Demonstration of Anti-asialo GM1 Antibody and Its Neurocytotoxicity in the Sera of Systemic Lupus Erythematosus Patients*

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SUMMARY **The presence of anti-asialo-GM1 antibody and its neurocytotoxicity in the sera of patients with SLE were studied. The antibody was detected in 55 per cent of patients with CNS complications. but not in healthy adult controls nor in patients without CNS complications. The exacerbation of CNS symptoms of CNS-SLE patients was accompanied by elevation of the anti-GA1 antibody level in the sera. In the presence of complement, the anti-GA1 antibody in the patients' sera showed cytotoxicity against nervous tissue cells bearing GA1 on their surface. The roles of these antibodies in the pathogenesis of CNS-SLE are suggested.**

ASIAN PACIFIC J ALLERG IMMUN 1984; 2:195-201.

As much as 30 to 50 per cent of patients with systemic lupus erythematosus (SLE) have central nervous system (CNS) complications.[1] Following renal insufficiency, CNS complications are the second most frequent cause of death of SLE patients and thus are deeply related to the pathogenesis of SLE. However, the pathogenesis of CNS complications has not been fully clarified and so diagnosis and treatment are still in dispute.

Since the early 1970s, there has been considerable interest in antineuronal antibodies in the sera and cerebrospinal fluid (CSF) of SLE patients in relation to the pathogenesis of CNS complications.[2-18] For example, antineuronal antibodies,[2,3,7,9,10] anti-glial antibodies,[7] anti-lymphocytes antibodies cross-reactive with brain tissue,[5,6] antibodies against the antigenic determinants shared between erythrocytes and the brain[9] and anti-glycolipid antibodies[11,19] have been reported to exist in the sera of SLE patients. Anti-brain tissue antibodies and IgG neurocytotoxic antibodies also have been demonstrated in CSF of SLE patients.[12,13] Recently, antineuronal antibodies of the IgG and IgA classes have been shown to exist in the necropsy brain tissue of an SLE patient with CNS complication (CNS-SLE).[16] Implantation of antibodies directed against various brain constituents into the ventricles or cerebral cortex of experimental animals caused convulsion, meningitis, impaired memory or motor dysfunction.[20-22] These observations suggest that antineuronal antibodies may play an important role in the CNS complications of SLE. Antineuronal antibodies in the sera of SLE patients have been recognised primarily by the immunofluorescence technique; hence, the physicochemical characteristics of the corresponding antigens have not been clarified. However, Hirano *et al*[11] detected antibody against the neutral glycolipid "asialo GM1 (GA1)" (ganglio-N-tetraosyl ceramide) in the sera of CNS-SLE patients. We also observed that exacerbation of CNS symptoms of a 10-year-old female CNS-SLE patient correlated with the elevation of the titre of the serum anti-GA1 antibody.[19]

Since GA1 exists in trace amounts in the human brain, anti-GA1 antibody could be one of the antineuronal antibodies in the sera of SLE patients. In the present

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This work was supported by a Research Grant from the Ministry of Health and Welfare of Japan.

study, we examined the neurocytotoxicity of anti-GAl antibody using established tissue culture cells derived from human brain tumours as targets.

MATERIALS AND METHODS

Patients and sera

Sera were obtained from 30 SLE patients (including 20 CNS-SLE patients) and 20 healthy adult controls and stored without preservative at -70°C until use. All the SLE patients met the criteria of SLE of the American Rheumatism Association. The criteria of neurologic disorders in SLE included definite neurologic (both central and peripheral) or psychiatric abnormalities not attributed to non-SLE aetiologies.

Cultured cell lines derived from human brain tumours

IMR-32 (human neuroblastoma)[23] 118 MGC (human glioblastoma)[24] and KG-1 (human oligodendroglioma)[25] were used as the targets of cytotoxicity assays. Cells were maintained in continuous culture in Minimal Essential Medium (Eagle's MEM, Nissui Seiyaku Co., Ltd. Tokyo, Japan) supplemented with L-glutamine and 5% foetal bovine serum. Viable cell suspensions were prepared by scraping adhesive cells with a rubber-policeman and suspending them in phosphate buffered saline (PBS: 8.0 g of NaCl, 0.2 g of KCl, 2.9 g of Na_2HPO_4 $12H_2O$ and 0.2 g of KH_2PO_4 in 1,000 ml of distilled water). They were used for tests when the viability was above 90 per cent.

