A One-step Purification of Membrane Proteins Using a High Efficiency Immunomatrix*

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A method is described by which an immunoaffinity matrix was constructed by binding antibody directly or indirectly to protein A-Sepharose 4B followed by cross-linking of the complex with dimethyl pimelimidate. This allows optimal spatial orientation of antibodies and, thus, maximum antigen binding efficiency. The affinity matrices were stable to high and low pH buffers without any significant antibody loss. The optimal conditions of antibody saturation, cross-linker concentration, and elution system were established and affinity columns made with the monoclonal antibodies J5, W6/32, and OKT9 for one-step isolation of the common acute lymphoblastic leukemia-associated antigen, HLA-AB antigens, and transferrin receptor, respectively, from cell lysates. The same methodology was also applied to immobilize transferrin with polyclonal anti-transferrin antibodies. This was then used to isolate the transferrin receptor from cell lysates.

The introduction and development of methods for producing monoclonal antibodies (1) have facilitated the detection of specific cell surface components and, in addition, made them more accessible to isolation and purification.

Conventional methods of immobilizing antibodies on solid matrices usually employ cyanogen bromide-activated Sepharose although other matrices have also been used (2). The use of cyanogen bromide-activated Sepharose often generates affinity columns with low antibody activity because of the multisite attachment and orientation of the immunoglobulin molecule, which reduces the efficiency of antibody/antigen interaction. Protein A from Staphylococcus aureus has the property of binding to the Fc portion of immunoglobulin molecules. A matrix employing protein A-Sepharose, therefore, offers optimal spatial orientation of antibodies. Gersten and Marchalonis (3) used this method to couple an antisera to Limulus hemocyanin, which they stabilized by chemical cross-linking.

In this report, we describe a method in which the advantages of this system are combined with those of monoclonal antibodies to produce a matrix which has optimal orientation of antibody molecules for high efficiency antigen binding on a stable matrix with no significant antibody leakage. The method of preparation is fast and permits microscale preparation of matrices for analysis of antigen as well as large scale one-step purification of membrane components from cell lysates for amino acid sequencing or functional studies. Furthermore, in cases where no antibodies are available, polyclonal antibodies against the ligand can be utilized as an indirect method to isolate the receptor.

**MATERIALS AND METHODS**

**Antibodies and Cells**

The monoclonal antibody W6/32 (IgG2a) (4) directed against HLA-AB antigens was kindly provided by Dr. W. Bodmer (Imperial Cancer Research Fund). It was purified from ascites by 50% ammonium sulfate precipitation and 2 successive Sephacyr S-200 fractionations. It was pure by isoelectric focusing criteria. OKT9 (IgG1) (5) which reacts with a cell surface receptor for transferrin (6) was kindly provided by Drs. G. Goldstein and P. Kung (Ortho Pharmaceutical Corp.). This monoclonal antibody was similarly purified from ascites by 50% ammonium sulfate precipitation, two successive Sephacyr S-200 fractionations, and preparative isoelectric focusing. Monoclonal antibody J5 (IgG2a), which reacts with the common acute lymphoblastic leukemia antigen (CALLA) (7), kindly provided by Dr. J. Ritz (Sidney Farber Cancer Institute) was from crude ascites. Rabbit anti-mouse Ig was prepared to a total mouse immunoglobulin fraction and the serum was affinity-purified on a mouse Ig-Sepharose 4B affinity column followed by cross-absorption on a human Ig-Sepharose 4B affinity column. Rabbit anti-transferrin was obtained from Dako (Denmark).

A cell line derived from a common acute lymphoblastic leukemia, Nalm-6 (8), was grown under standard conditions (9) in RPMI 1640 medium supplemented with 100 units of penicillin/streptomycin, 20 mM glutamine, and 10% fetal calf serum. This line was used as a source of CALLA and HLA-AB antigens. Fetal liver cells were used as a source of transferrin receptor as previously described (10).

**Preparation of Antibody-Protein A Matrix**

**Protein A-binding Monoclonal Antibodies (W6/32, J5)—Protein A-Sepharose CL-4B (Pharmacia) was mixed with antibody in 0.1 M borate buffer, pH 8.2, for 30 min at room temperature with gentle shaking, after which the Sepharose beads were washed with excess borate buffer. The Sepharose was washed with 0.2 M triethanolamine, pH 8.2, and then resuspended in 20 volumes of dimethyl pimelimidate dihydrochloride (Pierce) freshly made up in 0.2 M triethanolamine with the pH readjusted to pH 8.2. The mixture was agitated gently at room temperature for 45 min and the reaction was stopped by centrifuging the beads (500 × g for 1 min) and resuspending in an equal volume of ethanolamine, pH 8.2, of the same molarity as the dimethyl pimelimidate dihydrochloride. After 5 min, the cross-linked beads were washed three times with 0.1 M borate buffer, pH 8.2, supplemented with 0.02% sodium azide.

