Journal of Chromatography, 444 (1988) 13–20
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1292

ISOLATION OF SPECIFIC LYMPHOCYTE RECEPTORS BY HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

T. M. PHILLIPS* and S. C. FRANTZ
Immunochemistry Laboratory, Department of Medicine, The George Washington University Medical Center, 2300 Eye Street, N.W., Washington, DC 20037 (U.S.A.)

SUMMARY

Isolation of antigen-specific lymphocyte receptors can be achieved by high-performance immunoaffinity chromatography, using immobilized monoclonal antibodies directed against the antigen. Primed lymphocytes are allowed to react with their target antigen prior to disruption and membrane solubilization. The bound antigen acts both as a preservative for the receptor and as a ligand on which the immobilized antibody captures the antigen–receptor complex. Dissociation of the antigen–antibody complex also releases the antigen from the receptor, and both antigen and receptor can be separated and recovered for further study.

INTRODUCTION

The involvement of the immune system in peripheral nerve disease has been of interest to clinical immunologists for many years. This interest was hightened during the time of the Swine Flu vaccination program in 1976. Following vaccination, there was an increased incidence of immune-mediated peripheral nerve disease, called Guillian-Barre syndrome[1,2]. The involvement of the immune system in other forms of peripheral and central nervous diseases is now under investigation. The major antigenic component of peripheral nerves is myelin basic protein (MBP), of which an amino acid sequence, called P2, has been shown to induce immune-mediated peripheral nerve disease in animals[3,4]. The role of MBP-activated lymphocytes has been shown to be important in mediating the disease process[5].

Immunologists are focusing on the structure of specific antigen receptors on the membranes of specifically activated lymphocytes, such as the MBP-activated lymphocytes in peripheral nerve disease. The isolation of antigen-specific receptors from primed lymphocytes is a tedious process, in which the isolated receptor is often recovered in an inactive form[6]. This inactivation is due to many different factors, the most common of which is the time taken for the isolation procedure and the reagents used to achieve that isolation. Another approach to the problem has been to develop monoclonal antibodies (MAb) by immunizing with whole cells and then using the MAb to immunoprecipitate the membrane structures of interest[7]. In an attempt to resolve this situation, we have used high-performance immunoaffinity chromato-

0021-9673/88/$03.50

graphy[8,9], to isolate specific antigen receptors from the lymphocytes of MBP-immunized animals. We have developed a system based on the immobilization of biotinylated monoclonal antibodies, directed against the P2 sequence of the MBP, coupled to streptavidin-coated glass beads.

EXPERIMENTAL

Materials

Carbonyl diimidazole glycophase-derivatized controlled-pore glass beads (Pierce Chemicals, Rockford, IL, U.S.A.) were acid-washed prior to use and stored in double distilled water until required. Purified streptavidin was obtained as a lyophilized, pure product (Bethesda Research Labs., Gaitherburg, MD, U.S.A.) and reconstituted in 0.01 *M* phosphate buffer. Supplies for producing mouse monoclonal antibodies against the P2 protein (RPMI and HAT media) were obtained from Flow Labs. (McLean, VA, U.S.A.). Biotin hydrazine was obtained from Pierce Chemicals. Polyacrylamide gel supplies were purchased from BioRad Labs. (Rockville Centre, NY, U.S.A.). Radioisotopes were obtained from Amersham (Arlington Heights, IL, U.S.A.) and MBP was labelled via the Bolton–Hunter reaction[10]. The P2 antigen and MBP were gifts from Dr. E. Eylar of Ponce University, Puerto Rico.

Production of anti-P2 antibodies

Mice were immunized with the P2 sequence of bovine myelin basic protein by subcutaneous injection of 100 μg of antigen in complete Freund's adjuvant. The animals were rested for 2 weeks and booster-injected with the same dosage. Five days later the antibody titer was tested, and animals demonstrating high titers were again boostered, intravenously, with 300 μg of the antigen in 0.9% sodium chloride. Four days later, the animals were splenectomized, and the cells were used for hybridoma production. Fusion and cloning was performed by the technique of Campbell[11]. The clones were screened for the presence of antibody against P2 and isotypic expression by an enzyme-linked immunosorbent assay[12].

Biotinylation of antibodies

The anti-P2 MAb were biotinylated with biotin hydrazine. This reagent couples biotin to the carbohydrate moieties of the antibodies but does not attach itself to other parts of the molecule[13]. The majority of the carbohydrate portion of most antibodies is present in the Fc or tail region, and attachment of biotin to this area would ensure correct orientation of the antibody on the bead surface. In addition, the amount of functional binding groups, on the carbohydrate molecule, can be controlled by the amount of metaperiodate used to chemically activate the carbohydrate molecule and the length of the reaction time.

