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HIGH-PERFORMANCE AFFINITY ISOLATION OF LYMPHOCYTE MEMBRANE RECEPTORS ON BIOTINYLATED ANTIGEN AND AVIDIN-COATED BEADS

T. M. PHILLIPS* and S. C. FRANTZ
Immunochemistry Laboratory, George Washington University Medical Center, Washington, DC 20037 (U.S.A.)
J. V. BABASHAK
Kontes Scientific Glassware, Vineland, NJ (U.S.A.)
and
J. J. CHMIELINSKA
Immunochemistry Laboratory, George Washington University Medical Center, Washington, DC 20037 (U.S.A.)

SUMMARY

Isolation of lymphocyte membrane receptors can be achieved by high-performance liquid chromatography using immobilized streptavidin as the ligand and biotinylated antigen. Activated lymphocytes were allowed to react with biotin-labelled antigen prior to harvesting. The cells were disrupted and their membranes solubilized before passing the suspension through the avidin affinity column. The biotinylated antigen acted as an efficient receptor probe, which helped to maintain the integrity of the receptor during the isolation procedure. The biotin also acted as the substrate that attaches to the immobilized avidin. Recovery of the bound receptor was achieved by dissociation of the receptor from the antigen and recovery of the receptor in the effluent during the elution phase of the separation.

INTRODUCTION

Affinity chromatography, with a variety of different ligands, ranging from immobilized plant lectins[1–3] to receptor substrates[4–6], has been used to isolate cell membrane receptors. In a similar manner, immunoaffinity chromatography, using immobilized antibodies, can also be used to isolate specific receptors[7–9]. The latter technique accomplishes the isolation by means of antibodies directly against the receptor itself, or against the substrate, which is complexed to the receptor. This has led to an interest in using the biotin–avidin system for the isolation of receptors, with biotinylated probes and immobilized avidin as the ligand[10,11].

Streptavidin is a form of avidin, isolated from *Streptomyces avidinii*, which has the ability to bind up to four molecules of biotin[12]. This binding has been shown to be strong enough to withstand the conditions encountered during the elution phase of

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high-performance affinity chromatography (HPAC)[8,13]. We have previously reported the development of an avidin-coated glass bead that can be used to absorb biotinylated antibodies[7,9]. In this paper, we describe the use of immobilized avidin as an affinity ligand for the isolation of membrane receptors that have previously been allowed to bind biotinylated antigen.

EXPERIMENTAL

Materials

Glass beads (1 mm) were obtained from Kontes Scientific Glassware (Vineland, NJ, U.S.A.). Purified streptavidin was purchased as a lyophilized, pure product from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.) and reconstituted in 50 m*M* carbonate buffer (pH 9.0). The biotin derivatives and the laboratory chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). 3-Aminopropyltriethoxysilane and 1,1′-carbonyldiimidazole were obtained from Pierce (Rockford, IL, U.S.A.). All columns and column fittings were purchased from Jones Chromatography (Littleton, CO, U.S.A.).

Derivatization of the glass beads

The glass beads were washed by sedimentation in doubly distilled water, to remove manufacturing impurities from the beads surface, before preparing the beads for silanization and derivatization[14]. Briefly, this was performed by placing 10 g of the washed beads in 50 ml of 1 *M* hydrochloric acid and gently sonicating for 25 min. The beads were then washed by sedimentation in 200-ml portions of 1 *M* hydrochloric acid until the suspension became clear. The beads were removed, air-dried and refluxed for 30 min in 200 ml of 1 *M* nitric acid with constant agitation, then recovered, air-dried and suspended in 50 ml of 10% 3-aminopropyltriethoxysilane dissolved in toluene. This suspension was refluxed for 16 h with constant agitation.

Following silanilization, the beads were washed twice in 200 ml of 95% methanol, then refluxed for 20 min in 95% methanol to remove the excess of silanizing agent. The beads were recovered, washed three times in doubly glass-distilled water and air-dried prior to derivatization of the reactive side-groups.

The reactive carbonyldiimidazole (CDI) side-groups were attached to the bead surface by suspending the beads in 5 ml of dioxane and adding 100 mg of 1,1′-carbonyldiimidazole. The mixture was placed in a 15-ml capped glass tube and incubated for 6 h at room temperature in an overhead mixer. The beads were then recovered and washed thoroughly in dioxane by sedimentation and decantation. The beads were air-dried and used immediately for immobilization of the streptavidin.

