Solid-Phase Radioimmunoassay for Antibody to Myelin Basic Protein and Its Measurement in Sera from Patients with Neurological Diseases*

Mitsuhiro OHTA,^{1,**} Fumiyo MATSUBARA-MORI,¹ Kiyoe OHTA,¹
Hiroschi NISHITANI,² and Kyozo HAYASHI³

¹*Clinical Research Center, and* ²*Department of Neurology, Utano National Hospital,
Ondoyama-cho 8, Narutaki, Ukyo-ku, Kyoto 616, Japan*

³*Department of Biology, Gifu Pharmaceutical University,
Gifu 502, Japan*

(Received August 29, 1986)

Summary A solid-phase radioimmunoassay was developed for estimating antibody to myelin basic protein. Conditions of the various procedures were established for optimal specific antibody binding. Myelin basic protein was bound to microtiter plates, followed by incubation with diluted test serum. ¹²⁵I-protein A was used to measure the IgG bound to the antigen-coated well.

Sera from 47 patients with multiple sclerosis, cerebrovascular disease, neuro-Behçet's disease, and other neurological diseases were tested for antibody to myelin basic protein. Positive serum antibody was predominantly found in patients with significant basic protein antigen in their cerebrospinal fluids.

Key Words: myelin basic protein, anti-myelin basic protein antibody, solid-phase radioimmunoassay, multiple sclerosis, neurological diseases

Myelin basic protein (BP), or its fragment, has the ability to produce experimental allergic encephalomyelitis (EAE) in laboratory animals. This has been used as a model for studying human demyelinating diseases. In EAE, which is known to be mediated by T lymphocytes against BP [1-3], it has been suggested that major histocompatibility complex products are involved in the pathogenesis. BP-specific T-cell lines can adoptively transfer EAE in mice or rats [4, 5]. Auto-immune demyelinating diseases of the central nervous system (CNS) seem to be

*This work was supported in part by a research grant from the Research Committees of Intractable Neuroimmunological Disorders from the Ministry of Health and Welfare, and by Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

**To whom correspondence should be addressed.

primarily due to cell-mediated mechanisms, as measured by macrophage or leukocyte migration inhibition [6, 7] or by lymphocyte transformation assay [8–10]. However, the characterization of the humoral response to BP is also required for elucidating the pathogenic role of a humoral response to CNS antigens.

Antibody activity to BP has been investigated in several animal species by a variety of immunochemical techniques [11]. Since the levels of circulating anti-BP antibodies are quite low, the need for a more sensitive technique has arisen.

Solid-phase radioimmunoassays are widely used for measuring antibody against a variety of antigens. In these types of assays, species-specific radiolabelled anti-IgG is commonly used to measure the amount of specific antibody bound to insolubilized antigen. Protein A from *Staphylococcus aureus* binds to the Fc portion of IgG of most mammalian species [12, 13] and has been used as a solid-phase immunoabsorbent for precipitating radiolabelled antigen-antibody complexes in radioimmunoassays [14].

Elevated levels of BP were demonstrated in the cerebrospinal fluids (CSF) during exacerbation from patients with multiple sclerosis (MS) or in the acute active state from patients with myelopathy and cerebrovascular disease [15–20]. Determination of the antigen in the CSF of these patients is useful for clinical management. The appearance of BP in CSF may cause the production of anti-BP antibody, not only in the CSF, but also in the serum. In this paper we described a highly sensitive solid-phase radioimmunoassay to measure antibody to BP in the sera of rabbits hyperimmunized with BP. We have applied it to the detection of circulating antibodies in the sera of patients with various neurological diseases, especially those who have a high concentration of BP antigens in their CSF.

MATERIALS AND METHODS

Materials. Protein A from *Staphylococcus aureus* was purchased from Pharmacia, Uppsala, Sweden. Disposable flexible microtiter plates were purchased from Dynatech Laboratories Inc., Alexandria, VA. Human sera were obtained from 47 patients (MS, 15; cerebrovascular disease, 23; neuro-Behçet's disease, 3; polyneuropathy, 3; Guillain-Barré syndrome, 3). All samples were stored at -20°C until used.