Preparation of GAl

GMl was isolated from bovine brain tissue by a combination of anion-exchange chromatography and high performance adsorption chromatography.[26] GAl was prepared from GMl by treatment with 1N-formic acid at 100°C for one hour[27] and then purified by chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and Silica Gel 40 (Merck & Co., Inc., Rahway, N.J., U.S.A.).[28]

Preparation of antisera against GAl

Liposomes containing 1 mg of GAl, 2 mg of bovine serum albumin (BSA), 4 mg of cholesterol and 10 mg of lectithin were prepared and suspended in 0.5 ml of PBS. The same volume of Freund's complete adjuvant (Difco Lab., Detroit, Michigan, U.S.A.) was added to the suspension and the mixture was emulsified. Te emulsion was administered to a rabbit by intracutaneous injection at several sites on the foot-pads. Two to three weeks after the injection, blood with high titre anti-GAl antibody was obtained, and the plasma was heat inactivated (56°C for 30 minutes) and stored at -70°C with 0.05% sodium azide.

Detection of anti-GAl antibody

An enzyme linked immunosorbent assay (ELISA) was used.[29] A 50-μl volume of GAl solution (10μl per ml of 0.05% deoxycholate-PBS) was added to each well of Linbro E.I.A. 96-well flat-bottom microtitration plates (Flow Lab Inc, Maclean, Virginia, U.S.A.) and the plates were stored at 4°C overnight. After coating the plate with GAl in this way, sera serially diluted in 0.05% Twen 20 (v/v)-2% BSA (w/v)-PBS (dilution buffer) was added to each well. After incubation at room temperature for two hours, each well was washed three times with 0.05% Tween 20 (v/v)-PBS (washing buffer). A 50-μl volume of peroxidase-labelled anti-human IgG (Cappel Lab, Inc., U.S.A.) diluted 1:200 in dilution buffer was added to each well and the plate was incubated at room temperature for one hour. Excess conjugate was washed out and 160 μl of enzyme substrate solution was added; the latter was composed of 10 volumes of an aqueous solution containing 2,2'-azino-di (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (15 mg/ml), 1,000 volumes of citric acid monohydrate solution (10.5 mg/ml) adjusted to pH 3.9-4.1 with 5N-NaOH and 33 volumes of 35% hydrogen peroxide. After incubation at room temperature for 15 minutes, the reaction was stopped with the addition of 160 μl of stop solution, composed of 100 volumes of solution A (0.347 ml of 50% HF and 0.2 ml of 3N-NaOH in 100 ml of distilled water) and 1 volume of another solution B (2.92 g of EDTA and 1.6 g of NaOH in 100 ml of distilled water). The absorbance at 414 nm of the content of each well was measured against distilled water as a blank. As a control for each serum, we measured the absorbance of uncoated wells to which were added with patient's serum diluted 1:10. The antibody titre was determined by the end-point of the serum dilution giving an enzyme activity higher than that of each control.

Preparation of the IgG fraction from SLE patients' sera

Each patient's serum was applied to a Protein A-Sepharose CL-4B affinity column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). After elution of IgG bound to the column by 10 bed volumes of 1M – acetic acid, the eluate was dialysed, concentrated by AMICON-15 filtration to the same volume as the original serum and used as the IgG fraction.

Absorption of anti-GAl antibody

Anti-GAl antibody was absorbed from the sera or their IgG fraction by incubating the samples overnight at 4°C in microtitration plates coated with GAl.

