Neurocytotoxic antibodies in serum of patients with systemic lupus erythematosus

(human cells/neuroblastoma/glioblastoma/lymphocytotoxic antibodies)

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Communicated by J. Edwin Seegmiller, May 8, 1978

ABSTRACT  Sera from patients with systemic lupus erythematosus (SLE) have been tested for antibody to a human neuronal cell line, SK-N-SH, derived from a metastatic neuroblastoma. With a complement-dependent 51Cr-release cytotoxicity assay, 75% of SLE sera had antineuronal activity mediated by IgM antibody. Most of the sera containing this IgM neurocytotoxic antibody were also cytotoxic to the human glial cell lines A-172 and U-118MG. The sera did not mediate complement-dependent 51Cr release when tested against normal human fibroblasts or peripheral blood lymphocytes. IgG antineuronal antibody was detected in 17% of SLE sera by an antibody-dependent, cell-mediated cytotoxicity assay with SK-N-SH cells as targets.

The relationship of IgM and IgG antineuronal antibodies to the antilymphocyte antibodies present in SLE sera was evaluated by a series of crossabsorption experiments using SK-N-SH cells to remove neuronal antibodies and WI-L2 (human lymphoblasts) to remove antilymphocyte antibodies. Most of the complement-dependent neurocytotoxicity was not removed by multiple lymphoblast absorptions, although the WI-L2 cells readily removed lymphocytotoxic activity as assayed on normal lymphocytes. Absorption with SK-N-SH cells removed most, but not all, of the lymphocytotoxic antibody. Thus, although lymphocytotoxic antibodies reactive with membrane antigens shared by lymphocytes and brain may constitute a subset of the antibodies to neural cells, most of the antineuronal activity in SLE serum is directed at other cell surface antigens expressed on neuronal and glial cells. Should they gain access to the brain, these antibodies have the potential to produce neuropathology, but their presence in the neuronal system of patients with the neuropsychiatric manifestations of SLE is yet to be documented.

Lymphocyte membrane-reactive antibodies found in the serum of patients with systemic lupus erythematosus (SLE) also bind to central nervous system (CNS) tissue. Absorption of SLE sera with homogenized human brain reduces their lymphocytotoxic capacity more than 90%. Lymphocytotoxic activity has been eluted from the brain absorption and shown to be IgM with enhanced lymphocyte reactivity in the cold, characteristic of SLE lymphocytotoxic antibody (2, 3). IgG antibodies with leukocyte membrane reactivity also bind to CNS tissue. Brain absorption of SLE sera depletes their ability to suppress “killer” cell function in the antibody-dependent, cell-mediated cytotoxicity (ADCC) assay (4).

An analysis of the relationship between clinical manifestations of SLE neuronal cell lines and their circulating lymphocytotoxic antibodies revealed that the mean titer of lymphocytotoxic antibody was significantly higher in patients with CNS disease than in those with renal, serosal, or dermal manifestations (1). The observations of brain-reactive antibodies in the serum of patients with SLE, coupled with greater amounts of those antibodies in patients with CNS involvement, suggest the possibility of an autoantibody-mediated pathogenesis. Antibody reacting with neuronal membranes may interfere with their function, thereby producing some of the neuropsychiatric manifestations of SLE.

A prerequisite to attributing CNS manifestations to antineuronal activity is the demonstration that SLE patients have antibodies that react with neurons. To that end the tissue culture cell line, SK-N-SH, was obtained and used as a target in neurocytotoxicity assays. SK-N-SH was derived from a human neuroblastoma; its growth, biochemical, and cytogenetic properties confirmed its neuronal nature (5). The data to be presented demonstrates that SLE sera contain both IgM and IgG antibodies that react with human neuronal cells and mediate their lysis in the presence of complement (the IgM antibodies) or in an ADCC assay (the IgG antibodies). Most of the IgM neurocytotoxic antibody does not react with human lymphocytes, but the IgG antibodies for the most part are directed at antigens shared by neurons and lymphocytes. Some of the CNS manifestations of SLE may be produced by neuronal membrane-reactive antibodies, should they gain access to central nervous tissue.

