ISOLATION BY CELL-COLUMN CHROMATOGRAPHY OF IMMUNOGLOBULINS
SPECIFIC FOR CELL SURFACE CARBOHYDRATES*

By BEN-AMI SELA† AND GERALD M. EDELMAN

(From The Rockefeller University, New York 10021)

Studies of cell surface receptors have been carried out with a variety of reagents including lectins and antibodies to surface glycoproteins. However, aside from lectins (1), few macromolecular reagents specific for the carbohydrate portion of glycoproteins are available, and it has been particularly difficult to obtain antibodies against these determinants by direct immunization. Recently, we have shown that normal sera fractionated on a column of immobilized glycoproteins such as fetuin and thyroglobulin yield antibodies specific for the carbohydrate portion of glycoproteins and other cell surface carbohydrates (2). We now describe the more general approach of immobilizing whole cells on a solid support in order to fractionate specific cell-binding components from normal animal sera. We call this procedure cell-column chromatography.

Materials and Methods

Mouse lymphomas EL4.BU and P388 (The Salk Institute, La Jolla, Calif.) were grown in RPMI-1640 medium (Associated Biomedic Systems, Buffalo, N. Y.) with 5% fetal calf serum plus antibiotics. Chick retinal cells (3) and Dictyostelium discoideum cells in growing and developing stages (4) were prepared as described.

For a typical cell-column, 50 ml of swelled Sephadex G-50 (coarse) (Pharmacia Fine Chemicals, Piscataway, N. J.) was stirred for 30 min at room temperature with 50 mg of Concanavalin A (Con A) in phosphate-buffered saline (PBS). Excess Con A was decanted, and 1-4 ml of packed cells were allowed to adsorb to the Con A-coated Sephadex beads for 15 min with slow stirring. The mixture was poured into a column (4 x 20 cm), washed for 30 min with 3% glutaraldehyde in PBS at a rate of 10 ml/h and then for 2 h at 100 ml/h, allowed to stand in this solution without flow overnight at room temperature, and then washed extensively with PBS. Columns of immobilized cells could be used for several weeks without obvious morphological changes.

Pooled nonimmune sera were obtained from dogs, turkeys, chickens, goats, and rabbits (Grand Island Biological Company, Grand Island, N. Y.). Immune sera were obtained from rabbits after four intravenous injections of 5 x 10⁹ EL4 or P388 cells at weekly intervals. 100-150 ml serum was fractionated on a column at room temperature and eluted at a rate of 10 ml/h; the column was then washed with PBS until the absorbancy at 280 nm was below 0.005. Material bound to the column was then eluted in 0.05 M glycine-HCl, 0.5 M NaCl, pH 3.0. The eluate was immediately readjusted to pH 7.0 and then concentrated in Amicon cells with a Diaflo XM-50 membrane.

Column eluates were analyzed by complement-mediated cytotoxicity using ⁵⁹Cr-labeled cells incubated with agarose-absorbed guinea pig complement and known amounts of column eluate;

* This work was supported by U. S. Public Health Service grants AI-11378, AI-09273, and AM-04256 from the National Institutes of Health.
† Fellow in Cancer Research supported by Grant DRG-45-FT of the Damon Runyon-Walter Winchell Cancer Fund.
### Activity of Ig Fractions from Cell-Columns of P388 and EL4 Cell Lines

|                      | Fluorescent staining | Agglutination of | Complement-mediated cytolysis of: |
|----------------------|----------------------|------------------|-----------------------------------|
|                      | P388 cells | EL4 cells | P388 cells | EL4 cells | P388 cells | Neuramindase-treated P388 cells | EL4 cells |
| Ig (turkey) from:    |           |           |           |           |           |           |                         |
| P388 cell-column     | +         | -         | +         | +         | Not tested | Not tested | Not tested |
| EL4 cell-column      | -         | +         | -         | +         | Not tested | Not tested | Not tested |
| Ig (dog) from:       |           |           |           |           |           |           |                         |
| P388 cell-column     | +         | -         | +         | +         | 3         | 72        | 65                     |
| EL4 cell-column      | -         | +         | -         | -         | 5         | 68        | 68                     |
| Anti-P388 serum      | +         | +         | ++        | +         | 84        | 87        | 89                     |
| Anti-EL4 serum       | +         | +         | ++        | +         | 82        | 79        | 85                     |
| Serum from unimmunized rabbits, turkeys, or dogs: | 0 | 1 | 0 |

* After 30 min at room temperature, agglutination was scored on a scale of (-) to (+++) based on the lowest concentration showing agglutination: (-) no agglutination at 1 mg/ml; (+) 0.5 mg/ml; (+++) 0.1 mg/ml; (++++) 0.05 mg/ml; (++++) 0.01 mg/ml.