Briefly, the MAb was suspended in 1 ml of 0.1 *M* sodium acetate, pH 5.0 and cooled to 4°C. The MAb solution was mixed with 1 ml of 10 m*M* sodium metaperiodate and incubated for 20 min at 4°C in the dark. The reaction was stopped by adding 20 ml of 5% ethylene glycol, and the mixture was dialysed overnight against 0.01 *M* phosphate buffer at pH 7.0. The MAb solution was removed from the dialysis sac and placed in a capped, glass tube. Phosphate buffer (1 ml), containing 1 mg sodium cyanoborate and 1 mg biotin hydrazine, was added to the MAb solution and

incubated for 1 h at room temperature. The reaction was stopped by dialysis against 0.01 phosphate buffer overnight at 4°C.

Isolation of lymphocyte membranes

Prior to disruption and solubilization of their membranes, active lymphocytes primed to MBP were isolated by allowing them to interact and adhere to MBP, absorbed on plastic dishes[14]. The adherent cells and the antigen were resuspended by gentle sonication. The cells were washed three times in RPMI-1640 medium and pelleted by centrifugation at 600 *g* for 15 min.

The cell pellet, containing $1 \cdot 10^6$ MBP-reactive lymphocytes, was frozen and thawed three times and then sonicated for 2 min at maximum power. The membrane fraction was recovered by mixing the disrupted cells with 2 ml of 0.01 *M* phosphate buffer (pH 7.0) and centrifuging at 10 000 *g* for 30 min. The membrane-rich supernatant was mixed with an equal volume of 1% sodium deoxycholate and incubated for 30 min at room temperature. Finally, the mixture was centrifuged for 1 h at 10 000 *g*, and the supernatant was applied to the immunoaffinity column.

Construction of immunoaffinity column

The streptavidin was reconstituted in 10 ml of 50 m*M* carbonate buffer (pH 9.0) by gentle agitation. The glass beads were coated with the streptavidin by adding 2 g of derivatized beads to 1 mg of purified streptavidin. The beads and the avidin were placed in a 10-ml capped tube and mixed on an overhead mixer for 18 h at 4°C. Following this incubation, the beads were thoroughly washed by sedimentation in 0.9% sodium chloride (pH 7.0). The coated beads were mixed with 250 μg of biotinylated anti-P2 MAb, dissolved in 0.9% sodium chloride, and mixed on the overhead mixer for another hour. The beads were thoughly washed by sedimentation in 0.9% sodium chloride, and the unreacted biotin receptors on the streptavidin was blocked by incubating with 10 ml of a 10 mg/ml solution of biotin. This mixture was stirred in the overhead mixer for 30 min at room temperature. The beads were thoroughly washed in 0.9% sodium chloride–0.1 *M* sodium acetate buffer. Following the final wash, the beads were slurry-packed into a 10 cm × 4.6 mm I.D. stainless-steel chromatographic column at 500 p.s.i.[8,9].

Chromatography

The bead-packed column was installed in a Beckman 340 isocratic high-performance chromatography system (Beckman, Palo Alto, CA, U.S.A.), equipped with a Model 112 pump, a Model 160 UV detector (set at 280 nm), and a Shimadzu C-R1B peak integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The system was equipped with an Autochrom Model III OPG/S solvent selector/gradient controller (Autochrom, Milford, MA, U.S.A.), which automatically controlled the elution profile of the system. Samples were injected by means of an Altex 210 injection port with a 100-μl sample loop. Following the injection of the solubilized membrane sample, the column was isocratically developed with 0.9% sodium chloride–0.1 *M* sodium acetate buffer at pH 6.5 for 10 min at a flow-rate of 0.5 ml/min. During this time, the column was maintained at 4°C with a recycling ice-bath, connected to the column jacket. Following the initial 10 min, during which time the immobilized monoclonal antibody reacted with and retained its specific antigen and the unreacted

material had run through the column, an antigen elution phase was started. A chaotropic ion gradient of sodium thiocyanate from 0 to 2.5 *M* was generated over a 15-min period, and the highest concentration was maintained for a further 10 min before the column was recycled.

Recovery of the bound material was achieved either during the gradient phase or during the period when the upper level of the gradient was maintained. Peaks containing the eluted material were collected in 200-μl fractions in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, U.S.A.).