METHODS

Clinical Material. Sera were obtained from 40 patients with SLE and 14 with rheumatoid arthritis. All of the patients fulfilled the American Rheumatism Association diagnostic criteria (6, 7). Sera were stored at −20°C without preservatives and were heated at 56°C for 45 min prior to use.

Cell Lines. Human neuroblastoma line SK-N-SH (5) and glioblastoma lines A-172 (8) and U-118 MG (9) were provided by J. Biedler and J. Fogh from the Sloan Kettering Institute for Cancer Research. Human lymphoblast cell line, WI-L2 (10), and fibroblast line 594 from normal human skin were grown for us in the laboratory of J. E. Seegmiller of this department. WI-L2 was grown in suspension culture; all other cell lines were grown in monolayer culture. All of the cell lines were grown in Eagle’s minimal essential medium supplemented with 15% fetal calf serum.

Complement-Dependent Cytotoxicity. SK-N-SH, A-172, or U-118 MG cells grown to confluence in microtiter tray wells and labeled with 51Cr were incubated with 25 μl of a 1:2 dilution of test serum and 25 μl of SK-N-SH absorbed fresh rabbit serum as a source of complement. After 1 hr at 37°C, 50 μl of medium was added to each well and a 50-μl aliquot was removed for gamma counting. Cytotoxicity is expressed as the percent 51Cr specifically released by the test serum. The assay we used for measuring complement-dependent lymphocytotoxicity has been described (1).

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CNS, central nervous system; SLE, systemic lupus erythematosus.
significant serum supernatant killing centrifugation as target lymphocytes of the present obtained origin.

ADCC. Cr-labeled confluent monolayers of the cell lines were incubated at 37°C for 30 min with 50 μl of test serum diluted 1:2. The unbound serum was removed and 5 x 10^5 normal human peripheral blood lymphocytes obtained by density centrifugation (11) were added in 0.1 ml of medium. After 3 hr at 37°C, the Cr released into the supernatant was determined. Cytotoxicity is expressed as the % Cr specifically released by the test serum. Antilymphocyte activity was similarly detected with 4 x 10^5 Cr-labeled peripheral blood lymphocytes as target cells and 2 x 10^5 unlabeled peripheral blood lymphocytes as effectors.

Absorptions. SLE sera at 1:2 dilution in Eagle’s medium were absorbed with SK-N-SH or WI-L2 cells at a density of 4 x 10^6 cells/ml at 4°C for 1 hr. The cells were then removed by centrifugation at 600 x g for 10 min at 4°C. An aliquot of the serum supernatant was removed for testing, fresh cells were added to the same density, and the process was repeated for the number of absorptions indicated.

RESULTS
Approximately three-fourths (78%) of SLE sera exhibited complement-dependent killing of SK-N-SH neuroblastoma cells (Table 1). The 25 cytotoxic sera ranged from 15 to 78%. No significant Cr release was observed with any of the SLE sera in the absence of complement. Sera from patients with rheumatoid arthritis were much less cytotoxic to SK-N-SH cells, but 3 of 16 were cytotoxic at 16, 18 and 23% Cr release, respectively.

The ability of SLE sera to mediate complement-dependent cytotoxicity to cell line SK-N-SH was compared with their cytotoxic capacities against three other cell lines of human origin (Table 2). Twenty SLE sera were tested. These sera were obtained subsequent to the completion of the experiment presented in Table 1, but 12 of the 20 patients providing these sera also provided sera used in the earlier experiment. The mean percent cytotoxicity of these 20 sera to SK-N-SH cells (38.4%) and the percent of cytotoxic sera (75%) did not differ significantly from the original 32 serum samples tested. The SLE sera were also cytotoxic to the two glial lines. Thirteen of the 20 (65%) sera were cytotoxic to A-172. Those 13 sera were all included in the 15 sera cytotoxic to the neuronal cell lines. The mean percent Cr release produced by the SLE sera against cell line U-118 MG was considerably lower than that observed against lines A-172 or SK-N-SH. However, 60% (12 of 20) of the SLE sera were cytotoxic to U-118 MG. Those 12 sera were also included in the 15 SLE sera cytotoxic to SK-N-SH. Only 11 of the 12 sera toxic to U-118 MG, however, were cytotoxic to A-172. As a group, the 20 SLE sera were not more cytotoxic than the normal human sera to the human fibroblast line. Only 2 of 20 SLE sera produced greater than 10% Cr release against the skin fibroblasts, at 17 and 20%, respectively.