**Non-protein A-binding Monoclonal Antibody (OKT9)—If non-protein A-binding antibodies were to be used, protein A-Sepharose CL-4B beads were first precoated with rabbit anti-mouse Ig in 0.1 M borate buffer, pH 8.2, as described below. After washing the beads three times with borate buffer, the mouse monoclonal antibody was added. After further washing, the total complex was cross-linked as described above.

**Preparation of Ligand-Antibody Protein A Matrix**

Rabbit anti-transferrin antibodies were bound to protein A-Sepharose as described above and after washing, the matrix was divided into two aliquots and one was incubated with saturating amounts of transferrin. Both aliquots were then cross-linked with dimethyl pimelimidate dihydrochloride as above.
binds to protein A) or as two-layer systems using rabbit anti-neutrality (-pH 7.4) by the addition of washes).

using the anti-HLA-AB monoclonal antibody W6/32 (which sodium azide. The eluted material was immediately brought to near	beads with an equal volume of 0.05 M diethylamine, pH 11.5, contain-
times in borate buffer and stored in this buffer containing 0.02%
beads were removed by gentle centrifugation (500 x g for 1 min). The	was gently agitated with the matrix for 2 h at 4 C, after which the	Cell lysates prepared as described above, either freshly prepared	bovine serum albumin. After 15 min at 4 C, the lysate was centrifuged	2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, I mg/ml
variably described (10) and, after washing in fresh medium, lysed in a	buffer containing 0.15 m NaCl, 0.01 m Tris-HCl, pH 8.2, 1 mm EDTA, 2 mm phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1 mg/ml
bodies. This involved boiling in 0.05 M Tris-HCl, pH 6.8, containing 0.5% SDS; (ii) 0.15 m NaCl, 0.05 m Tris-HCl, pH 8.2, 0.5% Nonidet P-40; (iii) 0.15 m NaCl, 0.5% sodium deoxycholate (two washes).

Antigens specifically bound to the matrix were eluted from the	beads by an equal volume of 0.5 m diethylamine, pH 11.5, contain-
ing 0.5% sodium deoxycholate, for 2 min. This was then repeated and the two supernatants were pooled. The beads were washed several	times in borate buffer and stored in this buffer containing 0.02%
sodium azide. The eluted material was immediately brought to near

Centrifugation of /50 volume of a 10% suspension of antibody W6/32 at different percentages of saturation. After

TABLE I

<table>
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<th>Antibody</th>
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<th>After 0.5 m NaCl wash</th>
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<td>mM</td>
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<td>100</td>
<td>21705 (162)</td>
<td>19850 (146)</td>
<td>18910 (194)</td>
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Membrane Proteins and Receptor Purification

RADIOISOTOPE USE

Radial Isotope of Cells and Antibodies

Cells were surface-labeled by the 125I-lactoperoxidase method, previously described (10) and, after washing in fresh medium, lysed in a buffer containing 0.15 m NaCl, 0.01 m Tris-HCl, pH 8.2, 1 mm EDTA, 2 mm phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1 mg/ml bovine serum albumin. After 15 min at 4 °C, the lysate was centrifuged (100,000 x g for 30 min), and the supernatant was removed and brought to 0.5 m NaCl by the addition of 3.5 m NaCl. The lysate was then used for immune absorption on the affinity matrix.

Purified antibodies were radiolabeled by the chloramine-T method of iodination (11) using 125I.

SDS-Polyacrylamide Gel Electrophoresis

Samples were run on 1-mm thick polyacrylamide slab gels as described by Laemmli (12). After electrophoresis, gels were either visualized using the silver nitrate procedure for proteins (13) or dried directly onto a sheet of chromatography paper (Whatman) and detected by autoradiography.

Use of Immunoabsorbent Column

Cell lysates prepared as described above, either freshly prepared or stored at -70 °C, were pre-absorbed to remove nonspecifically binding components by incubation with 1% volume of a 10% suspension of Staphylococcus aureus Cowan I strain, which had been heat killed and formalin fixed, for 30 min at 4 °C, followed by centrifugation at 100,000 x g for 30 min. The specific affinity matrix was either used as a column or as a batch absorbent. In the latter case, the cell lysate was gently agitated with the matrix for 2 h at 4 °C, after which the beads were removed by gentle centrifugation (500 x g for 1 min). The centrifuged beads were washed in sequence with the following three buffers: (i) 0.5 m NaCl, 0.05 m Tris-HCl, pH 8.2, 1 mm EDTA, 0.5% Nonidet P-40; (ii) 0.15 m NaCl, 0.05 m Tris-HCl, pH 8.2, 0.5% Nonidet P-40; (iii) 0.15 m NaCl, 0.5% sodium deoxycholate (two washes).