Polyacrylamide gel electrophoresis

Identification of the eluted materials was performed by polyacrylamide gel electrophoresis[15]. Briefly, 25 μl of the isolated peak from the immunoaffinity column was mixed with an equal volume of 0.1% sodium dodecyl sulfate (SDS). The mixture was boiled for 5 min and allowed to cool to 4°C before use. A 10-μl sample was applied to a 10–30% linear gradient polyacrylamide gel and run at a constant voltage of 150 V for 3 h. The gels were then fixed in methanol–acetic acid (4:1) and silver stained[16].

Receptor–antigen binding studies

The functional ability of the immunoaffinity-isolated receptors to bind the original antigen was tested by incubating the immunoaffinity-isolated receptor fraction, conventionally isolated receptor fraction, and intact lymphocytes with ^{125}I-labelled MBP and plotting the binding efficiency against time[17].

RESULTS

The IgG_{2a} MAb, produced by immunizing the Balb/c mice with the P2 sequence of bovine myelin basic protein, was easily labelled with biotin via the hydrazine reaction with the carbohydrate moieties on the Fc portion of the IgG molecule.

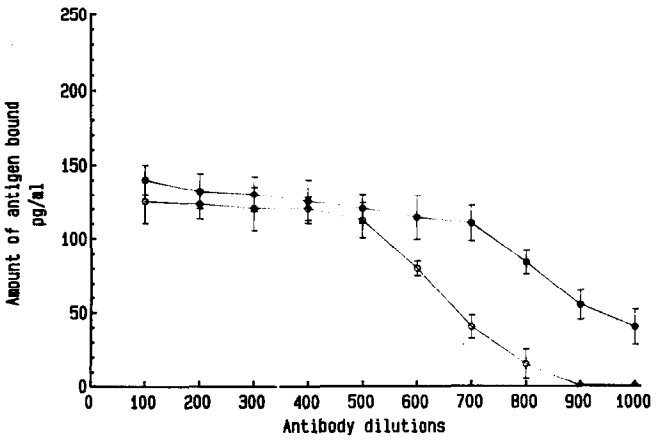

Fig. 1. Comparison of N'-hydroxysuccinimide and hydrazine biotinylation techniques in the efficiency of IgG to bind to labelled antigen. (○) N-Hydroxysuccinimide-labelled IgG; (●) hydrazine-labelled IgG. Points are the mean of ten labelling experiments ± the standard error of the mean.

Fig. 1 compares the hydrazine technique with the more conventional N-hydroxysuccinimide ester method of biotinylation. This hydrazine technique was more controllable and by titration with avidin, was shown to introduce less functional biotin groups than the N-hydroxysuccinimide method. Attachment of the biotin via the carbohydrate portions of the IgG appeared to prevent the biotin from becoming attached to the antigen receptors or binding sites of the IgG during the biotinylation process. In addition, this labelling technique orients all antibody molecules in a similar manner to Protein A. Attachment of the immobilized antibody via its Fc portion has the advantage that the antigen- binding sites are facing the mobile phase of the column, and this ensures maximum efficiency during the antigen–antibody capture phase of the immunoaffinity separation.

The elution profile obtained by using the P2 antigen as a probe for isolating the MBP receptor is shown in Fig. 2. This chromatogram was produced by passing 100 μl of the MBP-activated lymphocyte membranes through the anti-P2 immunoaffinity bead column. The primary large peak is the non-reactive membrane material, and the second peak is the MBP receptor. The third peak is the P2 antigen