To help identify the factor in SLE serum responsible for the neurotoxicity, we fractionated four of the most potent cytotoxic sera over Sephadex G-200. In each case virtually all of the cytotoxic activity to SK-N-SH cells was recovered in peak 1, the protein peak eluting with the void volume. The immunoglobulin concentrations and the cytotoxic activity of the Sephadex fractions of one of the SLE sera are presented in Table 3. Those results suggested that IgM antibody was responsible for neurotoxicity. To confirm this, we mixed each of the cytotoxic Sephadex fractions, peak 1 and 2, with rabbit antiserum to human immunoglobulins and tested the mixture for cytotoxicity against SK-N-SH. Peak 1 was diluted 1:5 with buffer and peak 2 with 1:25 with buffer.
human serum was assayed. Sera AF and CM were concentrated before it was mixed with the rabbit serum to bring its percent cytotoxicity down to the level of the undiluted peak 2. The neurocytotoxicity in both peaks 1 and 2 was completely inhibited with antisera specific for the heavy chain of human IgM (Table 4). Anti-IgG had only slight inhibitory activity against both fractions. Thus, the factor responsible for complement-dependent neurocytotoxicity in SLE serum is IgM antibody.

The complement-dependent cytotoxicity assay is not a sensitive test for IgG antibody. Therefore, we used an ADCC assay to test for IgG antibody to SK-N-SH cells. As a group, SLE sera were significantly more cytotoxic than normal or rheumatoid arthritis sera ($P < 0.02$) to the neuronal cells (Table 5). The upper limit of normal in this assay, calculated as the mean of the normal human serum samples plus 2 standard deviations, was 15% cytotoxicity. Only 4 of the 24 SLE samples induced a percent $^{51}$Cr release in the ADCC assay greater than the upper limits of normal. None of the normal human sera or the sera from patients with rheumatoid arthritis were cytotoxic by that criterion.

The four cytotoxic SLE sera were fractionated by Sephadex G-200 chromatography and the fractions were tested for activity in the ADCC assay. The pattern was the same in all four sera. The results for two of the SLE sera and a normal human serum run as a control are presented in Table 6. The cytotoxic activity in both of the SLE sera, AF and CM, was recovered in peak 2, which contains the bulk of the IgG antibody.

In order to investigate the relationship between the IgM and IgG antineuronal antibodies and the IgM and IgG antilymphocyte antibodies known to be present in SLE sera, we performed a series of absorption experiments to determine whether different antibody populations are responsible for antineuronal and antilymphocyte activities or whether the same antibodies react with both cell types. Antilymphocyte antibody was removed by absorption with the human lymphoblast cell line WI-L2. The lymphoid cells readily removed the ADCC-de

### Table 5. Antibody-dependent cell-mediated neurocytotoxicity induced by SLE sera

<table>
<thead>
<tr>
<th>Source</th>
<th>No.</th>
<th>$%$ cytotoxicity, mean ± SEM</th>
<th>No. cytotoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>24</td>
<td>$11.9 \pm 1.0$</td>
<td>4</td>
</tr>
<tr>
<td>RA</td>
<td>8</td>
<td>$6.0 \pm 1.1$</td>
<td>0</td>
</tr>
<tr>
<td>NHS</td>
<td>17</td>
<td>$8.6 \pm 0.8$</td>
<td>0</td>
</tr>
</tbody>
</table>

$^{51}$Cr-labeled SK-N-SH cells were preincubated with the test sera, the unbound serum was then washed off, and the cells were used as targets in the ADCC assay. Sera were considered to be cytotoxic if they induced greater than 15% cytotoxicity (NHS mean plus 2 standard deviations). RA, rheumatoid arthritis; NHS, normal human serum.

