Specific Separation of Cells on Affinity Columns*

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Abstract. Cell filtration is performed on affinity columns prepared by the chemical modification of large polyacrylamide beads. Affinity columns selectively bind cells that produce antihapten antibodies of corresponding specificity, while cells without the specific antihapten sites pass through freely and are recovered quantitatively in the eluate. The binding of the antihapten specific cells is inhibited by hapten. Such cell filtration affinity columns may be useful in specifically purifying cell populations with characteristic membrane-receptor proteins.

The study of differentiation in the mammalian immune response, and probably in other complex developmental systems, would be facilitated greatly if it were feasible to separate cells according to the specificity of receptor proteins situated on cell membranes. Could this be done efficiently with cells of the immune system, several possibilities would arise: (1) one could examine lymphocyte populations depleted of, or selected for, a single-antibody specificity and isolated at different stages of (or before) response to a particular antigen; (2) such specific cell populations could be isolated from different lymphoid tissues and examined separately or in recombined pools, *in vivo* or *in vitro*, to clarify their role in induction of the immune response; (3) specifically purified populations of antigen-sensitive cells might be prepared as the first step in attempts ultimately to isolate and characterize membrane-bound receptor antibody.

The use of immunoabsorbents should be uniquely suitable for such cell separations, and a few approaches toward the specific fractionation of cells on antigen-coated columns have been reported.^{1,2} However, severe problems have been encountered, since large numbers of cells adhere nonspecifically to glass beads and other chromatographic matrices utilized up to now, and the specific lymphocytes one would like to bind selectively occur at low frequencies in any cell population.

We report here the development of methods and materials for preparing affinity columns that bind cells with given antihapten antibody specificities, while permitting the free passage and virtually complete recovery of other cells. The columns are constituted from large polyacrylamide beads to which haptens have been covalently attached by several procedures, starting with chemical modifications of polyacrylamide recently worked out by Inman and Dintzis.³ In this paper, we describe the selective removal on affinity columns of antihapten

antibody-producing cells from cell populations obtained from the spleens of mice immunized against an azo-coupled phenyl- β -lactoside (*lac*) antigen.

Materials and Methods. Bio-Gel P-6, 16–25 mesh (over 600- μ m diameter), was a gift from Bio-Rad.⁴ Hydrazide, acyl azide, and aminoethyl derivatives of polyacrylamide beads were prepared according to procedures of Inman and Dintzis.⁵

Histamine dihydrochloride was obtained from Matheson, Coleman, and Bell. Glycoside derivatives were obtained from Cyclo. All other chemicals were reagent grade.

Preparation of affinity columns: Since aromatic amino compounds do not react readily with the acyl azide derivative of Bio-Gel, aminophenyl glycosides were covalently attached to polyacrylamide beads by way of a histamine-coupled Bio-Gel intermediate,⁴ in the following manner: (a) 5 g (dry weight) of Bio-Gel P-6 was converted to the hydrazide and then the acyl azide.⁵ (b) To the resulting 150-ml mixture, a 20-ml aqueous solution of 5 mmol histamine and sufficient triethylamine (about 3.75 ml) was added to bring the reaction solution to pH = 9.5. (c) After 1 hr at 0°C, 20 ml of ethanolamine were added to quench the remaining acyl azide groups. (d) After another hour, the gel was washed and equilibrated with 0.25 M sodium borate buffer, pH = 9.0. (e) 5.1 mmol of *p*-aminophenyl glycoside were dissolved in 45 ml 0.25 N HCl and a 5-ml aqueous solution of 5 mmol (0.34 g) sodium nitrite was added at 0°C.⁵ (f) After 15 min, the diazonium solution was added slowly, with stirring, to a 100-ml suspension of the histamine-coupled Bio-Gel in borate buffer, pH = 9.0, and the reaction mixture was allowed to stand at 4°C for several hours. (g) The azophenyl glycoside beads were washed several times with 1% NaCl by decanting, and were then poured into a siliconized 25 $\times$ 1.5 cm Pharmacia column with a 400-mesh nylon screen, and washed extensively with a 0.15 M sodium chloride, 0.02 M sodium phosphate buffer, pH 7.2. The capacity of a *lac* affinity column for binding purified anti-*lac* antibodies ranges from 0.2 to 0.5 mg antibody/ml gel.

