ANTIGENIC DETERMINANT SHARED BETWEEN MYELIN-ASSOCIATED GLYCOPROTEIN FROM HUMAN BRAIN AND NATURAL KILLER CELLS

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ABSTRACT

HNK-1, a monoclonal antibody to human NK cells, recognized myelin-associated glycoprotein (MAG) purified from human brain. Titration of the antibody and quantitative absorption tests were performed by enzyme-linked immunosorbent assay (ELISA) using purified MAG as an antigen. Titer of HNK-1 against MAG was measurable. HNK-1 was absorbed by purified MAG and myelin from human brain. Human myelin and MAG were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to nitrocellulose sheets (blots) and immunostained by HNK-1. HNK-1 stained mainly the position of MAG (M.W., 100 kDa) in the blots of myelin and purified MAG. After absorption by MAG, HNK-1 did not stain the blot of myelin.

The existence of antigens shared between lymphoid tissues and the brain has been reported (13, 16, 24, 28). Thy-1 antigens have been found in mouse thymocytes and brain (24), and the difference in the chemical characters between the Thy-1 in the thymocytes and that in the brain has been well studied (2). In the brain, Thy-1 is mainly distributed in gray matter (17), and its molecular weight is about 25 kDa (2).

It has been shown that monoclonal antibodies against human T cells, UCHT 1 and Leu-4, recognized human Purkinje cells (6) and that OKT8, a marker of human suppressor T cells, stained the surface of cultured oligodendrocytes of lamb (19). However, the chemical nature of those antigens has not yet been characterized.

A recent study has shown that F 10-44-2 monoclonal antibody, originated from immunization of human T-lymphocytes, recognizes the white matter of human brain (16). The F 10-44-2 antigen in the human brain is a glycoprotein with an apparent molecular weight of 90 kDa (16).

The anti-brain-associated T cell antigen (anti-BAT) is capable of killing the natural killer

cells (NK cells) of mice (7). Although a glycosphingolipid 'asialo GM₁' is known to be one of the antigens shared between the NK cells and brain in mice (13), its existence in human NK cells has not yet been reported.

The myelin-associated glycoprotein (MAG) is a myelin specific glycoprotein (21) rich in the white matter (11). The molecular weight of MAG is about 100 kDa, but incubation of purified myelin at pH 8 resulted in the conversion of MAG to a smaller derivative (dMAG) with an apparent molecular weight of about 90 kDa (26). Thus, the molecular weight of dMAG is similar to that of the F 10-44-2 antigen in brain, although it is not known whether or not dMAG exists in the brain *in situ*.

In this study, we demonstrate that human NK cells and human MAG from the central nervous system (CNS) myelin share a common antigenic determinant.

MATERIALS AND METHODS

Myelin Preparation

Myelin was purified from human white matter

essentially by the method of Norton and Poduslo (18). For MAG isolation, one-cycle myelin (26) was used, whereas 2-cycle myelin (26) was used for gel electrophoresis or immunoabsorption tests.

Extraction of MAG

MAG was extracted by the LIS-phenol method essentially according to the procedure of Quarles and Pasnak (23) with some modifications as previously described (26). Further purification for MAG was performed by gel filtration on a Sepharose CL-6B column equilibrated with 0.5% (w/v) sodium dodecyl sulfate (SDS), and then MAG fractions were dialyzed against deionized water (1).

Production of Antisera

MAG (250 μ g) was suspended in 0.8 ml of saline and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected intradermally at multiple sites on the back of a Japanese white rabbit (22). Similar injection was repeated at weeks 3 and 5 after the initial immunization.

Absorption of Antibody

HNK-1 (5 μ l; Becton Dickinson Monoclonal Center, CA) was diluted to 1:10 by 0.05% (v/v) Tween 20 and 2% (w/v) bovine serum albumin (BSA) in phosphate buffer saline (PBS), pH 7.1 (BSA-PBS), and 20 μ l of diluted HNK-1 was mixed with 10 μ g of purified MAG. The mixtures were incubated overnight at 4°C and diluted to various concentrations immediately before use for enzyme-linked immunosorbent assay (ELISA) (Fig. 1).

For immunostaining of nitrocellulose sheet (Fig. 4, lane 3), another absorbed HNK-1 was prepared. HNK-1 (10 μ l) was mixed with 10 μ g of purified human MAG and incubated overnight at 4°C. The absorbed solution was diluted to a final concentration of 1:200 before use.

Mononuclear cells in the peripheral blood were collected by the one step sodium metrizoate/Ficoll procedure (4). Briefly, 15 ml of heparinized blood were obtained from healthy adult human and mixed with an equal volume of PBS. The mixture was layered on Lymphoprep (Nyegaard & CO-AS-OSLO) and centrifuged for 30 min at exactly 400 g. The mononuclear cells at the interface between the blood and the

Fig. 1 Titration data. Untreated HNK-1, ●; HNK-1 absorbed by MAG, ○; and without the second antibody, ■. The absorbance of the content of two wells was read at 450 nm 1 h after incubation with a substrate solution.