** Percent specific 51Cr release. Antibody fractions at 0.5-1.0 mg/ml gave the highest cytotoxic response.

§ Fc fragment of avian IgG does not fix mammalian complement.

~ Ig's from sera of rabbits immunized with P388 or EL4 cells.

**Results and Discussion**

Columns made with the different cell types reproducibly bound a small fraction of the protein in normal sera of different animal species. This protein could be eluted with yields ranging from 2–3 mg/100 ml (chicken serum) to 5–6 mg/100 ml (dog serum). Thus, material comprising less than 0.1% of the total input protein could be isolated by the use of the cell-columns; we could not attain this efficiency using a batch adsorption procedure with fixed cells (5).

The components eluted from the cell-columns were shown to be Ig's by immunoelectrophoresis and polyacrylamide gel electrophoresis in SDS. As measured with 131I-labeled \( \mu \)- and \( \gamma \)-chains in SDS gels, the eluted Ig's from dog serum consisted of 65% IgM and 35% IgG; Ig's from turkey serum consisted of approximately 70% IgG and only 30% IgM. The eluates were devoid of any free Con A as tested by immunodiffusion with antibodies to Con A. Con A bound to the Sephadex was not eluted by \( \alpha \)-methylmannoside, indicating that no unfixed lectin remained after the glutaraldehyde treatment.

Ig's isolated on EL4 and P388 cell-columns were examined for their binding specificities to different cells (Table I). As shown by indirect fluorescent staining, Ig's from turkey, dog, and chicken sera fractionated on P388 cell-columns bound to more than 90% of the P388 cells. Of the stained cells, more than 90% showed a patchy distribution of the label and a minor fraction of the cells showed
caps. None of the cells stained diffusely. When the Ig's isolated on P388 columns were incubated with EL4 cells or with chick embryo retina cells, however, there was no fluorescent staining. Similarly, Ig's fractionated on EL4 columns specifically bound EL4 cells but not P388 cells. In contrast, the Ig's isolated on a cell-column containing chick embryo retinal cells bound both to isolated retina cells and to chick embryo fibroblasts (6).

The agglutinating activity of the Ig's isolated on the different columns was also specific. Dog anti-P388 Ig's (100 μg/ml) agglutinated P388 cells, but not EL4 or chick neural retina cells (Table I). Similarly, turkey (Table I) and chicken Ig's isolated from EL4 cell-columns agglutinated only EL4 cells. Anti-EL4 Ig's from the dog, goat, and rabbit failed, however, to agglutinate EL4 cells even at high concentrations despite the fact that the fluorescein-labeled Ig's were bound extensively to the surfaces of the EL4 cells.

Unfractionated normal sera did not stain cells in the fluorescence assay and showed no agglutinating activity even at protein concentrations of 10 mg/ml. Similarly, IgG fractions from chicken and rabbit sera had no agglutinating activity and did not stain the test cells at concentrations up to 5 mg/ml.

Complement-mediated cell lysis gave a complex picture of specificity that depended upon the cell type and its prior treatment (Table I). Although no cytolysis occurred with unfractionated dog serum or its IgG fraction, EL4 cells were lysed by dog Ig from both EL4 columns and P388 columns, while P388 cells were not lysed in the presence of either Ig. P388 cells pretreated for 30 min with 100 μl neuraminidase (Vibrio cholera) (Behring Diagnostics, Somerville, N. J.; 500 U/ml) were lysed to a similar extent (68-72%) by Ig's from dog serum fractionated on either EL4 or P388 cell-columns. Although neuraminidase treatment abrogated resistance to lysis (7), the enzyme-treated cells were not agglutinated or stained by fluorescein-labeled anti-P388 Ig at the sensitivity threshold of either test.

In sharp contrast to the specific cell binding properties of column isolated Ig's, immune sera obtained by inoculation of rabbits with EL4 or P388 cells stained, agglutinated, and lysed both cell lines equally well (Table I). This suggests that the Ig's isolated from normal sera by the affinity method recognized a narrower set of surface components, some of which were characteristic of each cell line. Nevertheless, as shown by the cytotoxicity tests, some portion of these Ig's was cross-reactive with each cell line (Table I).