### Table 6. Antibody-dependent cell-mediated neurocytotoxicity induced by SLE serum fractions

<table>
<thead>
<tr>
<th>Source</th>
<th>Serum</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AF</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>CM</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>NHS</td>
<td>5.6</td>
</tr>
</tbody>
</table>

The test sera were fractionated over Sephadex G-200 as described in the legend of Table 3. The unfractionated sera and their pooled and concentrated fractions (peak 1 at void volume, peak 2 containing the 7S IgG, and peak 3 containing albumin, etc.) were tested for their ability to prime $^{51}$Cr-labeled SK-N-SH cells to serve as targets in the ADCC assay. Sera AF and CM were from SLE patients. Normal human serum was obtained from a healthy donor. NT, not tested.

### Table 7. Lymphoblast absorption of antineuronal ADCC activity in SLE serum

<table>
<thead>
<tr>
<th>Cells</th>
<th>No.</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>0</td>
<td>16.9</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>2</td>
<td>7.8</td>
</tr>
<tr>
<td>WI-L2</td>
<td>4</td>
<td>4.1</td>
</tr>
<tr>
<td>WI-L2</td>
<td>6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Serum CM was absorbed sequentially with WI-L2 cells at a density of $4 \times 10^6$ cells per ml. Serum aliquots removed after the number of absorptions indicated were tested against normal human peripheral blood lymphocytes (PBL) and SK-N-SH cells by the ADCC assays. Two tested antineuronal activity from two of three sera tested (Table 7). The antineuronal activity in the third serum was not reduced by six sequential absorptions with WI-L2 cells. Thus, IgG antineuronal activity in SLE sera may be directed at antigens shared by neurons and lymphocytes or at neuronal antigens that are not expressed on lymphoid cells.

The relationship between the IgM antineuronal and antilymphocyte activity was also assessed in similar absorption experiments. As shown in Fig. 1 left, absorption with the lymphoblasts readily removed the lymphocytotoxicity against normal peripheral blood lymphocytes. The antineuronal activity in the lymphoblast-absorbed serum was partially diminished after one absorption, but continued absorptions with WI-L2 cells did not further reduce the neurocytotoxic activity. Although the results for only the first two absorptions are shown in Fig. 1, four sequential absorptions have been carried out, and the neurocytotoxicity remains over 70%. Three other sera tested in this way gave very similar results, with a partial depletion of antineuronal activity with the first WI-L2 absorptions and then no further removal of antineuronal antibody.

In contrast to the resistance of IgM antineuronal antibody to absorption with lymphoblasts, IgM antilymphocyte antibody is substantially removed by absorption with SK-N-SH neuronal cells (Fig. 1 right). In the example shown, antilymphocyte activity was decreased to 20% of its preabsorption activity after two absorptions with the neuronal cells. An additional two absorptions with the same cell line did not further reduce the lymphocytotoxicity. A similar pattern was observed with the

![FIG. 1. A comparison of the effects of absorption of SLE serum with lymphoblasts and neuroblasts on the complement-dependent cytotoxic activity against lymphocytes and neuronal cells. Serum AF was absorbed sequentially with (Left) WI-L2 or (Right) SK-N-SH cells at a density of $4 \times 10^6$ cells per ml. Serum aliquots removed after the number of absorptions indicated were tested against $^{51}$Cr-labeled SK-N-SH for neurocytotoxicity ($\bullet$) and against normal human peripheral blood lymphocytes for lymphocytotoxicity ($\Delta$ - - $\Delta$). The data are expressed as the residual cytotoxicity as a percent of the preabsorption activity.](attachment:image_url)
three other lupus sera absorbed with SK-N-SH cells, each of which had a residual lymphocytotoxic activity in the range of 10–25% of the preabsorption level. The bulk of the antilymphocyte antibody, therefore, appears to be directed at antigens present on both neuronal and lymphoid cells. However, a subset of the IgM lymphocytotoxic antibodies appears to be directed at an antigen(s) present on peripheral blood lymphocytes but not neuroblasts.