LYMPHOPREP were collected and washed twice by PBS. Erythrocyte membrane was purified essentially by the method of Ohnishi (20). Heparinized blood was washed twice with 0.15 M NaCl, and the erythrocytes were broken by osmotic shock, and the ghosts were washed with 0.07 M NaCl several times and finally with PBS. Postmortem human liver was stored at -80°C prior to homogenization. The tissue was manually homogenized in PBS using a glass homogenizer. The protein value of the tissue suspension including myelin, erythrocyte ghosts, lymphocytes or liver was measured (15), adjusted to 10 mg protein/ml, and then diluted to various concentrations, e.g., 2 mg/ml, 0.4 mg/ml and 0.08 mg/ml.

HNK-1 was diluted to 1:100 with BSA-PBS. Absorptions were performed by incubating equal volume (50 μ l) of diluted antibody with tissue suspensions at various concentrations overnight at 4°C. The tissue was then removed by centrifugation, and the absorbed sera were used immediately. The antibody solutions were diluted to a final concentration of 1:1,000 when used for ELISA (Fig. 2).

Enzyme-linked Immunosorbent Assay (ELISA)

The method for the detection of anti-basic protein (BP) antibody (8) was modified for HNK-1, using purified human MAG as an

Fig. 2 Quantitative absorption analysis of HNK-1 with the various human tissues; ●, myelin; ○, mononuclear cells; ■, erythrocyte ghosts; and □, liver homogenate. Fifty microliters of diluted (1 : 100) HNK-1 were mixed with 50 μ l of the tissue suspension in various concentrations. The final dilution of first antibody was 1 : 1,000. The absorbance of two wells was measured after 24 h incubation with a substrate solution.

antigen. Briefly, 100 μ l of PBS, pH 7.1, containing 1 μ g/ml of MAG were added to each well of a microplate (Immunoplate I, Nunc, Denmark). The plate was incubated at 25°C for 3 h. Then the wells were washed 4 times with washing buffer containing 0.05% (v/v) Tween 20 in PBS, pH 7.1. The untreated or the absorbed HNK-1 solutions were diluted to 1 : 1,000 with BSA-PBS immediately before use. Antiserum (100 μ l) was placed into each well and incubated for 1 h at room temperature. After the first antibody binding, the plate was washed with washing buffer. Peroxidase-conjugated anti-mouse IgM was diluted to 1 : 1,000 with BSA-PBS and 100 μ l of the solution were added to each well. The plate was again incubated at room temperature for 1 h. The substrate solution was prepared with 1 volume of 0.05% hydrogen peroxide and 9 volumes of aqueous 0.08% (w/v) 5-aminosalicylic acid (5-AS). The plates were washed and 200 μ l of the substrate solution were added to each well. After 1 or 24 h at room temperature, the absorbance at 450 nm of the content of each well was read against water using a microplate photometer (Corona Electric, MTP-12, Japan).

Gel Electrophoresis and Immunostaining for Blots

The methods of delipidation and solubilization of myelin have been described elsewhere (25, 26). Electrophoresis was carried out in 10% SDS-polyacrylamide slab gels prepared according to Laemmli and Favre (14). A part of the gel was cut off and stained with Coomassie Blue (25, 26).

After electrophoresis, the proteins in the gels were transferred electrophoretically to nitrocellulose papers (Bio-Red, Trans-Blot TM cell) essentially according to the method of Towbin *et al.* (27). The peroxidase anti-peroxidase (PAP) method for the immunohistochemical study (9) was modified to immunostain the blot. The blot was incubated with 3% (w/v) BSA/0.9% NaCl/10 mM Tris-HCl, pH 7.4 (BSA-saline) at 40°C for 2 h and subsequently incubated with the first antibody at room temperature for 1 h. Then the blot was rinsed with 0.9% NaCl/10 mM Tris-HCl, pH 7.4 (saline) for 1 h with the change of saline every 10 min. For the second antibody and/or third antibody and PAP binding, the procedure was the same as for the first antibody. Immediately before use, substrate solution containing 0.5 mg per ml diaminobenzidine (DAB), 0.01% (v/v) hydrogen peroxide/10 mM Tris-HCl, pH 7.4, was prepared, and then the blot was developed. After development, the blot was rinsed thoroughly to stop the reaction.

RESULTS

Titration and Absorption Analysis by ELISA

Titration of untreated and absorbed HNK-1 are shown in Fig. 1. The untreated HNK-1 gave a titer at 1 : 4,000 dilution, whereas no titer was seen by the absorbed HNK-1 even at 1 : 1,000 dilution.

Quantitative absorptions of HNK-1 with various human tissues including myelin, mononuclear cells, erythrocyte ghosts and liver are shown in Fig. 2. Though the mononuclear cells were expected to absorb HNK-1, the strongest absorption was seen with myelin. Erythrocyte ghosts and liver had no absorptive capacity even at high tissue concentration.