To clarify these differences in specificity, surface mapping procedures were used to compare the membrane components recognized by the natural antibodies fractionated on cell-columns and by antibodies to cell surfaces from immune sera. Cells were iodinated, extracted, and immunoprecipitated (8) by dog Ig's isolated from EL4 and P388 cell-columns. When EL4 cell extracts were treated with dog anti-EL4 Ig's, several cell surface molecules could be resolved (Fig. 1a). Immunoprecipitation with extracts of EL4 cells using dog Ig's fractionated on P388 cell-columns yielded much smaller amounts of radioactive material which showed a similar electrophoretic migration pattern. Dog anti-P388 Ig's also immunoprecipitated more label from surface molecules of P388 extracts than did dog anti-EL4 Ig's (Fig. 1a). These observations are consistent with the specificities of the cell-column Ig's indicated in Table I. The precipitation of small amounts of surface molecules from extracts of cells other than those used in
column fractionation may reflect limited cross-reactivity of the surface determinants on the two cell types. This is in accord with the cross-reactivity between EL4 and P388 cells in complement-mediated cytotoxicity assays (Table 1), inasmuch as the amount of binding required for cytotoxicity is likely to be below the amount required for fluorescent staining or agglutination of cells.

Unlike the naturally occurring Ig's, antibodies from sera of rabbits immunized with either EL4 or P388 cells gave electrophoretic profiles of EL4 surface molecules that were similar both in pattern and amount (Fig. 1b). This result
may account for the nonpreferential staining and agglutination of EL4 cells with antibodies from rabbits immunized with either EL4 or P388 cells (Table I).

A clearer understanding of the specificity patterns of the naturally occurring antibodies was obtained by examining the role of cell surface carbohydrates in antibody binding. P388 cells immobilized on a Sephadex column were treated with 4 liters of sodium periodate (0.005 M) in 0.1 M acetate, pH 4.5, at 4°C in the dark for 8 h under constant flow to destroy the terminal sugar residues of the carbohydrate chains of glycoproteins (9). After washing with PBS, the column was loaded with normal turkey or dog sera. Serum components eluted from this column did not stain or agglutinate P388 cells even at high concentrations. Incubation for 10 min of the anti-P388 Ig's with a pellet of $3 \times 10^6$ glutaraldehyde-fixed and periodate-treated P388 cells did not absorb these activities from the supernatant solution. The same amount of glutaraldehyde-fixed cells completely absorbed these activities.

The carbohydrate specificity of antibodies isolated from cell-columns was confirmed by the observation that either 0.02 M D-galactose or 0.1 M N-acetylneuraminic acid inhibited the agglutination of P388 cells by turkey anti-P388 Ig's. Similarly, a mixture of 0.1 M D-galactose and 0.1 M N-acetylneuraminic acid inhibited the fluorescent staining of P388 cells. When the turkey Ig's were eluted from a P388 cell-column treated first with neuraminidase and then with $\beta$-galactosidase, however, the agglutination of P388 cells was not inhibited by 0.2 M D-galactose. Partial inhibition of agglutination was obtained with L-fucose, N-acetyl-$D$-glucosamine, or $\alpha$-mannose, but not with D-glucose. In contrast to the results with column-isolated Ig's, agglutination or fluorescent staining of P388 cells by specific antisera from immunized rabbits was not inhibited by either D-galactose or N-acetylneuraminic acid at concentrations that completely inhibited both activities of cell-column-fractionated Ig's. This is in line with the differences in specificity of antibodies from cell-columns and from immune sera (Table I, Fig. 1).

The two maximally inhibiting sugars, D-galactose and N-acetylneuraminic acid, are the most abundant terminal sugars among surface glycoproteins; D-glucose, the noninhibitory sugar, is rarely found in surface glycoproteins and is present only in a nonterminal position in glycolipids (10). These results suggest that the majority of Ig's fractionated on cell-columns recognize saccharide termini extended from cell surfaces. We cannot, however, exclude the presence of small amounts of antibodies directed against noncarbohydrate-containing surface molecules.

Carbohydrate-specific antibodies isolated from cell-columns are useful in comparing the role of carbohydrates in cell-interactions at different developmental stages, as well as for detecting differences between carbohydrate-containing molecules on cell walls and cell membranes. For example, it has been reported that periodate-sensitive surface "contact sites" are involved in the cell aggregation of the slime mold, D. discoideum (11). We found that gel patterns of Nonidet-P 40 extracts of slime mold cells iodinated by the lactoperoxidase method showed differences between cells in the vegetative (nonaggregative) phase and those in the developing (aggregative) phase. When turkey Ig's isolated from a cell-column made with cells in the developing phase were absorbed with slime mold cells in the growing phase, the absorbed Ig fraction
enhanced the immunoprecipitation of a glycoprotein with a mol wt of about 110,000 daltons from an iodinated extract of developing slime mold cells; no evidence of this molecule was seen in growing slime mold cells. The absorbed Ig preparation is now being used to analyze the role of this surface component in the aggregation of developing cells. In a similar study, we found that different cell-columns containing the yeast *Saccharomyces cerevisiae* or spheroplasts obtained from the yeast by gluclulase treatment (12) each yielded Ig's specific for the corresponding cell form. No cross-reactivity was observed between the two Ig fractions, consistent with possible differences between yeast cell walls (13) and plasma membranes.

