Detection of Antilymphocyte Antibody with Two-Color Method in Systemic Lupus Erythematosus and Its Heterogeneous Specificities against Human T-Cell Subsets

KUNIO OKUDAIRA, HIDENORI NAKAI, TETSUO HAYAKAWA, TAKAMICHI KASHIWADO, KIYOKI TANIMOTO, YOSHIHICO HORUCHI, and TAKEO JIJU, Department of Internal Medicine and Physical Therapy, and the Transfusion Service, School of Medicine, University of Tokyo, Tokyo, Japan

ABSTRACT The two-color method originally described by Van Rood et al. (Van Rood, J. J., A. Van Leeuwen, and J. S. Ploen. 1976. Simultaneous detection of two cell populations by two-color fluorescence and application to the recognition of B-cell determinants. Nature (Lond.). 262: 795-797) for the typing of homologous leukocytic antibodies, D-region was used for the detection of antilymphocyte antibody (ALA) in systemic lupus erythematosus. In this method, surface immunoglobulin-bearing cells were identified with fluorescein isothiocyanate-labeled anti-immunoglobulin and nuclei of killed cells were stained with ethidium bromide. Therefore, cell type (T or B) of the target cells can be identified without fractionating them. ALA was detected in 87% of lupus sera and had a preferential reactivity with T cells. Its major immunoglobulin class was shown to be immunoglobulin (Ig)M.

The subspecificity of ALA was further analyzed using fractionated T-cell subsets as target cells. When T lymphocytes were separated into Fc receptor-bearing (Tγ) and lacking (Tγ(-)) cells, 64% of ALA showed preferential reactivity with Tγ cells and 14% with Tγ(-) cells. The remainder had no selective reactivity against Tγ or Tγ(-) cells. Tγ cells were shown to have suppressor activity, whereas Tγ(-) cells were indicated to contain helper cells. The above finding was in agreement with the observation that treatment of T cells with ALA that preferentially react with Tγ cells considerably enhanced immunoglobulin synthesis in vitro, whereas treatment of T cells with ALA reactive with Tγ(-) cells clearly suppressed the formation of immunoglobulins. Treatment of ALA with no selective reactivity showed variable effects on in vitro immunoglobulin synthesis.

These results indicate that ALA in lupus have heterogeneous specificities against human T-cell subsets.

INTRODUCTION

Antilymphocyte antibodies (ALA)1 are frequently detected in the sera from patients with systemic lupus erythematosus (SLE) (1, 2). The isotype of most ALA was shown to be immunoglobulin (Ig)M (3, 4). They are mainly reactive with T lymphocytes, and cold reactivity has been reported as one of their characteristic features (5, 6). The studies of natural thymocyte antibody in New Zealand Black mice and their F1 hybrids New Zealand Black/White (7–9) suggested a critical importance of ALA in the pathogenesis of SLE. The specificities of ALA against T-cell subsets in humans have not been established yet, and the pathogenetic role of ALA remains unclear. In the present study, the two-color fluorescent method, which was originally devised for the typing of homologous leukocytic antibodies, D-region antigen by Van Rood et al. (10), was introduced to explore the specificities of ALA. This method enabled us to identify the target cell type (T or B) of ALA without fraction-

1 Abbreviations used in this paper: ALA, antilymphocyte antibody; Con A, concanavalin A; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; PBL, peripheral blood lymphocytes; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.
ating the cells. The result indicated that ALA in the lupus sera were preferentially reactive with T cells.

Attempts were made to further define the specificity of ALA using fractionated Ty cells and Ty(−) cells (11). The effect of the treatment of T cells with different specificities of ALA on in vitro immunoglobulin synthesis was also examined.

METHODS

Subjects. Serum samples were obtained from 71 patients with SLE, 14 with rheumatoid arthritis (RA), 17 with Sjögren’s syndrome, and 19 with progressive systemic sclerosis in our clinic. 25 synovial fluid samples were aspirated from patients with RA and used in the same manner as serum samples.