Gel Electrophoresis and Immunostaining of Blots

A typical protein pattern of human CNS myelin is shown in Fig. 3, lane 3. Myelin in a lower

Fig. 3 A hundred nanograms of purified human MAG (lane 1) and 5 μ g of the 2-cycle human myelin (lane 2) were electrophoresed in 10% SDS-polyacrylamide slab gel, and transferred to a nitrocellulose sheet. The sheet was stained with HNK-1 (1:400 dilution) as the first antibody, anti-mouse IgM (rabbit; 1:100) as the second antibody, with anti-rabbit IgG (goat; 1:40) as the third antibody and finally a peroxidase anti-peroxidase complex (rabbit; 1:80). Twenty micrograms of the 2-cycle human myelin were electrophoresed in 10% SDS-polyacrylamide gel and stained with Coomassie-Blue (lane 3).

amount was electrophoresed, transferred to nitrocellulose paper and immunostained (Fig. 3, lane 2; Fig. 4, lanes 1–3).

A well-defined band was seen on the blot of human myelin with rabbit anti-human MAG serum (Fig. 4, lane 1). The molecular weight of this band was estimated to be around 100 kDa.

When a smaller amount of myelin protein (5 μ g) was electrophoresed, and a lower concentration of HNK-1 (1:400) was used, a broad band was clearly stained (Fig. 3, lane 2) around the position for the intact MAG (arrowhead in Fig. 3); a smaller molecule, dMAG, may also be stained in a similar position (26). A small amount (100 ng) of purified MAG was stained by HNK-1 (Fig. 3, lane 1). The banding pattern of the band was similar to that for myelin (Fig. 3, lane 2), except for the presence of faintly stained smaller molecules, likely to be degradation products of MAG. These findings show that HNK-1 recognized the intact MAG, and also probably the dMAG (26).

The nitrocellulose blot stained with HNK-1 (1:200) shows a broader band (with larger and smaller bands), probably because a larger amount of protein (15 μ g) was loaded on the gel. An amount of myelin protein same as in Fig. 3, lane 2 was loaded on the gel, and transferred to nitrocellulose paper (Fig. 4, lane 3). The immunostaining of two blots (Fig. 4, lanes 2 and 3) was performed simultaneously using the same procedure except for the first antibody. In lane 3, where an absorbed HNK-1 was used, no bands were stained. The result indicates that 10 μ g of purified MAG completely absorbed 10 μ l of HNK-1 under our experimental conditions.

DISCUSSION

Immunohistochemical study and radioimmunoassay have shown that MAG is decreased around the plaque in multiple sclerosis (MS) brain in an early phase of demyelination (10, 12). Incubation of human myelin resulted in the conversion of MAG to a smaller derivative (dMAG) with an apparent molecular weight of 90 kDa (26); dMAG was rendered soluble (R. H. Quarles; personal communication). If dMAG is formed *in situ*, it may be released from its natural site in the myelin membrane. Our recent study has demonstrated that dMAG is formed during incubation more rapidly in myelin from MS brains than in myelin from non-MS brains (unpublished data). These results suggest that

Fig. 4 Fifteen micrograms of the 2-cycle human myelin were electrophoresed and transferred to nitrocellulose sheet as in Fig. 3. The sheet was stained with the anti-human MAG serum (1:50) as the first antibody, anti-rabbit IgG (goat; 1:40) and peroxidase anti-peroxidase (rabbit; 1:80) complex (lane 1). In lanes 2 and 3, human myelin in the same amount as in lane 1 was electrophoresed and transferred to nitrocellulose paper. However, HNK-1 (1:200) was used in lane 2, and HNK-1 absorbed by purified MAG (1:200) was used as the first antibody in lane 3. Dilutions of the second, third antibodies and peroxidase anti-peroxidase complex were the same as in Fig. 3.

MAG plays an important role in the pathogenesis of MS.

Although the chemical nature of the antigen in NK cells in humans have not yet been fully understood, our present results have shown that MAG and NK cells may share an antigenic determinant. In case of Thy-1, antigenic determinant is shared between the thymocytes and the brain (24), and this determinant is likely to exist in the protein part of the molecule, because the carbohydrate composition of Thy-1 in the brain and the thymocytes is quite different (2). Little is known, however, whether the antigen in NK cells is identical with that in MAG, or whether only a part of the molecule shares a common antigenic site.

It has been shown that the activity of NK cells is depressed in patients with MS (3), though its mechanism is not known. It may be that an antibody to MAG released from the nervous tissue play a role at a certain stage of the demyelination. Recently, Braun *et al.* (5) reported that IgM M-proteins in some patients with polyneuropathy react with MAG, though the relation between anti-MAG antibody and polyneuropathy is not known and the NK activity of these patients has not yet been reported. We have so far been unable to detect MAG antibody in the serum of MS patients (unpublished data), which makes the relation between the depressed NK activity and the MAG antibody obscure. Whether the shared antigen shown in this report is relevant to the pathogenesis of demyelinating diseases such as MS, or whether its presence is merely incidental, remains to be known.

This study was supported by a grant from the Neuroimmunological Disorders Research Committee, the Ministry of Health and Welfare, Japan.

Received for publication 12 July 1983

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