**DISCUSSION**

The cell line SK-N-SH has been used as the target in assays for antineuronal antibody in the serum of patients with SLE. The neuronal origin of these cells has been confirmed by two observations: they contain high levels of dopamine β-hydroxylase activity, an enzyme distributed only in sympathetic nervous tissue (5), and they retain the action potential Na⁺ ionophore, as demonstrated by their response to the plant alkaloid veratridine (12, 13). Veratridine-dependent 22Na⁺ uptake is a characteristic of electrically excitable membranes (14). Thus, SK-N-SH retains differentiated features that enable its classification as a neuronal cell line.

Sera from the majority of patients with SLE have IgM antibody cytotoxic to SK-N-SH cells. The cytotoxic activity is not limited to cell lines of neuronal origin. Most of the sera reactive with SK-N-SH were also cytotoxic to cells of glial origin. Cell lines A-172 and U-118MG were both derived from human glioblastoma (8, 9). Both are unresponsive to veratridine (unpublished observations) and, thus, lack the action potential Na⁺ ionophore. Eighty percent of the neurocytotoxic SLE sera were also cytotoxic to A-172 and U-118MG. No sera were identified that killed the glial cells but not the neuronal cells. Differing sensitivities of the target cells to antibody-mediated complement-dependent cytotoxicity may account for the failure to lyse the glial cells by a few of the sera that were cytotoxic to SK-N-SH. The significantly lower mean percent cytotoxicity of SLE sera against cell line U-118MG suggests that that cell line may be less susceptible to complement-dependent lysis. Alternatively, since most of the sera that failed to kill the glial cells were not at the lower end of the cytotoxicity range against the neuronal cells, SLE sera may contain antibodies to several different cell-membrane antigens, some of which are shared by neuronal and glial cells and some of which are not. Analysis of membrane antigens on rodent neuronal and glial cell lines has revealed several determinants shared in various combinations (15).

Circulating antilymphocyte antibodies are commonly found in the serum of patients with SLE. Absorption of these sera with human brain tissue effectively depletes both IgM and IgG antilymphocyte immunoglobulin (1, 4). The relationship of the lymphocyte-reactive antibodies to IgM and IgG antineuronal antibodies was studied with a series of crossabsorption experiments by using an established human lymphoblast cell line to remove antibodies to lymphocytes and the SK-N-SH cell line to remove antibodies directed against itself. Although the antilymphocyte antibody present in SLE sera is not able to mediate complement-dependent 51Cr release, lymphocytotoxic antibody is readily detected by a dye-exclusion microcytotoxicity assay (2, 16). The lymphoblast cell line, WI-L2, is B-cell derived. However, most lymphocytotoxic antibodies present in SLE sera are directed at antigenic determinants that are shared by both T and B cells (1, 3), and thus it is not surprising that absorption with WI-L2 removed all of the lymphocytotoxic activity detectable when normal human peripheral blood lymphocytes were used as targets.

The absorption experiments demonstrate that most of the IgM antineuronal antibody is not directed at cell-surface antigens present on lymphoid cell membranes. In contrast, almost all of the lymphocytotoxic activity can be removed by absorption with the neuroblastoma cells. A small amount of residual lymphocytotoxic activity remains after extensive neuronal absorption, indicating the presence of a subset of lymphocyte reactive antibodies that are not directed at antigens shared with the neuroblastoma cells. Thus, at least three distinct populations of cytotoxic IgM antibodies have been identified: (a) those directed at antigens on neuronal cells but not on lymphoid cells; (b) a smaller population directed at antigens present on both neuronal and lymphoid cells; and (c) some reactive with lymphocyte surface antigens that are not found on neuronal cells.