Cytotoxicity of the IgG fraction of patients' sera and rabbit anti-GAl serum against cells of neural origin

Cultured cells which originated from a human neuroblastoma (IMR-32), a human glioblastoma (118 MGC) and a human oligoden-

droglioma (KG-1) were dislodged with a rubber policeman, dissociated with a Pasteur pipette and washed once with PBS. The cells were suspended in PBS at a concentration of $4x10^6$/ml. A 25 μl aliquot of each cell suspension was mixed with 50 μl of each patient's serum, IgG fraction or with rabbit anti-GAl serum diluted 1:10 in PBS. After incubation at room temperature for 30 minutes, 50 μl of fresh rabbit serum diluted 1:10 in PBS was added as a source of complement and then incubated at 37°C for 30 minutes. The reaction was stopped in ice bath. The 100 μl of 0.5% trypan blue-0.85% saline solution was added to each tube and the percentage of dead cells was calculated under a microscope. Dead cells in controls amounted to less than 10 per cent in all experiments. The cytotoxicities of the sera and their IgG fractions from which anti-GA1 antibody was eliminated were also examined.

Detection of GAl on the surface of neural cells by the indirect immunofluorescence method

By the above technique, $5x10^5$ cultured cells were suspended in PBS and pelleted by centrifugation (150xg for five mintues). A 50-μl volume of rabbit anti-GAl serum diluted 1:10 in PBS was added to each pellet and incubated at room temperature for 45 minutes. Cells were washed twice in PBS, and 50 μl of FITC-labelled goat anti-rabbit IgG (Cappel Lab, Inc., U.S.A.) diluted 1:5 in PBS was added and incubated at 4°C for 30 minutes. After again washing twice with PBS, another pellet was made by centrifugation. The pellet was dispersed in 50μl of 50% glycerine-PBS with a Pasteur pipette, and was put on a glass slide and observed under a fluorescence-microscope. As controls, pre-immune rabbit serum, rabbit anti-GAl serum after elimination of anti-GAl antibody and rabbit anti-BSA antibody were also used as reagents.

RESULTS

Anti-GAl antibody titres of the sera from patients with SLE

The anti-GAl antibody titres of sera from 30 patients, including 20 cases with CNS-SLE, were examined. Anti-GAl antibody titres of 1:40 or above were detected in 55 per cent of the patients with CNS-SLE. In all of the sera from the SLE patients without CNS complications and from 20 healthy adult, anti-GAl antibody titres were 1:20 or less (Table 1).

The anti-GMl antibody titres of sera from 24 SLE patients, including 14 cases with CNS-SLE, were also examined. In all of the sera from the SLE patients with or without CNS complications, anti-GMl antibody titres were 1:20 or less (Table 2).

Anti-GAl antibody titres and the CNS complications of SLE

In the observation of two cases of CNS-SLE, the exacerbation of CNS symptoms was accompanied by the elevation of the anti-GAl antibody titre (Fig. 1).

The correlation between anti-GAl antibody titres and their cytotoxicities against the cultured neural cells of the sera from SLE patients or rabbit anti-GAl serum

The sera of CNS-SLE patients having high titres of anti-GAl antibody and rabbit anti-GAl serum were strongly cytotoxic to IMR-32 cells. On the other hand, the sera from SLE patients without CNS complications and from healthy adults showed no cytotoxicity (Fig. 2). However, neither anti-GAl antibody-rich sera of CNS-SLE patients nor rabbit anti-GAl serum showed cytotoxicity to 118 MGC cells or KG-1 cells (data not shown).

The cytotoxicity against IMR-32 cells of the sera of SLE patients and rabbit anti-GAl serum after elimination of anti-GAl antibody

After the absorption of anti-GAl

Table 1 Anti-GA_1 antibody titre in the sera of 30 patients with SLE

	Cases	Anti-GA_1 antibody titre				Total positive %
		< x 20	x 20	x 40	x 80 <	
SLE with CNS involvement	20	3	6	3 (15.0%)	8 (40.0%)	55.0
SLE without CNS involvement	10	8	2	–	–	0.0
Healthy adult	20	20	–	–	–	0.0

Table 2 Anti-GM_1 antibody titre in the sera of 24 patients with SLE

	Cases	anti-GM_1 antibody titre				Total positive %
		< x 20	x 20	x 40	x 80 <	
SLE with CNS involvement	14	13	1	–	–	0.0
SLE without CNS involvement	10	8	2	–	–	0.0
Healthy adult	20	20	–	–	–	0.0

antibody, rabbit anti-GAl serum lost completely its cytotoxicity against IMR-32 cells. The sera of CNS-SLE patients lost most of the activity in that regard, although slight cytotoxicity remained (Fig. 3).