Preparation of BP antigen and antiserum to BP. BP from bovine brain was prepared according to the method of Deibler *et al.* [21]. In brief, an acid extract of brain delipidated with chloroform-methanol solvent was chromatographed on carboxymethyl-cellulose, pH 10.4, and BP fractions were eluted with a linear salt gradient. The preparation gave a single band on polyacrylamide gel disc electrophoresis by the method of Reisfeld *et al.* [22]. ^{125}I -BP and antiserum to BP raised in rabbits were prepared by our previously published method [19].

Radioiodination of protein A. Protein A was labelled with ^{125}I according to the method of Hunter and Greenwood [23]. In brief, 10 μg of protein A in 0.4 M phosphate buffer (20 μl), pH 7.2, was mixed with 10 μg of chloramine T (10 μl)

Scheme 1. Flow sheet of solid-phase radioimmunoassay procedure for measuring antibody to BP.

-
- (1) Coat microtiter plates with 100 μ l of BP antigen in Buffer I (borate-buffered saline, 0.2 M, pH 8.1) for 1–1.5 h at room temperature.
 - (2) Rinse three times with 200 μ l of Buffer II (Buffer I containing 0.5% BSA and 0.1% Tween 20).
 - (3) Incubate with 100 μ l of diluted test serum in Buffer I for 16 h at 4°C.
 - (4) Rinse plates as in (2).
 - (5) Incubate with 100 μ l of 125 I-protein A in Buffer I for 4 h at room temperature.
 - (6) Rinse plates as in (2).
 - (7) Count radioactivity.
-

and Na 125 I (0.5 mCi). After 1 min, 20 μ g of sodium metabisulfite (20 μ l) was added, and the mixture was immediately passed through a Sephadex G-50 column (1.0 $\times$ 20 cm) equilibrated with the above buffer containing 0.5% bovine serum albumin (BSA). The fractions of the 125 I-labelled protein A peak were pooled. Specific activity utilized in this assay ranged from 20 to 40 μ Ci/ μ g. The binding of 125 I-protein A to the insolubilized immune complexes on wells plateaued at 1 to 1.5 ng, so approximately 1 ng of 125 I-protein A was used.

Procedure for solid-phase radioimmunoassay. The procedure was performed as a modification of the methods described by Panitch *et al.* [24] and Linthicum *et al.* [25]. Several parameters were tested to establish optimal assay conditions. Scheme 1 shows the flow sheet of the procedure for quantitation of antibody to BP. Details of the experiments are described below.

RESULTS

Optimal conditions for incubation of test serum in antigen-coated wells

The effect of different conditions of BP binding on the final test results was examined. Microtiter plates coated with various BP concentrations were incubated with rabbit antiserum to BP in order to obtain the optimal binding of antibody to BP. With BP concentrations over 25 μ g/ml (100 μ l/well), the amounts of rabbit anti-BP antibody bound to wells almost reached a plateau at each antibody dilution (1 : 10 to 1 : 1,000) and significantly differed from the amount bound with preimmune rabbit serum. A concentration of 25 μ g/ml was chosen for the coating procedure. The amount of BP bound to the well at this concentration was about 1 μ g as measured by the use of 125 I-BP. Figure 1 shows the effect on the final results of the time of incubation of antigen-coated wells with rabbit anti-BP antibody (first incubation step) and of the time of incubation with 125 I-protein A (second incubation step). Increasing the first incubation time resulted in greater binding of the immunoglobulin of both hyperimmune and preimmune rabbit sera. In the second incubation, binding of 125 I-protein A to the insolubilized immune complexes reached a maximum after 4 h incubation. Thus we decided to use a 16 h incubation

Fig. 1. Effect of incubation times of the first step (sample to antigen-coated well) and the second step (^{125}I -protein A to insolubilized immune complex) on the sensitivity of solid-phase radioimmunoassay. Symbols represent the binding activities of the hyperimmune ($\text{O}—\text{O}$) and preimmune ($\times \cdots \times$) sera. First incubation time: A, 1 h; B, 4 h; C, 16 h.

period for the first step and a 4 h one for the second step. These conditions gave the greatest binding ratio of hyperimmune to preimmune rabbit sera.