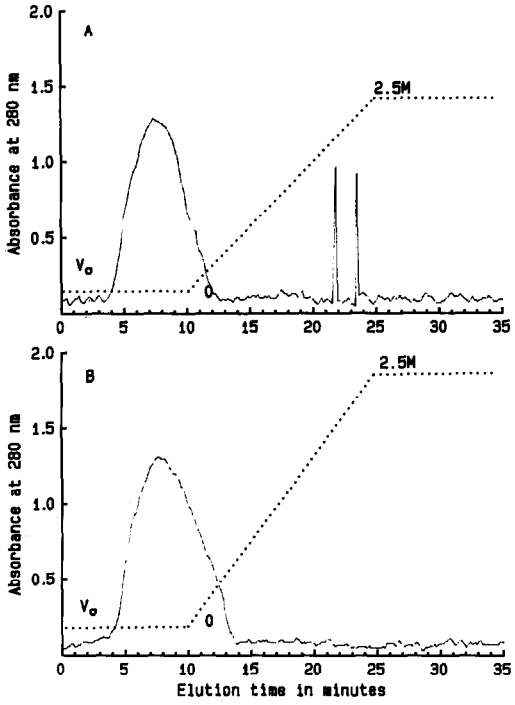

Fig. 2. (A) Chromatogram produced by passing 100 μl of the antigen–receptor complex through the immunoaffinity bead column. The chromatogram was developed with a chaotropic ion gradient elution from 0 to 2.5 *M*. The tracing was produced with a 10 cm × 4.6 mm I.D. immunoaffinity column containing immobilized anti-P2 MAb. The column was developed at a flow-rate of 0.5 ml/min with a running buffer of 0.9% sodium chloride, 0.1 *M* sodium acetate buffer (pH 6.5). The elution profile was monitored by absorbance at 280 nm with the detector set at 0.005 a.u.f.s. The column was maintained at 4°C. The dotted line indicates the chaotropic ion gradient profile. V_0 = void volume of the column. (B) Under identical conditions as in A unprimed lymphocyte membranes, treated in a similar manner, were chromatographed.

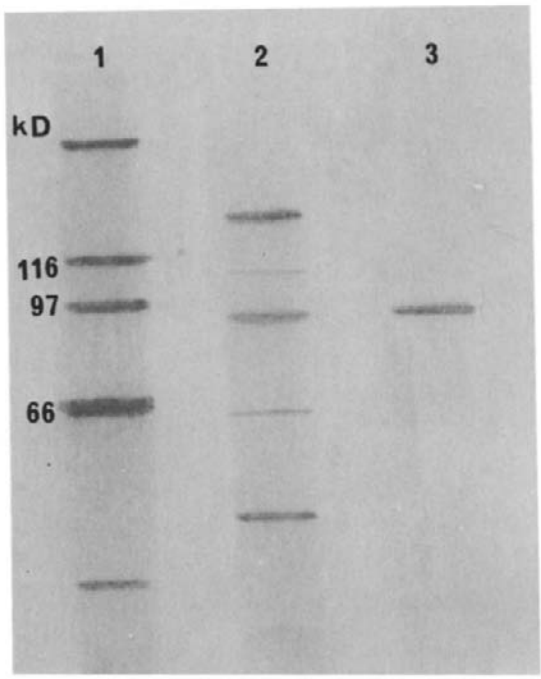

Fig. 3. Polyacrylamide gel electrophoretic analysis of the receptor peak isolated by immunoaffinity chromatography using immobilized anti-P2 MAb. Lane 1 = molecular weight standards, lane 2 = whole solubilized lymphocyte membrane before immunoaffinity chromatography, lane 3 = receptor peak, following immunoaffinity isolation.

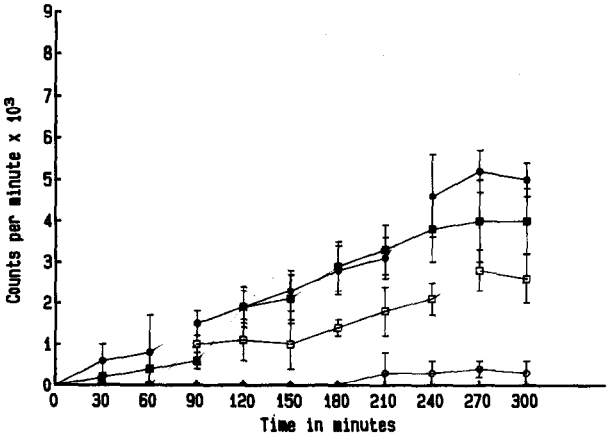

Fig. 4. Binding of labelled antigen by intact lymphocytes and purified receptors. (○) Intact, unprimed lymphocytes; (●) intact, primed lymphocytes; (□) conventionally isolated receptors; (■) immunoaffinity isolated receptors. Points are the mean of eight experiments ± the standard error of the mean.

used to probe the MBP receptor. The chromatogram reproduced in Fig. 2B is the control chromatogram obtained by passing the same amount of membrane preparation from unimmunized animals through the immunoaffinity bead column.

Polyacrylamide gel analysis of the second peak demonstrated the presence of only one protein band (Fig. 3) as compared with five bands, found in the whole, solubilized membrane. Comparison of the immunoaffinity isolated band with the molecular weight standards showed that the isolated band was approximately 90 kilodaltons (kD).

When radiolabelled antigen binding of intact lymphocytes obtained prior to their disruption and solubilization, is compared to that of the isolated MBP receptor, it is seen that the immunoaffinity isolation technique retained between 75 and 78% of the original binding capacity (Fig. 4).