Cytotoxicity against IMR-32 cells of the CNS-SLE serum IgG fraction

The IgG fractions were separated from the anti-GAl antibody-rich sera of CNS-SLE patients. The serum IgG fraction had as much cytotoxicity against IMR-32 cells as the original sera (Fig. 4).

The cytotoxicity against IMR-32 cells of the CNS-SLE serum IgG fraction, from which anti-GAl antibody had been absorbed, did not show any cytotoxicity against IMR-32 cells (Fig. 5).

Staining of neural cells for GAl antigen by immunofluorescence

Almost all the IMR-32 cells were fluorescence-stained on their cell surface membrane after reaction with rabbit anti-GAl serum (data not shown). However, no fluorescence was detected on the cell membrane of 118 MGC cells nor KG-1 cells. When the rabbit preimmune serum or rabbit anti-GAl serum with the anti-GA1 antibody removed by absorption, were used as reagents, no fluorescence was detected on the surface of these three cells lines.

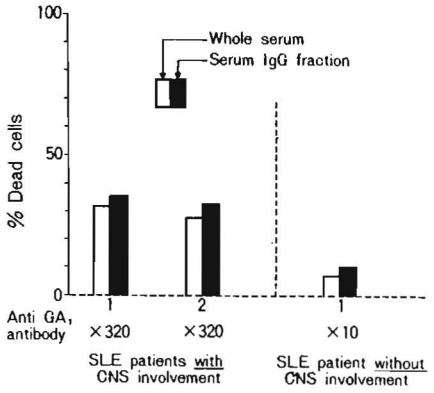

Fig. 4 Comparison of cytotoxicity against IMR-32 cells between whole serum and serum IgG fraction of SLE patients with and without CNS involvement

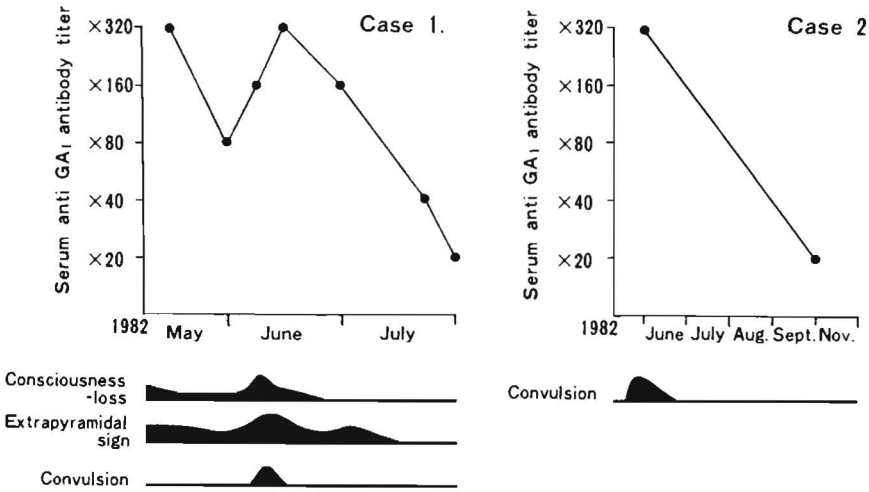

Fig. 1 Clinical course and anti-GA_1 antibody titre in 2 CNS-SLE patients.