Both of these examples and that of the mouse tumor cells (Fig. 1) suggest that antibodies fractionated on cell-columns can be used in identifying and comparing the glycoproteins of cell surfaces in a variety of systems. In addition, the method may be applicable to the isolation from body fluids or tissue extracts of any molecule capable of binding to the surface of suitably fixed cells.

**Summary**

A new method of affinity chromatography using glutaraldehyde-fixed cells immobilized on Sephadex beads has been used to isolate immunoglobulins (Ig's) specific for cell surface glycoproteins. Ig's that specifically bound and agglutinated the same cells as those originally fixed on the columns were isolated from nonimmune sera of various species. Periodate treatment of the cell-columns and the free cells destroyed their ability to bind the Ig's, and the binding of the Ig's to untreated cells was inhibited by monosaccharides such as D-galactose and sialic acid. The binding of antibodies directed against cell surfaces obtained by immunizing animals with the same mouse tumor cell lines used on the columns (P388 and EL4) was not inhibited by various saccharides. Surface glycoproteins obtained from the mouse tumor cells by immunoprecipitation with the column-isolated Ig's yielded specific electrophoretic patterns that differed from those obtained using Ig's from the sera of rabbits immunized with the tumor cells. The data suggest that the Ig's isolated by cell-column chromatography were directed against carbohydrates, probably those in terminal positions of the polysaccharide portions of the tumor cell surface glycoproteins. Column-isolated Ig's specific for carbohydrates were also useful in studies of cell interactions in nonmammalian systems including *Dictyostelium discoideum* and *Saccharomyces cerevisiae*. The cell-column method appears to be adaptable to the isolation of a variety of molecules in addition to antibodies.

The authors are grateful to Dr. Robert Brackenbury for valuable discussion and to Ms. Elizabeth Leibold for excellent technical assistance.

*Received for publication 1 October 1976.*

**References**

1. Sela, B-A., J. L. Wang, and G. M. Edelman. 1975. Isolation of lectins of different specificities on a single affinity adsorbent. *J. Biol. Chem.* 250:7535.
2. Sela, B-A., J. L. Wang, and G. M. Edelman. 1975. Antibodies reactive with cell surface carbohydrates. *Proc. Natl. Acad. Sci. U. S. A.* 72:1127.
3. Rutishauser, U., J-P. Thiery, R. Brackenbury, B-A. Sela, and G. M. Edelman. 1976. Mechanisms of adhesion among cells from neural tissues of the chick embryo. *Proc. Natl. Acad. Sci. U. S. A.* 73:577.
4. Sussman, M. 1966. Biochemical and genetic methods in the study of cellular slime mold development. *In Methods in Cell Physiology, II.* D. Prescott, editor. Academic Press, Inc., New York. 397.
5. Reitherman, R. W., S. D. Rosen, and S. H. Barondes. 1974. Lectin purification using formalinized erythrocytes as a general affinity adsorbent. *Nature (Lond.)*, 248:599.
6. Edelman, G. M., and I. Yahara. 1976. Temperature-sensitive changes in surface modulating assemblies of fibroblasts transformed by mutants of Rous sarcoma virus. *Proc. Natl. Acad. Sci. U. S. A.* 73:2047.
7. Tompkins, W. A. F., P. Seth, S. Gee, and W. E. Rawls. 1976. Neuraminidase reversal of resistance to lysis of herpes simplex virus-infected cells by antibody and complement. *J. Immunol.* 116:489.
8. Henning, R., R. J. Milner, K. Reske, B. A. Cunningham, and G. M. Edelman. 1976. Subunit structure, cell surface orientation, and partial amino acid sequences of murine histocompatibility antigens. *Proc. Natl. Acad. Sci. U. S. A.* 73:118.
9. Marshall, R. D., and A. Neuberger. 1972. Periodate oxidation methods. *In Glycoproteins. Part A.* A. Gottschalk, editor. Elsevier Publishing Co., Amsterdam. 2nd edition. 331.
10. Wiegandt, H. 1971. Glycosphingolipids. *Adv. Lipid Res.* 9:249.
11. Beug, H., G. Gerisch, S. Kempff, V. Riedel, and G. Cremer. 1970. Specific inhibition of cell contact formation in Dictyostelium by univalent antibodies. *Exp. Cell Res.* 63:147.
12. Kuo, S-C., and J. O. Lampen. 1971. Osmotic regulation of invertase formation and secretion by protoplasts of Saccharomyces. *J. Bacteriol.* 106:183.
13. Ballou, C. 1976. Structure and biosynthesis of the mannan component of the yeast cell envelope. *Adv. Microbiol. Physiol.* In press.