Lymphocyte preparation. Peripheral blood lymphocytes (PBL) were isolated from the heparinized blood of normal individuals by differential centrifugation with Ficoll-Conray solution (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (12, 13). Mononuclear cells on the interface were aspirated, washed three times with Hanks’ balanced salt solution (HBSS), and used as lymphocyte preparations for the following experiments.

Procedures of two-color fluorescent method in the lymphocyte cytoxicity test. The method described by Van Rood et al. (10) was essentially followed. 100 µl of 6 × 10⁶ cells/ml of separated lymphocytes were incubated with an equal volume of fluorescein isothiocyanate (FITC)-labeled rabbit anti-human immunoglobulin purchased from Behring-Werke AG Marburg/Lahn, West Germany, at 37°C for 10 min. They were washed three times with HBSS and the cell number was adjusted to 8 × 10⁶/ml. 0.05 µl each of the lymphocyte suspensions and undiluted heat-inactivated serum samples were incubated in a Kissmeyer tray at 15°C for 30 min. Then, 2.5 µl of rabbit complement were added to each well in the tray. The mixtures were further incubated at 15°C for 150 min. At the end of the incubation, 0.5 µl of 2 µg/ml of ethidium bromide was added to the tray, and the stainings by FITC and ethidium bromide were observed under fluorescein microscopy (Vanox, AHB-LB, Olympus, Tokyo).

Concanavalin A (Con A) treatment of SLE serum. Immunoglobulins other than IgG were removed from SLE sera by using Con A (14, 15). 1 ml of serum samples was incubated with 10 mg of Con A coupled to Sepharose beads (Pharmacia Fine Chemicals) at 37°C for 60 min. The supernates were separated from the beads by centrifugation at 1,500 rpm for 10 min and assayed for the cytotoxicity test. Con A treatment of the serum resulted in maximal reduction of IgM to undetectable levels and IgA to ≤40% of original serum value as assessed by single radial immunodiffusion, whereas the level of IgG remained unchanged (15).

Separation of Ty and Ty(−) lymphocytes. Sheep erythrocytes were treated with neuraminidase and E rosettes were formed with normal PBL by the standard method (16). E rosette-forming cells were separated from nonrosette-forming cells by the differential centrifugation on Ficoll-Conray solution as described above. In this procedure E rosette-forming cells sank to the bottom and sheep erythrocytes contained in the E rosette-forming fractions were lysed with Tris-ammonium chloride buffer at 37°C for 20 min (17). The cell suspensions were washed three times with HBSS and used as T cells. Non-E rosette-forming cells were recovered from the interface and used as B cells. Ox erythrocytes were sensitized at 37°C for 20 min with rabbit IgG antibody separated from IgM antibody by Sephadex G 200 gel-filtration (Pharmacia Fine Chemicals). The sensitized ox erythrocytes (EAOx) were washed three times with HBSS. EAOx and separated T cell preparations were incubated at 37°C for 30 min to form EA rosettes. The reaction mixtures were applied to Ficoll-Conray solution and the rosette-forming cells and nonrosette-forming cells were separated in the same manner as described above. Ox erythrocytes in the EA rosette-forming fractions on the bottom were lysed with Tris-ammonium chloride buffer and used as Ty cells. The fractions at the interface were washed three times with HBSS and used as Ty(−) cells. These cell fractions were used as the targets of the lymphocyte microcytotoxicity test (18).

Treatment of T lymphocytes with SLE serum and complement. Equal volumes of 8 × 10⁶/ml of T-cell preparations and serial dilutions of heat-inactivated lupus sera were incubated at 15°C for 30 min. Fivefold volume of rabbit complement was added to the mixture and incubated further at 15°C for 150 min. The treated cells were washed three times with HBSS and used for in vitro immunoglobulin synthesis experiments.

In vitro immunoglobulin synthesis. 1 million PBL in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 300 µg/ml of l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.05 µg/ml of pokeweed mitogen 10 µg/ml (Grand Island Biological Co.) at 37°C in the presence of humidified 5% CO₂ atmosphere for 7 d in 13 × 10 mm plastic tubes (Falcon Labware, Div. Becton, Dickinson & Co. Oxnard, Calif.). After 7 d, the supernates were separated from the cells by centrifugation at 400 g for 10 min, and the levels of IgG and IgM in the supernates were measured.