Column construction

CDI-derivatized beads (10 g) were suspended in 5 ml of doubly distilled water and 5 ml of 50 m*M* carbonate buffer (pH 9.0) containing 2.5 mg of streptavidin were added. The mixture was placed in a 15-ml capped glass tube and incubated for 18 h at 4°C in an overhead mixer. The beads were then allowed to settle and washed ten times in 0.01 *M* phosphate buffer by sedimentation and decantation. Attachment of the streptavidin to the beads was checked by incubating a 25-μl drop of the bead

suspension, obtained from the last wash, with fluorescein-labelled biotin and examining 100 beads under a fluorescence microscope. Following satisfactory coating of the beads, they were sedimented, recovered, resuspended in 5 ml 0.01 *M* phosphate buffer and slurry-packed into 10 cm × 4.6 mm I.D. high-performance liquid chromatographic (HPLC) columns at 250 p.s.i.

Biotinylation of antigen

The antigen was biotinylated with four different forms of biotin: (a) N-hydroxysuccinimide biotin, (b) long-chain N-hydroxysuccinimide biotin, (c) cleavable iminobiotin and (d) hydrazine biotin. The first three biotinylations were performed in a similar manner by incubating 100 μg of the antigen with 1 mg of the biotin derivative in 1 ml of 0.05 *M* carbonate buffer (pH 9.0). The mixture was placed in a rotating mixer for 2 h at room temperature, and the reaction was stopped by overnight dialysis at 4°C against 0.01 *M* phosphate buffer (pH 7.0).

The hydrazine biotinylation required modification of the carbohydrate portion of the glycoprotein antigen, which was performed by suspending 100 μg of antigen in 1 ml of 0.1 *M* sodium acetate buffer (pH 5.0) and cooling to 4°C. A 1-ml volume of a 10 m*M* solution of cold sodium metaperiodate was added to the antigen before incubation for 20 min at 4°C in the dark. The reaction was stopped by adding 10 ml of 5% ethylene glycol and dialyzing the solution against 0.01 *M* phosphate buffer for 18 h at 4°C, with five changes of the dialysate. The antigen was then removed from the dialysis tubing and placed in a capped glass tube. To this was added 1 ml of phosphate buffer containing 1 mg/ml of sodium cyanoborohydride and 1 ml/ml biotin hydrazine, and the mixture was placed in a rotating mixer for 1 h at room temperature. The reaction was stopped by dialysis against 0.01 *M* phosphate buffer overnight at 4°C.

Isolation of lymphocyte membrane receptors

Prior to disruption and solubilization of their membranes, active lymphocytes were isolated from whole blood by centrifugation at 400 *g* for 15 min in a Ficoll gradient. The lymphocyte band was recovered and the specific receptors were localized by allowing them to interact with the biotinylated antigen for 1 h at 37°C. Following this incubation, the cells were collected, washed twice in 0.01 *M* phosphate buffer (pH 7.0) and pelleted by centrifugation at 600 *g* for 15 min. The pellet, containing 10^8 lymphocytes, was frozen and thawed three times and then sonicated for 2 min at maximum power. The sonicated pellet was resuspended in 2 ml of 0.01 *M* phosphate buffer and the membrane fraction was isolated by centrifugation at 10 000 *g* for 30 min. The membrane-enriched supernatant was mixed with an equal volume of 1% sodium deoxycholate solution and incubated for 30 min at room temperature. Finally, the solubilized membrane sample was centrifuged for 1 h at 10 000 *g* and the supernatant was applied to the avidin columns.

Chromatography

The avidin bead-packed column was installed in a Beckman (Palo Alto, CA, U.S.A.) Model 340 isocratic HPLC system equipped with a Model 112 pump, a Model 160 UV detector (set at 280 nm) and a Shimadzu C-R1B recording peak integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The elution profile was automatically controlled by a Model III OPG/S solvent selector/gradient controller

(Autochrom, Milford, MA, U.S.A.). Samples were introduced into the system by injection through an Altex 210 injection port, equipped with a 100-μl sample loop. The column was developed isocratically in 0.01 *M* phosphate buffer, containing 10 m*M* sodium deoxycholate at pH 7.0 for 15 min at a flow-rate of 0.5 ml/min. Throughout the entire run, the column temperature was maintained at 4°C by a glass column jacket attached to a recycling ice-bath.

Following the initial 15-min run, during which the biotinylated antigen–membrane receptor complex was absorbed to the immobilized avidin, an elution recovery phase was started. In all instances except for iminobiotin, a chaotropic ion gradient was developed by adding 0–2.5 *M* sodium thiocyanate to the running buffer over a further 15 min. Recovery of the cleavable iminobiotin complex was achieved using a pH gradient from pH 7.0 to 3.0[15], developed by the addition of 0.5 *M* hydrochloric acid over the same 15-min period.

Recovery of the bound materials was accomplished during the gradient phase, and the upper limits of both gradient types were maintained for a further 5 min before recycling the column by returning it to the initial running conditions. Fractions of the eluted material were collected in 500-μl Beckman microfuge tubes, in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, U.S.A.). The recovered receptors were dialyzed overnight at 4°C against 0.01 *M* phosphate buffer, to which 25 m*M* sodium deoxycholate had been added. The purity of the isolated receptors was checked by polyacrylamide gel electrophoresis[16] and their activity was measured by radiolabelled antigen binding studies[17].