DISCUSSION

High-performance immunoaffinity chromatography with immobilized antibodies is a rapid technique for isolating antigen-specific lymphocyte receptors in an active form. The use of the antigen to protect the molecular shape and structure of the receptor during isolation appears to be a convenient method and provides additional antigenic sites for attachment to the immobilized antibody. In this way the receptor does not have to suffer from both the solubilization and interaction with an antibody. We speculate that this latter factor helps to maintain the integrity of the receptor shape, but, to date this has not been proven. Although the technique is rapid and produces receptors which can be shown to retain up to 78% of the original binding capacity, loss of receptor function does occur during elution from the immobilized antibody. It has been shown that both acid and chaotropic ion elution interfere with the tertiary structure of proteins[18,19]. New elution techniques need to be investigated to improve the recovery of the immunoaffinity-isolated materials. Even with these problems, the gel electrophoresis analysis of the isolated peak demonstrated that it contained a single protein of *ca.* 90 kD. This size has been shown to compatible with other studies which have isolated primed T cell receptors[20,21].

The development of the streptavidin-coated bead provides a better immunoaffinity packing than Protein A, especially now that biotinylation can be controlled and the attachment site specified[13]. The growing repertoire of commercially available biotinylated monoclonal antibodies makes this technology more interesting to all investigators who use practical immunoaffinity techniques to isolate biological materials in viable form.

CONCLUSION

The isolation of membrane receptors by immunoaffinity chromatography is a rapid, easy technique which gives yields of functional receptor which is comparable with most biochemical techniques and preserves the functional aspect of the receptor better than detergent solubilization techniques.

REFERENCES

1 B. G. W. Arnason, in P. J. Dyck, P. K. Thomas and E. H. Lambert (Editors), *Peripheral Neuropathy*, Vol. 2, Saunders, New York, 1975, Ch. 56, p. 1110.
2 P. Y. Paterson, *Rev. Infect. Dis.*, 1 (1979) 468.
3 E. H. Eylar, *Proc. Natl. Acad. Sci. U.S.A.*, 67 (1970) 1425.
4 S. W. Brostoff, S. Levit and J. M. Powers, *Nature (London)*, 268 (1977) 752.
5 A. A. Vandenbark, H. Offner, T. Reshef, R. Fritz, C.-H. J. Chou and I. R. Cohen, *J. Immunol.*, 135 (1985) 229.
6 S. Lapanje, *Physicochemical Aspects of Protein Denaturation*, Wiley, New York, 1978, p. 56.
7 J. J. Weis and D. T. Fearon, in T. A. Springer (Editor), *Hybridoma Technology in the Biosciences and Medicine*, Plenum, New York, 1985, Ch. 2, p. 207.
8 T. M. Phillips, N. S. More, W. D. Queen, T. V. Holohan, N. C. Kramer and A. M. Thompson, *J. Chromatogr.*, 317 (1984) 173.
9 T. M. Phillips, W. D. Queen, N. S. More and A. M. Thompson, *J. Chromatogr.*, 327 (1985) 213.
10 A. E. Bolton and W. M. Hunter, *Biochem. J.*, 133 (1973) 529.
11 A. M. Campbell, *Monoclonal Antibody Technology*, Elsevier, Amsterdam, 1984, p. 120.
12 A. M. Campbell, *Monoclonal Antibody Technology*, Elsevier, Amsterdam, 1984, p. 186.
13 D. J. O'Shannessy, *Immunol. Lett.*, 8 (1984) 273.
14 H. Wigzell, *Transplant. Rev.*, 5 (1970) 76.
15 A. Chrambach and D. Rodbard, in B. D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, Washington, DC, 1981, p. 93.
16 D. W. Sammons, L. D. Adams and E. E. Nishizawa, *Electrophoresis*, 2 (1981) 135.
17 J. Haberman, C. R. Pickardt and P. C. Scriba, *Acta Endocrinol. (Copenhagen)*, 2324 (1980) 26.
18 E. Rouslahti, in E. Rouslahti (Editor), *Immunoadsorbents in Protein Purification*, University Park Press, Balmitore, 1976, p. 3.
19 T. M. Phillips, W. D. Queen and M. G. Lewis, in R. A. Reisfeld and S. Ferrone (Editors), *Melanoma Antigens and Antibodies*, Plenum, New York, 1982, p. 289.
20 K. Haskins, R. Kubo, J. White, M. Pigeon, J. Kappler and P. Marrack, *J. Exp. Med.*, 157 (1983) 1149.
21 E. Reinherz, S. Meuer, K. Fitzgerald, R. Hussey, J. Hodgdon, O. Acuto and S. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 4104.