In earlier column preparations, diazoniumphenyl glycosides were added directly to Bio-Gel hydrazide at pH 9 in order to form a tetrazole ring linkage.⁶ Columns prepared in this manner appeared to lose antibody-binding capacity over a period of weeks, whereas the azohistamine-coupled affinity columns remain stable indefinitely.

Trinitrophenyl (TNP) or dinitrophenyl (DNP) groups were linked to beads by reacting the corresponding benzenesulfonates with Bio-Gel hydrazide.⁸ It was noted that trinitrobenzenesulfonate reacts also with unmodified Bio-Gel, yielding an unstable product and resulting in graduate release of yellow material into storage buffers. Therefore, TNP and DNP bead preparations were washed consecutively with 0.1 N acetic acid, water, 0.1 N ammonia, and phosphate-saline buffer. With this washing procedure, no leaching of color into storage buffers was observed.

TNP and DNP gels were also prepared by reacting the appropriate benzenesulfonate with the aminoethyl derivative of Bio-Gel.⁸ The TNP-aminoethylated beads tend to be fragile, whereas other modified polyacrylamide beads remain intact with repeated use.

Cell filtration through affinity columns: All cell filtrations were performed on the Pharmacia columns at 4°C in the phosphate saline buffer, pH 7.2. A 1- or 2-ml cell suspension was layered on and allowed to penetrate the column. Two 0.5-ml portions of buffer were added successively to wash the cells into the column. Buffer was then passed through the column at a very rapid rate (40–60 ml/min). The column void volume was 7–9 ml, and cells emerged in the remaining 15 ml of the eluate. For counting of cells, and for assaying antibody secreting cells, a corresponding 1- or 2-ml aliquot of the original cell suspension was diluted to 15 ml and compared with the eluate cell suspension. Cells were diluted in 0.15 M NaCl containing 0.1% saponin and counted in a Coulter counter, model A. *Lac* affinity columns were washed after use with 100 ml 0.5 M lactose and then reequilibrated with phosphate saline buffer. Columns were stored at 4°C in 0.02% sodium azide.

Assay for antibody-producing cells: A modified form of the Jerne assay⁷ for detecting plaque-forming cells (PF cells) was used.⁸ After incubation for 3 hr at 37°C, complement and amplifying anti-IgG antisera were added. For the assay, hapten was

coupled to sheep red blood cells essentially by the method of Ingraham:⁹ 1 ml of a solution of 5×10^{-3} M diazoniumphenyl glycoside in 0.014 N HCl⁵ was added at 4°C to 1 ml of a 50% suspension of sheep erythrocytes in 0.4 M phosphate buffer, pH 7.8, 0.3 M in NaCl; the mixture was rotated at room temperature for 1 hr; the modified red cells were centrifuged and washed three times with balanced salt solution⁸ containing 1% fetal bovine serum.

Sheep red blood cells, guinea pig complement, and amplifying antiserum used in plaque assays were provided by Dr. R. I. Mishell.

Immunization: Highly coupled azoconjugates of aminophenyl glycosides and key-hole limpet hemocyanin¹⁰ were used for immunization. Swiss Webster mice, 3 months old, were injected in the foot pads with 50- μ g antigen in complete Freund's adjuvant. After 3–5 weeks, the animals were injected intraperitoneally with 25- μ g antigen in saline. Five days later, the mice were killed and the cells of the spleen suspended and washed in balanced saline solution for immediate use.

Immunization of mice with sheep red blood cells was performed as described by Mishell and Dutton.⁸

Results. Table 1 summarizes the results of 11 experiments in which cells from mice immunized against *lac* antigen were passed through *lac* affinity columns.

TABLE 1. Removal of anti-*lac* PF cells on *lac* affinity columns.