A titration curve obtained as a result of serial dilutions of hyperimmune serum showed 48% of the total ^{125}I -protein A to be bound at a dilution of 1 : 100, whereas, only 25% was bound at a dilution of 1 : 1,000.

Radioimmunoassay of anti-BP antibody in sera of neurological patients

Experiments were carried out in order to define the optimal dilution of positive and control sera in this procedure. Mean and standard deviation (SD) values of control sera obtained from 19 normal subjects are shown in Fig. 2. The non-specific binding decreased with higher dilutions of samples. From these data, we chose to use a serum dilution of 1 : 160, because the ratio of positive sera to control sera at this dilution was maximal.

Levels of anti-BP antibodies in the sera of patients with various neurological diseases were determined using the above procedure (Fig. 3). Test samples were considered positive if the binding ratio exceeded the normal mean plus 3SD. All 19 sera from normal control subjects were negative, whereas 27 out of 47 sera from patients with neurological diseases were positive. The positive percentages of MS, cerebrovascular disease, neuro-Behçet's disease, and polyneuropathy were 67%, 57%, 67%, and 67%, respectively. All Guillain-Barré syndrome samples

Fig. 2. Effect of serum dilution on the amount of ^{125}I -protein A bound to insolubilized immune complexes. Dilution was carried out with Buffer I (see text). Samples were obtained from patients with cerebrovascular disease (case 1), MS (case 2), and neuro-Behçet's disease (case 3). The normal curve represents the mean value of 19 control sera, with $\pm 1\text{SD}$ indicated by the vertical bar.

Fig. 3. Levels of antibodies to BP in sera from patients with various neurological disease. The dotted line represents the normal upper limit. Horizontal axis shows the binding ratio of ^{125}I -protein A to the normal mean measured at a serum dilution of 1 : 160. Closed circles in the column of MS indicate the samples obtained from active MS.

were negative. Of 3 cases of MS in exacerbation, 2 cases were positive and the third showed a borderline value.

Figure 4 shows the serial clinical courses of neurological dysfunctions and the serial determinations of BP antigen in the CSF and its antibody in sera from 3 patients with typical MS. The elevated amounts of BP in the CSF were detected during early stages of the neurological attacks and returned rapidly to normal levels (1.0 ng BP/ml CSF). On the other hand, the serum level of anti-BP antibody

Fig. 4. Clinical courses of neurological dysfunctions and the levels of BP in CSF and antibodies to BP in sera from 3 female patients with MS: case A, 50-year-old; case B, 41-year-old; case C, 51-year-old. Shaded areas indicate the severities of neurological attacks. The levels of BP in CSF (○---○) and of antibodies to BP in sera (●—●) are shown in the lowest panel. Normal upper values are 1.0 ng BP/ml CSF and 1.70 of antibody titer.

was increased a few weeks after the attack in case A, but no marked change was observed in cases B and C.

DISCUSSION

The appearance of BP antigen in the CSF from MS patients has been used as a marker of active demyelination [15–17]. There are some other neurological diseases in which BP occurs in the CSF such as cerebrovascular disease, neuro-Behçet's disease [19, 26], brain trauma following brain surgery [27], Guillain-Barré syndrome, brain tumors, neurosyphilis [28], and retrobulbar neuritis [29]. The presence of specific antibody to BP in the serum or CSF means that a humoral autoimmune response has occurred against free BP or its fragments.

Radioimmunoassay based studies on the antibody to BP have produced conflicting results. Many workers have shown no evidence of antibody to BP using various methods [30–32]. Some investigators have shown the presence of antibody in the CSF from MS and subacute sclerosing panencephalitis patients [24, 33, 34] and in extracts of MS brain [35]. These conflicting results may be attributed to differences in antigen source and purity, the population of patients examined, and the sensitivity of the assay method.