Measurements of IgG and IgM levels in the culture supernates. The method of Gleich et al. (19) was used. 100 µl of culture supernates were mixed with an equal volume of 20 ng/ml of 125I-labeled human IgM in pH 7.5, 0.01 M phosphate buffer containing 1% of bovine serum albumin and 1:3,000 diluted rabbit anti-human IgG (Behring-Werke AG), and incubated at 4°C for 48 h. Then, 100 µl each of 1:6 diluted goat anti-rabbit IgG and 1:150 diluted normal rabbit serum were added to the mixtures and incubated at 4°C for an additional 24 h. After the reaction was completed the mixtures were centrifuged at 4,000 g for 30 min, and the supernates were separated from the precipitates. The radioactivities in the supernates and precipitates were counted in a gamma scintillation counter. Percent radioactivity of the precipitates was calculated, and the values of IgG concentration were figured out in comparison with the standard curve made from purified IgG preparations. The IgM concentrations in the culture supernates were measured in the same manner as IgG except for the use of 125I-labeled human IgM preparations and rabbit anti-human IgM antiserum.

RESULTS

A photograph of normal PBL stained with the two-color method is presented in Fig. 1. Surface immunoglobulins of a lymphocyte at the upper left were capped and stained with FITC-labeled anti-human immunoglobulin. The nucleus of it was stained with ethidium bromide indicating that it is a nonviable B cell. Another lymphocyte at the lower left showed positive staining of nucleus with ethidium bromide but lacked surface immunoglobulin staining. Thus, the cell could be recognized as a nonviable T cell. A lymphocyte at the
right bore a capped surface immunoglobulin but was not stained with ethidium bromide. This cell could be identified as a viable B cell. The result of this experiment illustrates the validity of the two-color method which informs both cell type and viability in the exploration of ALA in various connective tissue diseases.

Using this two-color method, the incidence of ALA was studied in various connective tissue diseases (Table I). In SLE, 62 of 71 cases (87.3%) were positive, and in 29 the cytotoxic activities exceeded 70%. In RA, 7 of 14 serum samples showed moderate ALA activity. In synovial fluid from patients with RA, only 12% were positive, and their titers were low. In Sjögren's syndrome and progressive systemic sclerosis, the incidences of ALA were 47.1% and 36.9% respectively. Their average activities were lower than those of SLE, but higher than those of RA.

The specificities against lymphocyte subpopulations were examined. All seven ALA-positive serum samples from lupus patients showed preferential cytotoxicity against T cells at 15°C (Fig. 2). At 37°C, T-cell dominant

**TABLE I**

*Incidence of ALA in Connective Tissue Diseases*

<table>
<thead>
<tr>
<th>Disease</th>
<th>(+) 0-30%</th>
<th>(++) 30-50%</th>
<th>(+++) 50-70%</th>
<th>(++++) 70%</th>
<th>Total positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>(71)†</td>
<td>9</td>
<td>14</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>RA (serum)</td>
<td>(14)</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>RA (SF)§</td>
<td>(25)</td>
<td>22</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>SjS¶</td>
<td>(17)</td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PSS¶</td>
<td>(19)</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

* The cytotoxic activity was graded as 0–30%, 30–50%, 50–70%, and >70%. The first group (<30%) was judged as negative and the others (>30%) were regarded to be positive.
† Numbers of samples in parentheses.
§ SF, synovial fluid.
¶ SjS, Sjögren's syndrome.
¶ PSS, progressive systemic sclerosis.
reactivities as well as cytotoxic activities against B cells were greatly but not completely diminished (data not shown in the figure).

The immunoglobulin class of ALA was studied. Eight ALA-positive SLE sera were treated with Con A. As shown in Fig. 3, Con A treatment markedly reduced their cytotoxic activities in all the samples. The results indicated that the major immunoglobulin class of ALA is IgM because all the immunoglobulins except IgG were removed by Con A and complement fixing cytotoxic antibody mainly belonged to the IgM or IgG class.