RESULTS

Even coating of the glass beads with the streptavidin was achieved, as demonstrated by microscopic examination of the localization of fluorescein-labelled biotin. The biotin binding capacity of the columns were found to be between 500 and

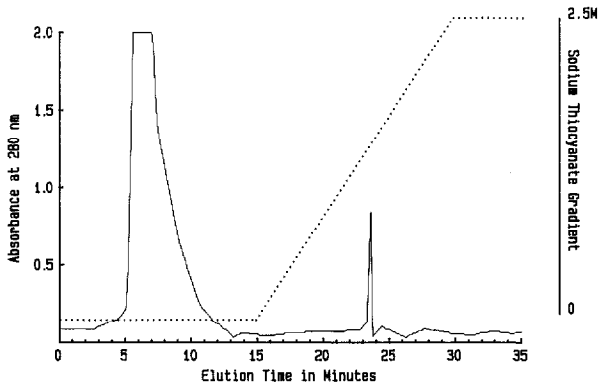

Fig. 1. HPAC isolation of lymphocyte membrane receptors with N-hydroxysuccinimide biotin-labelled antigen as the probe. The chromatogram was produced by passing 100 μl of biotinylated antigen receptor complex through a 10 cm × 4.6 mm I.D. column containing immobilized streptavidin as the ligand. The column was developed in running 0.01 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min. The elution was monitored at 280 nm with the detector set at 0.005 a.u.f.s. The column was maintained at 4°C throughout the experiment. The dotted line indicates the 0–2.5 *M* sodium thiocyanate elution gradient.

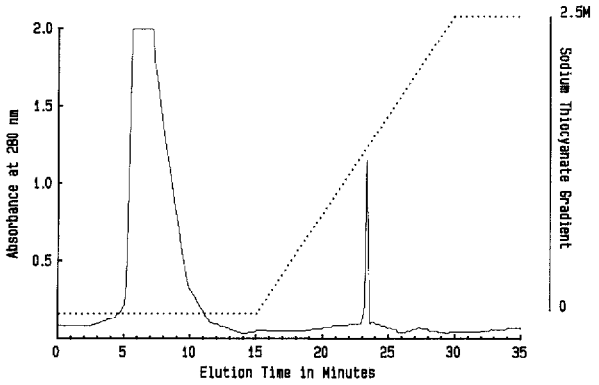

Fig. 2. HPAC isolation of membrane receptors with long-chain biotin-labelled antigen as the probe. Chromatographic conditions as in Fig. 1.

545 μg per column. This coating was stable over ten runs at flow-rates of up to 1.5 ml/min and storage at 4°C for 3 months. However, studies have shown that the CDI-activated beads need to be coated within 48 h of derivatization of the bead surface because the diimidazole side-groups are unstable and the protein binding efficiency will decrease drastically with time.

Affinity isolation of specific receptors, with either the N-hydroxysuccinimide biotin or the hydrazine biotin-labelled antigen, could easily be achieved by using immobilized streptavidin as the ligand. Both biotinylations had the same degree of efficiency and produced similar elution profiles. Fig. 1 shows a typical chromatogram produced by this technique. The primary peak contains the non-reactive membrane components and the sharp, secondary peak contains the isolated receptor eluted from the biotin-labelled antigen. The antigen remains in the column, attached to the immobilized avidin.

Similar results were obtained with long-chain biotin as the antigen label. Fig. 2 shows a typical chromatogram of receptor isolation with this form of biotinylation.

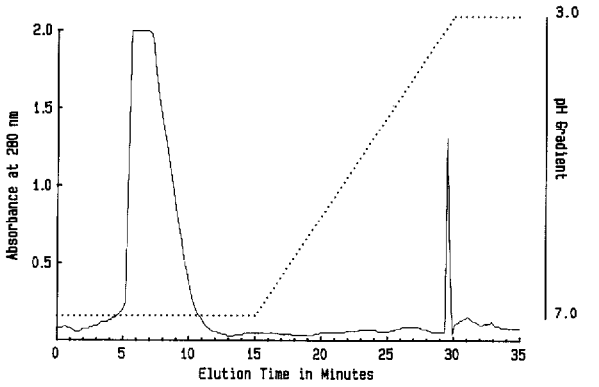

Fig. 3. HPAC isolation of receptors with cleavable iminobiotin as the antigen probe label. Chromatographic conditions as in Fig. 1. The dotted line indicates the pH 7–3 elution gradient.

The secondary peak, which contains the receptor, is slightly larger than that produced by standard N-hydroxysuccinimide or hydrazine biotin labels.