In the previously developed solid-phase assay systems, test tubes [24, 36], microtiter plates [25, 34], or polystyrene beads [33] were used as solid-phase media for coating with antigen. After a comparative study of the above media, we preferred the use of microtiter plates as the solid-phase because of sensitivity, simplicity, and reliability. In this paper, we have reported the development of a sensitive radioimmunoassay for the detection of antibody to BP that involves the combination of flexible microtiter plates, bovine BP, and ^{125}I -protein A, according to the method of Panitch *et al.* [24] and Linthicum *et al.* [25]. Protein A reacts with the Fc portion of most classes of IgG in many species, and it has advantages over a specific labelled anti-IgG. Human IgG binds to protein A, except for its IgG3 subclass [12].

BP is known to bind to α_2 -macroglobulin [37], the Fc portion of aggregated IgG, soluble immune complexes [38], and other molecules [39, 40], which may yield false positive results. We tried to minimize these effects by the following precautions. No difference in binding activity of normal serum specimens was observable over IgG concentrations from 800 to 1,800 $\mu\text{g/ml}$, so the serum samples were tested only after a dilution 1 : 160 without adjusting IgG concentrations. To prevent the effect of non-specific IgG binding, BSA and Tween 20 were added to the rinse buffer [25, 41]. The effects of aggregated IgG and soluble immune complexes may be negligible since preliminary data on 6 sera from patients with rheumatoid arthritis and 5 sera from those with systemic lupus erythematosus showed values within the normal range. These effects must be considered in evaluating the significance of antibody to BP in future studies on a large number of sera from patients with various autoimmune diseases.

We applied this radioimmunoassay to sera of patients with MS and other neurological diseases. To establish the fact that the binding of IgG to BP represents

a genuine and immunologically specific reaction, we prepared an F(ab')₂ fragment of the MS IgG with the highest titer, and confirmed that its activity reflected an immunospecific antigen-antibody reaction. Not only the active MS patients, but also more than half of the inactive ones had a significantly elevated antibody level. Moreover, many cases of cerebrovascular disease and neuro-Behçet's disease who were in the chronic inactive state had high levels of antibodies. The elevated antibody level in the sera of 2 patients with polyneuropathy may be related to the presence of a protein like CNS BP in peripheral nerves. Most of the diseased patients with significant antibodies to BP also had elevated BP antigen in their CSF, as shown by our previous work [19]. In addition, a similar elevation was observed in the CSF antibody level in these neurological patients (data not shown).

In a sequential study of BP antigen in the CSF and antibody in the serum during 3 typical clinical courses of MS, the elevated BP seen in the CSF during the early stages of exacerbations decreased rapidly to the normal level. A correlation was found between the clinical state and the level of BP in CSF. Whereas, the antibodies to BP fluctuated with no correspondence to the clinical state or to changes in the CSF BP levels.

Characterization of the humoral response to BP may be useful in evaluating the pathogenesis of demyelinating and other neurological diseases, and such studies should accompany those on cellular immunity to myelin and BP.

The authors acknowledge with gratitude to Dr. Tetsuro Konishi for the kind supply of case materials.

REFERENCES

1. Driscoll, B.F., and Kies, M.W. (1979): Transfer of experimental allergic encephalomyelitis with guinea pig peritoneal exudate cells. *Science*, **203**, 547-548.
2. Richert, J.R., Driscoll, B.F., Kies, M.W., and Alvord, E.C., Jr. (1979): Adoptive transfer of experimental allergic encephalitis—Incubation of rat spleen cells with specific antigen. *J. Immunol.*, **122**, 494-496.
3. Pettinelli, C.B., and McFarlin, D.E. (1981): Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after *in vitro* activation of lymph node cells by myelin basic protein—Requirements for Lyt⁺2-T lymphocytes. *J. Immunol.*, **127**, 1420-1423.
4. Ben-Nun, A., and Cohen, I.R. (1982): Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines—Process of selection of lines and characterization of the cells. *J. Immunol.*, **129**, 303-308.
5. Richert, J.R., Lehky, T.J., Muehl, L.A., Mingioli, E.S., and McFarlin, D.E. (1985): Myelin basic protein-specific T cell lines and clones derived from SJL/J mice with experimental allergic encephalomyelitis. *J. Neuroimmunol.*, **8**, 129-139.
6. Sheremata, W., Cosgrove, J.B.R., and Eylar, E.H. (1976): Multiple sclerosis and cell-mediated hypersensitivity to myelin A1 protein. *J. Neurol. Sci.*, **27**, 413-425.
7. Kallen, B., Nilsson, O., and Thelin, C. (1977): Effect of encephalitogenic protein on migration in agarose of leukocytes from patients with multiple sclerosis: variable effect of the antigen in a large dose range with a literature review. *Acta Neurol. Scand.*, **55**, 33-46.
8. Webb, C., Teitelbaum, D., Abramsky, O., Arnon, R., and Sela, M. (1974): Lymphocytes

