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Abstract

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KEYWORDS: antilymphocyte antibody, systemic lupus erythematosus, purification

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PURIFICATION OF ANTILYMPHOCYTE ANTIBODY (ALA) FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)-IMMUNOABSORPTION AND ELUTION

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Abstract. Purification of antilymphocyte antibody (ALA) from patients with systemic lupus erythematosus (SLE) was achieved by immunoabsorption and elution. Human tonsil cells or thymocytes were used as absorbents. Complement dependent microcytotoxicity tests showed that, in comparison to the parent sera, the eluate from tonsil cells was eight times, and that from thymocytes four times, more active. Antinuclear activity was eliminated by elution. The ALA was almost entirely IgM, IgG being involved in only a few cases. IgA lacked cytotoxic activity. ALA was directed at both T- and B-cell surface determinants, which suggests that, in SLE, it has a heterogeneous biological composition.

Key words: antilymphocyte antibody, systemic lupus erythematosus, purification

Antilymphocyte antibody (ALA) is an autoantibody which is complement dependent (1) and cytotoxic at lower temperatures (2). It is thought to be mainly IgM (3), with its activity directed at surface determinants on T-cells (4). In systemic lupus erythematosus (SLE), however, affinity for both T- and B-cell determinants has been demonstrated (3), moreover, ALA activity is present on all immunoglobulin classes (5). This suggests that the ALA is actually more heterogeneous than previously thought. IgG antibodies, for example, are occasionally lymphocytotoxic and may react selectively with PBL (peripheral blood lymphocyte) from different individuals (6). In these cases, the IgG antibody appears to be more specific for B-cells. This discrepancy in SLE-ALA is probably related, in part, to the presence of various other autoantibodies in the circulation.

In view of the importance of ALA in the pathogenesis of SLE, therefore, the present study was an attempt at its purification by immunoabsorption and elution using either human thymocytes or tonsil cells as absorbents. Purified antibodies from which antinuclear antibodies could be excluded were examined

for their biological characteristics. The results and a discussion of their significance are presented.

MATERIALS AND METHODS

Clinical material. The patients studied all had SLE as diagnosed by the criteria of the American Rheumatism Association. Pooled sera for absorption and elution experiments had strong lymphocytotoxic activity (1 : 4 or greater by Terasaki's microcytotoxicity test), antinuclear antibodies, a low CH50 level (less than 10), increased native DNA binding activity, and positive anti-RNP and anti-Sm antibodies. A mixture of unequal amounts of serum from six healthy adult donors was used as a control serum source (normal human sera, NHS). Sera were stored at −20°C without preservatives. Before use, all sera were heated at 56°C for 45 min.

Cytotoxicity assay. Cytotoxicity in the SLE sera was determined by microdroplet dye exclusion cytotoxicity assay using a slightly modified Terasaki's method (2). Target lymphocytes were obtained from the peripheral blood of nine healthy adult volunteer donors using the Conray-Ficoll sedimentation technique. The lymphocyte rich population was washed twice with Hanks' balanced salt solution (Hanks' BSS). One thousand cells from each donor were mixed with 1 μl of test serum in a well of a microtiter tray (Falcon plastic microassay tray). This was incubated for 30 min at 4°C, 5 μl of rabbit serum (prescreened for lack of cytotoxicity to human lymphocytes) was added to each well and the incubation continued for an additional 3 h at 15°C. Eosin (3 μl of a 5% aqueous solution) was then added, followed within a few minutes by the addition of 8 μl of 40% formaldehyde adjusted to pH 7.0. One hundred cells were examined in each well and the number of cells stained with eosin counted. The mean percentage of dead cells in triplicate assays was expressed as percent cytotoxicity. A cytotoxicity titer greater than 20% was classified as positive.

Preparation and iodination of xenogenic antilymphocyte serum. Modification of the method of Levey and Medawar (7) was used for the preparation of xenogeneic antilymphocyte antibody (X-ALS). Briefly, 1×10^9 cells of BALB/c mouse thymocytes were injected into an albino rabbit intravenously on each of two occasions four teen days apart. Blood was collected seven days after the second injection. Serum was stored at −20°C before use.

The IgG fraction of X-ALS was obtained by DEAE cellulose column ion-exchange chromatography. Crude X-ALS globulin was separated out by the use of 40% saturated ammonium sulphate. The crude globulin was applied to a DEAE cellulose column equilibrated with 0.0175 M phosphate buffer to pH 8.0 and eluted at a 20–30 ml/h flow rate. Approximately four ml fractions were collected and monitored by extinction at 280 nm. The IgG fraction thus obtained was concentrated and iodinated with ^{125}I (sodium iodide, Code No IMS30, Radiochemical Centre, Amersham, England) using the Chloramin-T method of McConahey & Dixon (8). The IgG fraction of sera from three patients with SLE was isolated similarly.