Total cells put on column	PPM* before filtration	PPM* in eluate	Cells recovered (%)	PPM* recovered (%)
6.0×10^7	200	43	100	22
1.2×10^6	750	210	100	28
1.2×10^6	750	230	100	30
1.6×10^7	760	127	100	16
1.6×10^7	760	132	100	17
4.0×10^7	1200	77	97	9
4.0×10^7	1200	102	97	12
4.0×10^7	1200	77	100	9
4.0×10^7	1200	230	100	29
1.5×10^8	300	30	95	10
2.0×10^6	700	119	96	17
Average recoveries:			99 $\pm$ 2	18 $\pm$ 7

* PPM = plaque formers per million cells.

Cell filtrations reported in this table were performed on columns prepared from several different batches of *lac*-modified polyacrylamide beads. See *Materials and Methods*.

Of the total number of cells (10^6 – 10^8) put on a column in any run, the average recovery of cells in the eluate was $99 \pm 2\%$ while $18 \pm 7\%$ of the initial total of anti-*lac* PF cells appeared in the eluate.

To test the specificity of the *lac* affinity columns, several types of experiments were performed (Table 2). Cells were passed through a *lac* column equilibrated

TABLE 2. Specificity of *lac* affinity columns.

PF cell specificity	Concentration of PNP- <i>lac</i>	Total cells put on column	PPM before filtration	PPM in eluate
Anti- <i>lac</i>	0	4.0×10^7	1200	102
Anti- <i>lac</i>	1×10^{-6} M	3.7×10^7	1240	1270
Anti-sheep red blood cells	0	5.2×10^6	430	430
Anti-azophenyl- β -glucoside	0	7.2×10^7	30	30

All cell filtrations reported in this table were performed on the same *lac* column. See *Materials and Methods*.

with 1×10^{-5} M *p*-nitrophenyl- β -lactoside (PNP-*lac*), and then assayed for PF cells after dilution to a hapten concentration of 2.5×10^{-7} M; all anti-*lac* PF cells were recovered in the eluate. Cells from mice immunized against sheep red blood cells passed freely through *lac* affinity columns, all anti-sheep red cell plaque-forming cells being recovered in the eluate. Similarly, there was no retention of antiglucoside PF cells when cells from mice immunized against *p*-azophenyl- β -glucoside antigen were passed through *lac* affinity columns.

The inhibition by PNP-*lac* of plaque formation in cell populations before and after passage through a *lac* affinity column was investigated. The results of one such comparison are shown in Figure 1, demonstrating that anti-*lac* PF cells not

FIG. 1.—Inhibition of anti-*lac* PF cells by PNP-*lac* hapten. —, inhibition of PF cells prior to cell filtration. ---, inhibition of PF cells in the eluate after cell filtration on a *lac* affinity column. Percentages of PF cells values are based on the amount found in the original cell population before the addition of hapten.

retained on the column were considerably less sensitive to inhibition by hapten than the unfractionated PF cells in the original cell population. In assays of cell populations prior to filtration on a *lac* column, 10–20% of the plaque formers were still detectable at concentrations of PNP-*lac* in excess of 10^{-5} M.

Since *lac* hapten inhibits the binding of anti-*lac* PF cells to a *lac* affinity column (Table 2), the feasibility of eluting bound anti-*lac* PF cells with PNP-*lac* was examined. Experiments with this objective were hampered by the fact that the ability to detect anti-*lac* PF cells falls off rapidly when cell suspensions are very dilute. Furthermore, when cells from *lac*-immunized mice are maintained in the presence of 5×10^{-4} M PNP-*lac* at 4°C for 1 hr, no anti-*lac* PF cells can be detected even after the hapten concentration is reduced to 10^{-6} M. The latter effect seems to be specific, in that when the cells are treated in the very same manner

with *p*-nitrophenyl- β -glucoside instead of with PNP-*lac*, over 90% of the anti-*lac* PF cells can still be demonstrated in plaque assays. In order to test whether anti-*lac* PF cells are actually bound specifically to *lac*-modified Bio-Gel beads and whether they can be removed by elution with hapten, the following "batch" experiments were performed:

To each of two tubes, one containing 0.2 ml (decanted) unmodified Bio-Gel P-6, 300- μ m beads and the other containing 0.2 ml *lac*-modified beads, a 1.5 ml suspension of 2×10^7 spleen cells from *lac*-immunized mice was added while mixing. After the beads settled, 25- λ aliquots were removed from each supernatant for subsequent determination of the number of anti-*lac* PF cells. The remaining mixture in each tube was made 1×10^{-5} M in PNP-*lac*, and aliquots were removed from the supernatants at various times after gentle mixing and allowing the beads to settle. The cells were maintained at 4°C throughout. The results of assays for anti-*lac* PF cells in each of the aliquots (diluted 1:40

to reduce the hapten concentration to 2.5×10^{-7} M) are shown in Figure 2. It can be seen that the *lac* affinity beads initially removed about 70% of the number of anti-*lac* PF cells observed in the control tube containing unmodified beads.

FIG. 2.—Elution by PNP-*lac* hapten of anti-*lac* PF cells from *lac* affinity beads. —, the number of plaque formers per 10^6 cells (PPM) in a cell suspension mixed with unmodified polyacrylamide beads. — · —, the number of PPM in an equivalent cell suspension mixed with *lac* affinity beads. After determining the initial values (0 min) for PPM, PNP-*lac* is added to both cell suspensions to a concentration of 1×10^{-6} M; aliquots of cells are removed at various times for assay (at a 1:40 dilution) of anti-*lac* PPM.

After addition of PNP-*lac*, a substantial release of anti-*lac* PF cells from the beads, continuing over at least a 45-min interval, is evident. As indicated previously, there is a progressive loss in the number of anti-*lac* antibody-producing cells detectable when the cells are maintained for a period of time in the presence of hapten.

Several additional experiments, which will be reported in full separately,¹¹ tested the possibility that cells involved in antibody production or in other aspects of the immune response might be adversely affected by passage through Bio-Gel affinity columns. Spleen cells from nonimmunized mice were passed through sterilized *lac* affinity beads and then stimulated in culture with trinitrophenylated sheep red blood cell antigen. The resulting primary immune responses against both sheep erythrocytes and TNP determinants were as described by Kettman and Dutton,¹² and the response levels could not be distinguished from those obtained with cells not first exposed to modified Bio-Gel.

Columns constituted from other Bio-Gel derivatives were compared to *lac* affinity columns with respect to the free passage and recovery of spleen cells. From 95 to 100% cell recoveries were obtained with columns prepared by the coupling of either ortho-diazoniumphenyl- β -glucoside or trinitrobenzenesulfonate to Bio-Gel hydrazide. Cell recoveries in the eluates of columns prepared by reacting trinitrobenzenesulfonate or dinitrobenzenesulfonate with the ethylene diamine derivate of Bio-Gel averaged 70–80%. Cells from *lac*-immunized mice were passed through each of the various glucoside, trinitrophenyl, and dinitrophenyl affinity columns, and in no case was there a diminution in the ratio of anti-*lac* PF cells to other cells.

Discussion. The filtration of cells through modified polyacrylamide affinity columns results in the highly selective binding of cells which have on their surfaces proteins of complementary specificity. In these studies, the specific receptor molecules are on antihapten antibody-producing cells generated by immunization against an azophenyl- β -lactoside conjugated antigen.

Specificity in the removal of anti-*lac* antibody-producing cells on *lac* affinity columns is established in the following manner (Tables 1 and 2): (1) while enumeration of cells on a Coulter counter indicates that spleen cells put on a *lac* Bio-Gel column are all recovered in the eluate, 70–90% of the anti-*lac* PF cells are in fact filtered out by the column; (2) in the presence of hapten, 10^{-5} M PNP-*lac*, all anti-*lac* PF cells are recovered in the eluate; and (3) the number of antibody-producing cells of other specificities, including anti-sheep red blood cells and anti-azophenyl- β -glucoside PF cells, is not at all reduced in cell populations passed through a *lac* affinity column. Furthermore, anti-*lac* PF cells are not selectively removed from cell populations passed through polyacrylamide beads to which glucoside, TNP, or DNP groups, rather than *lac* groups, have been covalently attached.