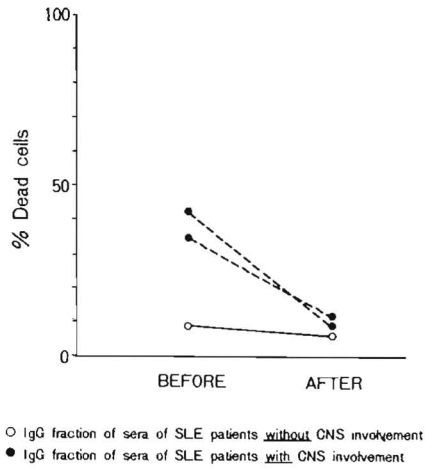

Fig. 5 Alteration of cytotoxicity of IgG fraction against IMR-32 cells after elimination of anti-GA_1 antibody

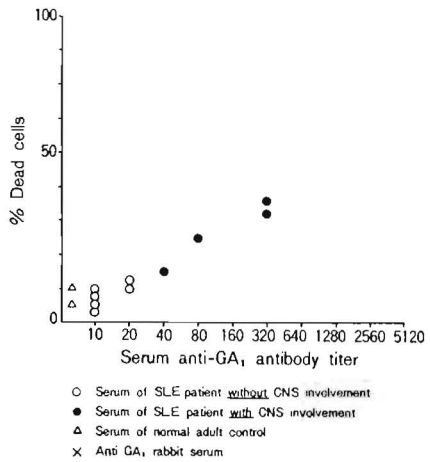

Fig. 2 Relationship between cytotoxicity of sera against IMR-32 cells and serum anti-GA_1 antibody titre

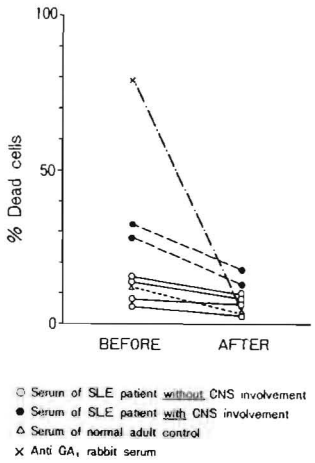

Fig. 3 Alteration of cytotoxicity of sera against IMR-32 cells after elimination of anti-GA_1 antibody

DISCUSSION

It was shown in this study that the sera of CNS-SLE patients had cytotoxic activity against IMR-32 cells (a cell line derived from a human neuroblastoma) and that the activity was reduced after the elimination of anti-GAl antibody from the sera. These results suggest that serum anti-GAl antibody of CNS-SLE patients has a role to play in the cytotoxicity. In contrast to rabbit anti-GAl serum, which lost its cytotoxicity completely after the absorption of anti-GAl antibody, the sera of SLE patients retained activity to some extent after the absorption of anti-GAl antibody. This indicates that some unknown neurocytotoxic factors other than anti-GAl antibody could be present in the sera of SLE patients. Because anti-GAl antibody had been reported to be mainly of the IgG class,[11] we exmained the cytotoxicity of the IgG fractions of the sera. A large proportion of the IgG neurocytotoxic antibodies in CNS-SLE sera was determined to be anti-GAl antibody in that the original sera and their IgG fractions showed almost the same cytotoxicity; IgG fractions with the anti-GA1 antibody removed were no longer cytotoxic against IMR-32 cells. Bluestein *et al* reported that 75 per cent of SLE sera had cytotoxic activity to SK-N-SH cells, a line also of human neuroblastomal origin, and that this cytotoxicity derived from IgG and/or IgM antibodies.[7] Our results were consistent with their report. It should be emphasised that, in the present study, one of the most important antigens reacting with antineuronal antibodies in the SLE sera was demonstrated to be an unique chemical substance, GAl. The SLE sera were not cytotoxic against 118 MGC cells and KG-1 cells probably because of the absence of GAl on their cell membrane. Using as targets three cell lines of neural origin (SK-N-SH, LA-N-1 and IMR-32) and two of glial origin (A-172 and U-118 MG), Bluestein *et al* demonstrated that 40 per cent of the SLE sera were cytotoxic to all five cell lines and 25 per cent reacted against at least one neural and one glial cell line, while 15 per cent had antineural but not anti-glial reactivity.[8] The differences between their conclusions and ours may arise from the different cell lines, sera and/or assays used.

