 × 10^7 cells from A/J mice. Spleen cell populations had been passed through cotton wool; portions of the same pool of cells were used for the first five experiments. After treatment with hapten and centrifugation very few cells could be seen on any tube.

crease in the amount of radioactivity present on the cells. Values were corrected for the relatively small amount of radioactivity absorbed by spleen cells from nonimmune A/J mice. This experiment indicates that most receptors in the recovered cells were not occupied.

It was found that only a small fraction (<5%) of the labeled KLH-*p*-azobenzoate was released from the tube upon exposure to 1 × 10^{-3} M *p*-nitrobenzoate for 30 min, followed by centrifugation at 200 *g*. This observation is discussed below.

DISCUSSION

The method described should provide a means for isolating, at neutral pH, B cells with a variety of specific receptors. It is possible to recover about 1 to 3 × 10^5 antigen-specific cells per polystyrene tube. The data reported were obtained with spleen cells from mice immunized with KLH. We have carried out preliminary experiments with ovalbumin as antigen, with similar results, and have used an antiglobulin reagent to isolate B cells. In the latter instance benzoate groups were conjugated to the IgG fraction of rabbit anti-mouse IgG having activity against both Fab and Fc fragments.

In theory, the technique should be applicable to the isolation of T cells, through the use of an anti-theta reagent, or to cells bearing various other specific antigens. A possible disadvantage derives from the presence of antigen on the isolated cells. However, as indicated above, most receptors remain unoccupied.

An unexpected result was the failure of 1 × 10^{-3} M *p*-nitrobenzoate to release a substantial fraction of the ^{125}I-labeled KLH-*p*-azobenzoate from the

tube, despite the fact that antibenzoate antibodies can readily be recovered from immunoadsorbents containing bound hapten under these conditions. A possible explanation is that the antigen, of high molecular weight, is held through multiple bonds to antibodies on the tube. An antibody, in contrast, can be linked to immobilized hapten through a maximum of two bonds.

The preferential release of cells may be attributable to a weakening of the noncovalent bonds, joining KLH benzoate molecules on the cell to the anti-benzoate antibodies, by the action of gravitational force on the relatively massive lymphocyte during centrifugation. In any event, the relative ease of elution of cells, as compared to KLH-*p*-azobenzoate, tends to reduce the amount of antigen present on the isolated cells.

Antihapten antibodies other than antibenzoate should be effective in the procedure. We used antibenzoate antibodies because they were available and because the haptens tested (*p*-nitrobenzoate and *p*-aminobenzoate) were not lethal to cells, even at concentrations much higher than those required in the procedure.

Adoptive transfer experiments are in progress to determine the activity of cells isolated by this procedure.

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