The specificities of ALA against human T-cell subsets were analyzed using IgG Fc receptor as a marker. Normal T-lymphocyte preparations were separated into IgG Fc receptor-positive (Ty) and negative (T\(\gamma(-)\)) populations. The cytotoxicity test was carried out against these populations. As illustrated in Fig. 4, their specificities were mainly classified into three groups. One group showed a preferential reactivity against Ty-enriched fractions. A typical example is presented in the upper panel of the figure. Another group was preferentially reactive with T\(\gamma(-)\) populations (middle panel). The third showed no preferential reactivity with either Ty or T\(\gamma(-)\) populations (bottom). Table II is a summary of the results in lupus sera. T\(\gamma\)-dominant type was the most frequent and 64.3% were included in this group. T\(\gamma(-)\)-dominant type had the lowest frequency (14.3%) and "no difference" type was seen in 21.3%.

To examine the effect of ALA on the T-cell function, preliminary experiments were carried out to establish a system in which immunoglobulins are synthesized in vitro. Normal peripheral blood B-lymphocyte fractions were co-cultured with normal Ty cells or T\(\gamma(-)\) cells in the presence of pokeweed mitogen. Results represented in Fig. 5 showed that Ty cells had suppressor activity, whereas T\(\gamma(-)\) cells enhanced immunoglobulin production considerably. The result indicated that suppressor T cells and helper T cells belong to Ty and T\(\gamma(-)\) cell populations, respectively. Normal T cell-depleted fraction alone did not produce a significant amount of immunoglobulin. Depletion of adherent cells contained in B-lymphocyte preparations resulted in considerable reduction of immunoglobulin synthesis in the co-culture experiment.

When normal T lymphocytes were pretreated with ALA reactive with Ty cells and rabbit complement and co-cultured with B lymphocytes, the synthesis of immunoglobulin was enhanced dose dependently. A representative example is presented in the upper panel of Fig. 6. In contrast, treatment of T cells with

| Ty dominant* | 18/28 | 64.3 |
| T\(\gamma(-)\) dominant† | 4/28 | 14.3 |
| No difference§ | 6/28 | 21.3 |

* ALA with specificity mainly directed to Ty cells.
† ALA with specificity mainly directed to T\(\gamma(-)\) cells.
§ ALA reactive with both Ty and T\(\gamma(-)\) cells.
Cytotoxic activity before and after Con A treatment. Eight lupus sera were treated with Con A to remove IgM and assayed for cytotoxic activity. Marked reductions of the cytotoxic activity were observed after Con A treatment in all cases.

**FIGURE 3**

ALA reactive with Ty(−) cells resulted in the suppression of immunoglobulin synthesis (middle panel). By using one of the "no difference" type ALA, immunoglobulin synthesis was also enhanced but the dose dependence was unclear (bottom panel). Samples of this type of ALA did not always enhance immunoglobulin synthesis and some showed suppression of synthesis.

**DISCUSSION**

The two-color method used in the present study is quite useful for the study of ALA. It is a simple method and does not require the fractionation of T and B cell for the identification of target lymphocytes. In the present study using this method, it was shown that specificity of ALA is essentially directed to T cells.
directed to T cells bearing IgG Fc receptor. Our experimental results (Fig. 5) and the finding of Moretta et al. (11) support the possibility that most suppressor T cells bear IgG Fc receptors although a recent report suggested that some suppressor T cells are derived from non-Ty cells (25). Because normal T-cell fraction treated with ALA of this group plus complement showed increased immunoglobulin synthesis, this type of ALA was considered mainly directed to suppressor T cells. The inhibition of suppressor T-cell activity by Ty-specific ALA is consistent with the observations that Con A-induced suppressor activity was diminished in SLE compared with normal healthy individuals in the production of antibodies and immunoglobulins (26–30) and in cellular immune responses (31). Furthermore, treatment of normal T cells with serum from SLE patients decreased the Con A-induced suppressor activity in mixed lymphocyte reaction (32). Suppressor T cell-specific ALA in SLE was reported recently (22–24). Reduced Ty cell numbers were described by several investigators (29, 33, 34). These findings are consistent with the present observation that Ty-specific ALA that impair suppressor T cells are frequently found in SLE.