The chromatogram shown in Fig. 3 is representative of the elution profile produced by using cleavable biotin-labelled antigen as the receptor probe. In this chromatogram, the biotinylated antigen receptor complex is eluted from the avidin column by a pH gradient. The second peak, which contains the complexed receptor and antigen, is eluted later in the chromatogram. It was found that further dissociation by chaotropic agents and/or detergents was necessary in order to dissociate the receptor from the antigen probe.

Polyacrylamide gel electrophoresis of the second peak, produced by all of the biotinylated antigen probes, demonstrated the presence of a single protein band, except for the cleavable biotin probe, which also produced a second minor band. This second band was subsequently found to correspond to the biotinylated antigen. All of the major bands were found to consist of 90–92 kDa material by comparison with molecular weight. markers. No comparable bands were found in the first peak of the chromatograms.

Radiolabelled antigen binding studies (Fig. 4) demonstrated differences in the binding efficiency of the different receptor isolates. The receptors isolated by the long-chain biotinylated probe demonstrated the highest degree of antigen binding, which was shown to be approximately 60% of that demonstrated by intact lymphocytes. Receptors isolated by both the standard N-hydroxysuccinimide ester and hydrazine biotin-labelled antigen probes demonstrated comparable binding characteristics. These receptors bound *ca.* 50% of the antigen bound by intact cells. Receptors isolated by cleavable biotin probes appeared to have lost a large amount of their antigen-binding capacity. These receptors demonstrated binding capacities of *ca.* 30% of that shown by intact cells. We postulate that the additional step required to remove the antigen probe from the receptor caused damage to the receptor structure, although we have not been able to prove this.

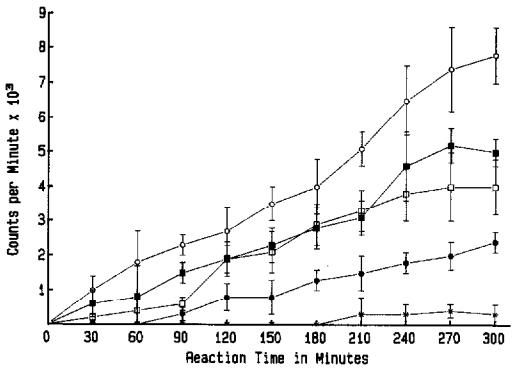

Fig. 4. Comparison of antigen-binding capacities of HPAC-isolated receptors. Points represent the mean of ten experiments ± the standard error of the mean. Receptors isolated by N-hydroxysuccinimide biotin-labelled antigen (□), long-chain biotin-labelled antigen (■) and ...inobiotin labelled antigen (●). The binding of the isolated receptors was corrected for the amount of receptor present in each sample and compared a similar number of intact stimulated (○) and normal, unstimulated lymphocytes (★). All cells and receptors were incubated with 50 μg of ^{125}I-labelled antigen.

DISCUSSION

HPAC on avidin-coated glass beads is a rapid technique for isolating membrane receptors that have been complexed with biotinylated antigen. The use of large glass beads (1 mm diameter) was found to have an advantage over smaller packing media, such as controlled-pore glass beads. The larger solid beads were found to help to maintain reasonable flow-rates, reduce column running pressures and provide a large surface area for avidin attachment. However, the advantage of this technique over conventional lectin or biochemical techniques is questionable. In most instances, once the biotin-labelled antigen has attached itself to the biotin receptors on the immobilized avidin, this material is bound so tightly that its removal often damages the avidin coat. This means that the HPAC column is usable only once and must then be discarded. Even when cleavable biotin derivatives are used, the column is reusable, but the isolated membranes are often damaged during the second step, which is required to rid the receptor of the biotinylated probe. The loss of antigen-binding capacity in the receptors isolated with the cleavable biotin-labelled probe was probably caused by a combination of the pH and the chaotropic agent used for elution of the bound antigen. The effects of both of these factors on protein structures and function have been reported previously[18,19]. Steric hindrance also plays a part in the efficiency of biotin binding on the immobilized avidin. In this study, this was seen when long-chain biotinylation was used.

However, the material isolated by all four biotin-labelled probes demonstrated a single band in polyacrylamide gel electrophoresis in the region of 90 kDa, which is comparable to that found by other workers who isolated T cell receptors by other means[20,21].

In conclusion, we believe that the biotin–avidin system is useful for the isolation of membrane receptors, but care must be taken in planning the isolation procedure. The disadvantage of the technique is that, in most instances, the column packing can be used only once and great difficulty is experienced in recycling the packing media. However, the materials isolated by this technique are reasonably pure and in many instances still active. Long-chain biotin derivatives were found to give the most reproducible results and the highest yields of bioactive receptors. Cleavable biotins are not as useful as reported because they require another step for removal of the antigen.

The avidin-coated beads are useful and provide an excellent support for the immobilization of biotinylated ligands or for the isolation of biotin-labelled biological material.

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