IMR-32 cells were determined to have GAl on their surface, because they were fluorescein-stained by rabbit anti-GAl serum but not by pre-immune rabbit serum or rabbit anti-GAl serum with the anti-GAl antibody removed by absorption. The unequivocal identification of the antigenic substance of these cells as GAl requires further biochemical analysis. Our preliminary biochemical analysis using thin-layer chromatography suggested that the substance was truly GA1 (data not shown). On the other hand, 118 MGC cells and KG-1 cells showed no immunofluorescence and therefore, the absence of GAl on these cells was suggested.

Anti-GAl antibody would be cytotoxic only to GAl-positive cells among the various nerve tissue cells. In order for the circulating anti-GAl antibody to be pathogenetic to CNS tissues, destruction of the blood-brain barrier would be required to allow the antibody to enter the CNS so as to bind directly with brain tissues. In this regard, we demonstrated anti-GAl antibody in the CSF of two cases of CNS-SLE patients (in preparation). Bluestein *et al* similarly found that CSF from CNS-SLE patients had elevated IgG antineuronal activity.[13] As previously mentioned, it was reported that antineuronal antibody (IgG or IgA) was bound directly to brain tissue in one autopsied CNS-SLE case.[16]

In terms of correlation between antineuronal antibody and CNS symptoms of SLE, some authors have reported affirmateively[3,9,10] while others negatively.[17] This is probably due to the fact that antineuronal antibodies are heterogeneous. In our investigation, the exacerbation of CNS symptoms was accompanied by the elevation of the titre of serum anti-GAl antibody. These results indicated that anti-GA1 antibody titre in the CNS-SLE sera could be a good indicator of its CNS involvement.[19] Hirano *et al* reported that anti-GAl antibody decreased concurrently with periods of convulsive disorders and the titres recovered some time later.[11] This discrepancy between their results and ours probably arises from the difference in the assays used.

Recently, elevation of anti-GAl antibody was demonstrated also in the sera of patients with Neuro-Behcet's disease and AIDS (acquired immune deficiency syndrome.)[30] Only low titre of anti-GAl antibody in the sera of neurological diseases other than CNS-SLE and autoimmune diseases was detected in our studies (in preparation). Anti-GM1 antibody was not found in the sera of our CNS-SLE patients; antibodies to asialo GM2 and to galactocerebroside were reported not to exist in the sera of CNS-SLE patients.[11] Therefore, the marked elevation of anti-GAl antibody titre in the sera was rather characteristic of CNS-SLE.

GAl is known to be an antigen shared by immunocytes and nervous tissues. Sera of rabbits immunised with mouse brain homogenate have marked anti-Natural Killer (NK) activity and contain high titres of anti-GAl antibody. In the presence of complement, anti-GAl antibody inhibits mouse NK activity and this anti-NK activity is eliminated after absorption with mouse brain homogenate or GAl.[31-33] These findings indicate that, in mice, GAl is common to NK cells and nervous tissues. GAl was reported to exist on the surface of suppressor T cells as well as NK cells in mice,[34] but it has not been demonstrated to be on the surface of guinea pig or human NK cells. In rats, GAl was demonstrated on

the surface of thymocytes, peripheral T cells, granulocytes and macrophages.[35] The distribution of GA1 thus seems quite different from species to species. A small amount of GA1 is derived from human brain tissue, but the distribution of GA1 in nervous tissues is not yet understood. The existence and the distribution of GA1 on the surface of human lymphocytes have been in dispute. Normally, human lymphocytes are thought not to have GA1 on the surface, but lymphocytes from thymoma patients with myasthenia gravis or acute lymphoblastic leukaemia cells are reported to have GA1 on their surface.[36] Shinomiya *et al* reported that Con A-induced suppressor activity was inhibited by anti-GA1 antibody in the presence of complement,[37] presumably indicating that suppressor T cells induced by Con A possessed some substance which reacted with anti-GA1 antibody.

Although the distribution of GA1 and the effects of anti-GA1 antibody on the host are thought to be quite different among humans conpared with other species, GA1 seems to be an antigen common to both immunocytes and nervous tissues in various animals. The definition of the effects of anti-GA1 antibody may lead to great progress in clarifying the pathogenesis of neuroimmunological disorders.

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