Antigens specifically bound to the matrix were eluted from the beads with an equal volume of 0.5 m diethylamine, pH 11.5, containing 0.5% sodium deoxycholate, for 2 min. This was then repeated and the two supernatants were pooled. The beads were washed several times in borate buffer and stored in this buffer containing 0.02% sodium azide. The eluted material was immediately brought to near neutrality (pH 7.4) by the addition of 1/10 of 0.5 m NaH2PO4.

RESULTS AND DISCUSSION

Efficiency of Chemical Cross-linking

Affinity matrices were made either as one layer systems using the anti-HLA-AB monoclonal antibody W6/32 (which binds to protein A) or as two-layer systems using rabbit anti-mouse Ig and the anti-transferrin receptor monoclonal anti-

body OKT9. Radiolabeled antibodies were used for this purpose and, after the initial binding to protein A-Sepharose, were cross-linked with increasing concentrations of dimethyl pimelimidate dihydrochloride up to 100 mM. Aliquots of beads at each dimethyl pimelimidate dihydrochloride concentration were washed with elution buffer (1 containing 0.5 m NaCl and then either with an acidic buffer system (0.2 m citrate buffer, pH 3.0, containing 0.5% Nonidet P-40) or an alkaline buffer (0.05 m diethylamine, pH 11.5, containing 0.5% sodium deoxycholate). The beads could then be examined for antibody leakage by counting the 125I bound to the beads after each wash (Table I).

After the first high salt wash, when some nonspecific counts are removed, subsequent washes do not remove any significant amounts of antibody at any of the dimethyl pimelimidate dihydrochloride concentrations used. However, in the absence of cross-linker, the antibody was almost totally eluted. This demonstrates in addition that no significant hydrolysis of the cross-linker occurs at pH 11.5. Aliquots of cross-linked matrix were subjected to harsher conditions to try and remove antibodies. This involved boiling in 0.05 m Tris-HCl, pH 6.8, containing 1% SDS for 2 min. Under these conditions, no significant loss of antibody was observed, except in the non-cross-linked aliquots. The results were essentially identical for one-layer and two-layer systems.

If acidic buffer systems were used for washing, no significant loss of antibody occurred at any of the dimethyl pimelimidate dihydrochloride concentrations used. However, antibody was eluted in the absence of dimethyl pimelimidate dihydrochloride (data not shown). Acidic buffers were not routinely used because of their inability to remove bound antigen (see below).

Assessment of Antibody Concentration on Matrix Efficiency

One-layer System Using the Monoclonal Antibody W6/32—The monoclonal antibody W6/32 was labeled with 125I using the chloramine-T method and used to measure the amount of labeled antibody able to saturate a fixed volume of protein A-Sepharose beads. Once the saturation value was known, the amount of antibody, in milligrams, required to saturate the beads was measured by competition with increasing amounts of unlabeled W6/32 of known concentration. The saturation value for W6/32 is 24 mg of monoclonal antibody/1 ml of packed Sepharose CL-4B-protein A. Using the binding curve, it was possible to make affinity matrices containing antibody W6/32 at different percentages of saturation. After
antibodies were bound to the Sepharose-protein A, they were stabilized by chemical cross-linking and aliquots of beads were incubated with saturating amounts of 125I-labeled cell lysates. After appropriate washing procedures, aliquots of beads at varying concentrations of W6/32 were counted (Fig. 1A). It was found that optimal antigen binding occurred when the antibody was present at about 50% saturation (i.e. ~12 mg/1 ml of packed beads for W6/32).

Two-layer System Using Rabbit Anti-mouse Ig and the Monoclonal Antibody OKT9—Matrices were prepared by coating protein A-Sepharose beads at 50% saturation with rabbit anti-mouse Ig (11 mg/ml of packed beads) and varying the amount of OKT9 added as a second layer antibody. After cross-linking, the affinity matrices were assessed for their ability to bind antigen, as described above. Fig. 1B shows that a similar result was obtained with the two-layer system in that maximum antigen binding was achieved at approximately 50% saturation of OKT9 (i.e. rabbit anti-mouse Ig and OKT9 were present in a 1:1 (w/w) ratio; 11 mg of OKT9/11 mg of rabbit anti-mouse Ig/1 ml of packed Sepharose-protein A.

Effect of Cross-linker Concentration on Antigen Binding Characteristics

One- or two-layer matrices were made and chemically cross-linked with dimethyl pimelimidate dihydrochloride concentrations from 10 to 80 mM. Saturating levels of 125I-labeled cell lysate containing the appropriate antigen were incubated with a fixed amount of antibody-coupled beads and, after washing with the relevant buffers, were counted to assess the amount of antigen bound (Table II). Variation in the concentration of cross-linker over the range 10 to 80 mM did not affect the amount of antigen bound in either the one-layer or the two-layer systems. Thus, one can assume that dimethyl pimelimidate dihydrochloride does not interfere with the antigen binding site of the antibody molecule.