The depletion of anti-*lac* PF cells from cell populations filtered through *lac* Bio-Gel is the direct result of the reversible binding of these cells to the *lac* beads. This is demonstrated by the fact that anti-*lac* antibody-producing cells, which have been removed from cell suspensions by mixing with *lac* Bio-Gel, can be recovered from the beads as active anti-*lac* PF cells by the addition of hapten (Fig. 2). Major difficulties confront attempts to examine directly isolated populations of specifically purified anti-*lac* antibody-producing cells. Nevertheless, the results shown in Figure 2 suggest that anti-*lac* PF cells are not damaged functionally when bound to the *lac* beads, and, under the conditions of the experiment, may even be somewhat protected.

The major precondition we have sought to meet in developing materials for receptor-specific cell separations is that these materials be inert to the characteristic "sticky" properties of cells that are readily adsorbed on glass and on many other charged or hydrophobic surfaces. Recent major advances in the specific purification of enzyme and antibody molecules on affinity columns prepared by modification of neutral polysaccharides¹³ or polyacrylamide Bio-Gels³ seemed to us to provide a possible basis for specific chromatographic separation of cells. While we have had some success in the removal of antihapten antibody-producing cells in "batch" experiments with modified Sepharose,¹⁴ the prerequisite activation of the original polysaccharides with cyanogen bromide¹³ fractures Sepharose or Sephadex beads, making cell filtration on columns impossible. On the other hand, Bio-Gel beads are not broken in the course of the chemical modification reactions utilized by Inman and Dintzis,³ nor by the additional reaction procedures which we have applied.

With the use of large polyacrylamide beads, cells can be filtered without loss through a variety of modified Bio-Gels. Even when TNP groups were attached by the reaction of trinitrobenzenesulfonate with Bio-Gel hydrazide, no non-specific adsorption of filtered cells was observed. Attachment of TNP or DNP groups to aminoethyl Bio-Gel, however, did reduce cell recoveries by 20–30%, possibly because the aminoethyl chain permits association of proximal aromatic groups and the consequent formation of hydrophobic patches on Bio-Gel bead surfaces.

Numerous categories of small molecules can be covalently bonded to polyacrylamide beads, affording a potential for the possible use of affinity columns in

studies with cellular receptors for various antigens, hormones, neuroeffectors, and chemotactic sites. Compounds with aromatic amino groups, which react very slowly with Bio-Gel acyl azide, can be coupled readily as diazoniums to the histamine derivative of Bio-Gel which we have used as an intermediate in the preparation of our *lac* affinity columns.

For studies with antibody-producing cells and, even more, with antigen-sensitive precursor cells, the number of determinant groups of a given kind that can be attached chemically to a small volume of polyacrylamide beads far exceeds the frequency with which the cells of any particular antibody specificity occur in a general lymphocyte population. Since antibody molecules cannot penetrate Bio-Gel P-6, the amount of anti-*lac* antibody specifically bound by a P-6 *lac* affinity column provides a minimum approximation of the number of *lac* groups on the external surface of the modified beads. Since our P-6 *lac* preparations bind from 0.2 to 0.5 mg of antibody/ml decanted gel, there must be about 10^{15} surface *lac* groups per milliliter gel. It is likely that affinity columns modified to a far lesser extent would still be effective in depleting cell populations of substantial proportions of antigen-reactive cells. Thus, for example, it may be feasible to form affinity columns with protein antigens coupled in amounts insufficient to negate the free flow through the beads of all cells other than those with the appropriate receptor sites.¹⁵

In view of the relatively high number of equivalents of *lac* groups per milliliter of modified beads used in this study, a question arises as to why from 10 to 30% of anti-*lac* PF cells are not bound to *lac* affinity columns. The results obtained in inhibiting anti-*lac* plaque formation with hapten (Fig. 1) indicate that the cells which escape the column are probably those whose antibody is of low affinity for the phenyl- β -lactoside group. Presumably, some of the antibody on the surface of antibody-secreting cells is loosely held and may be stripped away as the cells pass through the *lac* beads. If this is so, antigen-reactive precursor cells may be sequestered on affinity columns more readily than antibody-producing cells. Experiments to test this possibility are in progress.

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