The studies described in this report demonstrate the existence of circulating antibodies reactive with membrane antigens on cells derived from central nervous tissue. The existence of such antibodies had been suggested by the previous observation from this laboratory that antilymphocyte antibodies could be removed from the serum by absorption with brain tissue (1). That observation led to the speculation that the interaction of those antibodies with cells in the CNS may be responsible for some of the neuropsychiatric manifestations seen in patients with SLE. Autoantibodies to CNS components have been demonstrated in a number of neurologic disorders, including multiple sclerosis (17–20), ataxia telangiectasia (21), and chorea (22).

Implication of antineuronal antibodies in the pathogenesis of the neuropsychiatric manifestations of SLE requires (a) a demonstration that such antibodies exist in patients with SLE, (b) that those antibodies gain access to the CNS, and (c) that the antibodies can interact with neuronal function. This study satisfies requirement (a) by demonstrating the existence of antineuronal antibodies. It also partially satisfies requirement (c) by demonstrating that the antibodies can mediate neurocytotoxicity. Studies in animals have demonstrated alterations in behavior and neuropathological changes after injection of antibodies to CNS components into the brain. Antiserum to homogenized brain tissue placed into the ventricles of normal rabbits produced foci of cerebral edema and meningeal inflammation which were accompanied by motor discoordination and epileptic seizures, with epileptiform activity on electroencephalography (23). Epileptiform activity was also produced in rats after injection of small amounts of antiserum to brain ganggliosides into the sensorimotor cortex (24), and antibodies to synaptosomal plasma membranes introduced into the rat brain caused a significant impairment in memory (25).

The mechanisms by which antibodies to CNS components induce the neuropathologic changes have not been elucidated. The neuronal antibodies found in SLE sera were detected by cytotoxicity assays. However, though they have cytotoxic potential, the antibodies could affect neuronal function by alternate mechanisms. Antiserum to cell-surface antigens can inhibit the responsiveness of cells to stimuli delivered at their membrane. This may occur via antibodies to cell-membrane receptors, such as the insulin receptor autoantibodies in some insulin-resistant diabetics (26) or the antibodies to acetylcholine receptors found in the circulation of patients with myasthenia gravis (27, 28). Alternatively, antibodies to cell-membrane antigens that are not part of receptor-combining sites may also prevent cell activation by external stimuli. In vivo studies with animal cells have demonstrated that mitogen-induced B-cell activation is blocked by antibody to immunoglobulin (29), antigen-induced T-cell proliferation is inhibited by antiserum to histocompatibility region antigens (30), and the induction of in vivo colony-forming units in bone marrow cells treated with colony stimulating factor can be inhibited by antibody to brain (31).
Although we have shown that antibodies capable of reacting with neuronal cells are present in the circulation of patients with SLE and that those antibodies can mediate neurocytotoxicity, it is yet to be demonstrated that those antibodies gain access to the CNS. Approximately 75% of patients have neuronal antibodies, but most studies suggest that only slightly more than half that percentage of patients with SLE have neuropsychiatric manifestations. The feature distinguishing those patients with CNS manifestations may be accessibility of the nervous tissue to the antibodies. Most of the neurocytotoxic antibody detected thus far is of the IgM class and would not be expected to cross the blood-brain barrier under normal circumstances. A lesion at that barrier, such as immune complex-mediated vascular injury, may allow the antibody to reach the CNS. In this regard, it is interesting to note the clinically recognized relationship between neuropsychiatric manifestations and vasculitis in other organ systems in patients with SLE (32).

The valuable technical assistance of Cheryl Ogden-Bell, Roberta Dupre, and Maria Cullen, and the excellent secretarial support by Deborah Ann Frank are gratefully acknowledged. I am grateful to Drs. June Biedler and Jorgen Fogh of the Sloan Kettering Cancer Research Institute, and Dr. J. E. Seegmiller of the University of California, San Diego, School of Medicine for supplying the tissue culture cell lines used in this study. These studies were supported in part by Research Grants AI-10931 and AM-14916, and Training Grant AM-07062 from the National Institutes of Health; Contract 76-57087 from the State of California Department of Health; and a grant from the Kroc Foundation.