The Use of a Transferrin-Anti-transferrin Matrix for the Isolation of Its Cellular Receptor

125I-labeled fetal liver lysate, which contains both transferrin receptor and endogenous transferrin, was absorbed with either (i) anti-transferrin/protein A-Sepharose or (ii) transferrin/anti-transferrin/protein A-Sepharose. The material eluted from the first matrix contained both transferrin and transferrin receptor (Fig. 2, track A) whereas that eluted from the second matrix contained transferrin receptor alone (track B). A control track (track C) of transferrin receptor plus transfer-

![Fig. 1. Antigen binding as a function of antibody saturation. A, saturating amounts of cell extract containing 125I-labeled antigen were added to 1 ml of Sepharose CL-4B beads coupled to increasing amounts of antibody. 100% saturation was previously calculated to be 24 mg of W6/32/ml of beads; 75% = 19 mg of W632, 50% = 12 mg, 25% = 6 mg, 10% = 2.4 mg/ml of beads. The inset shows antigen bound at each concentration point after SDS-polyacrylamide gel electrophoresis. Values are means of three separate experiments. B, Sepharose CL-4B beads were coupled with rabbit anti-mouse Ig at 50% saturation (11 mg/ml beads) and increasing amounts of antibody OKT9 as above. 100% saturation was calculated as a 2:1 ratio (w/w) of monoclonal OKT9 to rabbit anti-mouse Ig respectively; 100% = 22 mg of OKT9, 75% = 16.5 mg of OKT9, 50% = 11 mg of OKT9, 25% = 5.5 mg of OKT9, 10% = 2.2 mg of OKT9/ml of beads. The inset shows transferrin receptor after SDS-polyacrylamide gel electrophoresis. 45K, 12K, and 90K, 45,000, 12,000., and 90,000-dalton.

![Fig. 2. Isolation of the transferrin receptor using a transferrin-anti-transferrin matrix. Track A, transferrin receptor (90,000 daltons) and transferrin (80,000 daltons) eluted from rabbit anti-transferrin/protein A-Sepharose CL-4B. Track B, transferrin receptor (90,000 daltons) eluted from transferrin/rabbit anti-transferrin/protein A-Sepharose CL-4B. Gel was run on a polyacrylamide gradient (7-15%), dried, and autoradiographed. Standard molecular weight markers are represented on the right.

<table>
<thead>
<tr>
<th>Dimethyl pimelimidate dihydrochloride concentration</th>
<th>125I-labeled HLA-AB antigen bound to W6/32</th>
<th>125I-labeled transferrin receptor bound to OKT9</th>
</tr>
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<tr>
<td>mM</td>
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<td>cpm</td>
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Membrane Proteins and Receptor Purification

After neutralization with 0.5 M NaH₂PO₄, aliquots of the eluted material were run on SDS-polyacrylamide gels and either processed for autoradiography or visualized with the silver nitrate stain (Fig. 3). The gel pattern shows that it is possible to process a large amount of antigen in this way and obtain a virtually purified protein in one step. ¹³¹I-Labeled material shows only a single band for each antigen and silver nitrate staining shows that the only significant unlabeled contaminant was bovine serum albumin, which was used in some of the buffers. If [³⁵S]methionine-labeled cells were used, again only a single band corresponding to the relevant antigen was observed (data not shown).

One of the major problems using cyanogen bromide-activated Sepharose is its unpredictability in making an efficient affinity matrix with different antibodies. The method described here utilizes the specific interaction of antibody Fc regions with protein A, thus giving a reproducible and simple method for constructing a matrix with correct orientation for maximal antigen binding. Chemical cross-linking stabilizes the complex, thus overcoming another problem, namely that of antibody leakage. The high efficiency of this method allows maximum activity from the minimum amount of antibody. For example, 1 ml of protein A-Sepharose (coupled at 12 mg of W6/32/ml of packed beads) can remove all the HLA-AB (~500 μg) from 5 x 10⁶ Bristol 8 cells. In addition, one can construct affinity matrices on a microscale which can be used analytically if the antibody is in short supply and for immunoprecipitations instead of conventional methods which sometimes need extensive antibody titration.

If several matrices with different antibodies are used sequentially, it is possible to completely absorb out antigens in a stepwise manner which is useful when preparing several antigens from a single source. In addition, S. aureus can be used as a support instead of Sepharose CL-4B for such procedures as cascade immunoprecipitation.

REFERENCES


² F. Harlow, personal communication.
A one-step purification of membrane proteins using a high efficiency immunomatrix.
C Schneider, R A Newman, D R Sutherland, U Asser and M F Greaves