ALA was cold reactive in nature, belonged largely to IgM isotype, and was frequently found in lupus patients. Those results are in agreement with the findings in earlier reports (3–6).

Studies on human T-cell subsets and the specificity of ALA are accumulating. Natural thymocytotoxic antibody in New Zealand Black or New Zealand Black/White F1 mice was demonstrated to have preferential reactivity with suppressor T cells (20). Peculiar ALA found in patients with juvenile rheumatoid arthritis were also reported to have specificity against suppressor T cells (21). Several reports suggested that ALA in lupus patients reacted mainly with suppressor T cells (22–24). However, reactivity of ALA with fractionated human T-cell subsets has never been studied.

The present study clearly demonstrates that ALA have at least two different specificities against human T-cell subsets. Specificity of one group of ALA was

**FIGURE 4** Cytotoxic activity of ALA in lupus sera against human T-cell subsets. Human T lymphocytes were fractionated into Ty-enriched and Ty-depleted fractions and used as target cells for the cytotoxicity test. The specificities were classified into three groups. One group had specificity essentially directed to Ty cells, and another had specificity directed mainly to Ty(-) cells. The rest were reactive with both Ty and Ty(-) cells.

**FIGURE 5** Function of Ty and Ty(-) cells in in vitro immunoglobulin synthesis. Normal PBL were fractionated into T- and B-cell fractions. T-cell fraction was subdivided into Ty-enriched and Ty-depleted fractions. Those T-cell subsets were co-cultured with B cells and IgG and IgM concentrations in the culture supernates were measured after 7 d. The Ty-enriched fraction had suppressive activity, whereas the Ty-depleted fraction increased in helper activity for immunoglobulin synthesis.
The second group of ALA in the present study had a preferential reactivity with \( Ty(\neg) \) cells. This group of ALA appears to be reactive with helper T cells because in vitro immunoglobulin synthesis was suppressed by the T lymphocytes pretreated with this group of ALA. This, in our knowledge, is the first report of helper T cell-specific ALA in lupus sera. However, their pathogenetic role is unknown. ALA of helper T cell-specific type may be merely the result of multiple autoantibody productions in SLE with no relevant biological significance. This speculation awaits further evaluation.

The nature of ALA of the nonselective group is largely unknown. This group may represent the mixture of ALA of \( Ty \)-dominant and \( Ty(\neg) \)-dominant specificities. Alternatively, ALA in this group may have an independent specificity against an unknown antigen expressed on both \( Ty \) and \( Ty(\neg) \) cells. One example of ALA of this type presented in Fig. 6 shows an augmenting effect on immunoglobulin synthesis. The reason for the augmentation is unclear. Although no difference in the cytotoxic activity against \( Ty \) and \( Ty(\neg) \) cells was detected, suppressor T cells might be more susceptible to this particular ALA than helper T cells.

Collectively, the data in the present study indicate that ALA in SLE have heterogeneous specificities against human T-cell subsets. Revillard et al. (35) recently reported the presence of anti-\( \beta_2 \)-microglobulin lymphocytotoxic autoantibody in SLE. It may be reasonable to speculate that anti-\( \beta_2 \)-microglobulin reacts with both T and B cells because \( \beta_2 \)-microglobulins are present on both T and B cells. Therefore, it is unlikely that anti-\( \beta_2 \)-microglobulin has a direct relationship with ALA directed to T cells studied in the present report.

ACKNOWLEDGMENTS

The authors express their thanks to Miss Akiko Shimizu for her excellent technical assistance and to Miss Megumi Fukazawa for the preparation of the manuscript.

This work was supported by a research grant for “Autoimmune Disease” from the Ministry of Health and Welfare, Japan.

REFERENCES