- sensitized to basic encephalitogen in patients with multiple sclerosis unresponsive to steroid therapy. *Lancet*, **2**, 66–69.
9. Lisak, R.P., and Zweiman, B. (1977): *In vitro* cell-mediated immunity of cerebrospinal fluid lymphocytes to myelin basic protein in primary demyelinating diseases. *N. Engl. J. Med.*, **297**, 850–853.
 10. Ben-Nun, A., and Cohen, I.R. (1981): The rapid isolation of clonable antigen specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.*, **11**, 195–199.
 11. Wallace, A.D., Shapira, R., and Fritz, R.B. (1978): Isolation and characterization of rabbit antibodies to bovine myelin basic protein. *Immunochemistry*, **15**, 47–54.
 12. Goding, J.W. (1978): Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods*, **20**, 241–253.
 13. Kronvall, G., Grey, H.M., and Williams, R.C. (1970): Protein A reactivity with mouse immunoglobulins: structural relationship between some mouse and human immunoglobulins. *J. Immunol.*, **105**, 1116–1123.
 14. Langone, J.J. (1978): ¹²⁵I-Protein A—A tracer for general use in immunoassay. *J. Immunol. Methods*, **24**, 269–285.
 15. Cohen, S.R., Herndon, R.M., and McKhann, G.M. (1976): Radioimmunoassay of myelin basic protein in spinal fluid; an index of active demyelination. *N. Engl. J. Med.*, **295**, 1455–1457.
 16. Cohen, S.R., McKhann, G.M., and Guainieri, M. (1976): A radioimmunoassay for myelin basic protein and its use for quantitative measurements. *J. Neurochem.*, **25**, 371–376.
 17. Whitaker, J.N. (1977): Myelin encephalitogenic protein fragments in cerebrospinal fluid of persons with multiple sclerosis. *Neurology*, **27**, 911–920.
 18. Whitaker, J.N., Lisak, R.P., Bashir, R.M., Fitch, O.H., Seyer, J.M., Krance, R., Lawrence, J.A., Ch'ien, L.T., and O'Sullivan, P. (1980): Immunoreactive myelin basic protein in the cerebrospinal fluid in neurological disorders. *Ann. Neurol.*, **7**, 58–64.
 19. Ohta, M., Matsubara, F., Konishi, T., and Nishitani, H. (1980): Radioimmunoassay of myelin basic protein in cerebrospinal fluid and its clinical application to patients with neurological diseases. *Life Sci.*, **27**, 1069–1074.
 20. Carson, J.H., Barbarese, E., Braun, P.E., and McPherson, T.A. (1978): Components in multiple sclerosis cerebrospinal fluid that are detected by radioimmunoassay for myelin basic protein. *Proc. Natl. Acad. Sci. USA*, **75**, 1976–1978.
 21. Deibler, G.E., Martenson, R.E., and Kies, M.W. (1972): Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem.*, **2**, 139–165.
 22. Reisfeld, R.A., Lewis, U.J., and Williams, D.E. (1962): Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, **195**, 281–283.
 23. Hunter, W.M., and Greenwood, F.C. (1962): Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature*, **194**, 495–496.
 24. Panitch, H.S., Hooper, C.J., and Johnson, K.P. (1980): CSF antibody to myelin basic protein: measurement in patients with multiple sclerosis and subacute sclerosing panencephalitis. *Arch. Neurol.*, **37**, 206–209.
 25. Linthicum, D.S., Jones, S., Horvath, L., and Carnegie, P.R. (1981): Detection of antibodies to myelin basic protein by solid-phase radioimmunoassay with ¹²⁵I-protein A. *J. Neuroimmunol.*, **1**, 17–26.
 26. Ohta, M., Nishitani, H., Matsubara, F., and Inaba, G. (1980): Myelin basic protein in spinal fluid from patients with neuro-Behçet's disease. *N. Engl. J. Med.*, **302**, 1093.
 27. Alling, C., Karlsson, B., and Vållfars, B. (1980): Increase in myelin basic protein in CSF after brain surgery. *J. Neurol.*, **223**, 225–230.
 28. Prange, H., Kohlschütter, A., and Ritter, G. (1980): Basisches myelin protein im liquor cerebrospinalis bei neurosyphilis. *Dtsch. Med. Wochenschr.*, **105**, 1119–1121.

29. Biber, A., Englert, D., Dommasch, D., and Hempel, K. (1981): Myelin basic protein in cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases. *J. Neurol.*, **225**, 231–236.
30. Lennon, V., and Mackay, I.R. (1972): Binding of ¹²⁵I-myelin basic protein by serum and cerebrospinal fluid. *Clin. Exp. Immunol.*, **11**, 595–603.
31. Gutstein, H.S., and Cohen, S.R. (1978): Spinal fluid differences in experimental allergic encephalomyelitis and multiple sclerosis. *Science*, **199**, 301–303.
32. Lisak, R.P., Zweiman, B., Waters, D., Koprowski, H., and Pleasure, D.E. (1978): Cell-mediated immunity to measles, myelin basic protein, and central nervous system extract in multiple sclerosis: a longitudinal study employing direct buffy coat migration inhibition assays. *Neurology*, **28**, 798–803.
33. Ruutiainen, J., Arnadottir, T., Molnár, G., Salmi, A., and Frey, H. (1981): Myelin basic protein antibodies in the serum and CSF of multiple sclerosis and subacute sclerosing pan-encephalitis patients. *Acta Neurol. Scand.*, **64**, 196–206.
34. Wajgt, A., and Górný, M. (1983): CSF antibodies to myelin basic protein and to myelin-associated glycoprotein in multiple sclerosis. Evidence of the intrathecal production of antibodies. *Acta Neurol. Scand.*, **68**, 337–343.
35. Bernard, C.C.A., Randell, V.B., Horvath, L.B., Carnegie, P.R., and Mackay, I.R. (1981): Antibody to myelin basic protein in extracts of multiple sclerosis brain. *Immunology*, **43**, 447–457.
36. Randolph, D.H., Kibler, R.F., and Fritz, R.B. (1977): Solid-phase radioimmunoassay for detection of antibodies to myelin basic protein. *J. Immunol. Methods*, **18**, 215–224.
37. McPherson, T.A., Marchalonis, J.J., and Lennon, V. (1970): Binding of encephalitogenic basic protein by serum alphaglobulins. *Immunology*, **19**, 929–933.
38. Sindic, C.J.M., Cambiaso, C.L., Masson, P.L., and Laterre, E.C. (1980): The binding of myelin basic protein to the Fc region of aggregated IgG and to immune complexes. *Clin. Exp. Immunol.*, **4**, 1–7.
39. Mullin, B.R., Decandis, F.X., Montanaro, A.J., and Reid, J.D. (1981): Myelin basic protein interacts with the myelin-specific ganglioside GM4. *Brain Res.*, **222**, 218–221.
40. Iwasa, Y., Iwasa, T., Matsui, K., Higashi, K., and Miyamoto, E. (1981): Interaction of calmodulin with chromatin associated proteins and myelin basic protein. *Life Sci.*, **29**, 1369–1377.
41. Macklin, W.B., and Lees, M.B. (1982): Solid-phase immunoassay for quantitation of antibody to bovine white matter proteolipid apoprotein. *J. Neurochem.*, **38**, 348–355.