Absorption and elution. Tonsils were obtained at operation. Thymus tissue known to be free of pathology was obtained at post-mortem examination within 24 h of death. After removal of attached connective tissue, the tissue was gently disrupted with forceps in Hanks' BSS and passed through a 100 μ copper wire mesh covered by eight layers of cotton gauge to remove debris. Washing with Hanks' BSS was performed twice, then an equal volume of 0.2% formaldehyde was added. The mixture was incubated for 15 min to fix the cell membranes. Heat inactivated SLE sera, 25 ml diluted two-fold in phosphate buffered saline (PBS), was added to each cell pellet (2×10^{10} cells). This was followed by overnight incubation at 4°C. After removal of the serum supernatant, the pelleted cells used for the first serum absorption were washed at least 4 times by centrifugation in more than 100 volumes of PBS maintained at 4°C. The pelleted cells were resuspended in 20 ml PBS and incubated in a water bath for 60 min at 37°C. This process was repeated. After centrifugation, the supernatant eluate was collected and passed through a millipore filter membrane (pore size 0.6 μ) to remove visible particles. This gave approximately 150 fold concentration. Eluate from the thymus and tonsil tissue was expressed as Thy-e and Ton-e respectively.

As a different elution system, elution was carried out as described above for 15 min using 0.12 M sodium citrate buffer at a pH of 3.0 (9).

Miscellaneous. Antinuclear antibodies were detected by an indirect immunofluorescent technique using nuclei from human peripheral blood neutrophils. Complement activity was assessed by 50% hemolysis of sheep erythrocytes (CH50). Native DNA binding activity was measured by Actinomycin D-DNA labelled with ^{3}H-thymidine. Anti-RNP and Sm antibody were assessed by both precipitin reactions and passive hemagglutination tests using extracts of young albino rabbit thymus as the antigen. Immunoglobulin in the eluate was detected by the Ouchterlony test. The binding ability of ALA to surface determinants on lymphocyte membrane was demonstrated by an indirect membrane immunofluorescent technique. Protein concentration was measured by the Folin-Lowry method, together with radial immunodiffusion.

RESULTS

Potent lymphocytotoxic antibody was effectively recovered from the surface of lymphocytes. To achieve this, preliminary experiments were required.

Elimination of non-cell bound antibody. BALB/c mouse thymocytes (1×10^{8}) were incubated with excess ^{125}I-labelled IgG fractionated from anti-BALB/c mouse thymocyte serum. The incubation was performed at room temperature for 30 min. After removal of the supernatant by centrifugation, pelleted cells were washed 5 times (using 4 ml Hanks' BSS solution each time). Each supernatant was collected and the ^{125}I-IgG was counted. After 4 washings, the cpm reached a level equivalent to the background, indicating that morethan four washings were necessary to remove non-cell bound free IgG. In this elution

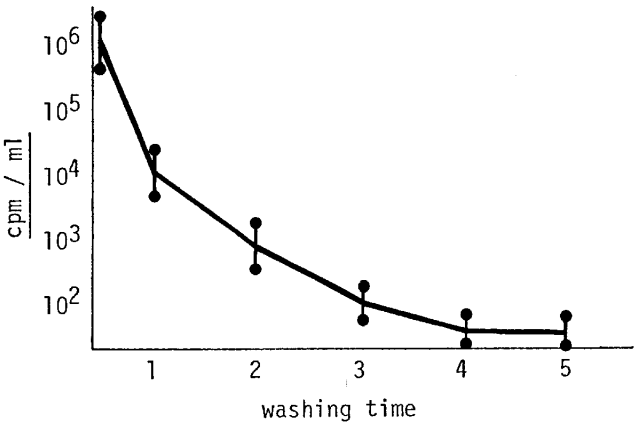

Fig. 1. Determination of washing time necessary for complete removal of non cell-bound antibody. BALB/c mouse thymocytes, 1×10^8, were incubated with excess ^{125}I-labelled anti-thymocyte antibody (IgG fraction). In each washing, 4 ml PBS were used.

experiment, therefore, 4–5 washings were required (Fig. 1).

Effects of fixative on cell surface antigenic determinants. Repeated washings of absorbent cells were essential for elution with the batch-method (more efficient than the column method). Therefore, conditions which did not affect the antigenic determinants on absorbent cells were studied. BALB/c mouse thymocytes as absorbent cells were fixed for 5 min with different concentrations of either formaldehyde or glutaraldehyde. Rabbit anti-BALB/c thymocyte serum was then applied to these fixed cells and the result studied by indirect membrane immunofluorescence.

TABLE 1. EFFECT OF FIXATIVE ON CELL SURFACE ANTIGENIC DETERMINANTS

Treated with	Conc. of fixative (%)	Antibody	Intensity of immuno-fluorescent staining
Formaldehyde[a]	4.0	Rabbit-ALS	+
〃	1.0	〃	++
〃	0.1	〃	+++
〃	0.01	〃	+++
Glutaraldehyde[b]	0.25	Goat-ALS	+
〃	0.125	〃	+
〃	0.625	〃	+
〃	0.1	Goat normal serum	−

a Best staining was obtained when 0.1% formaldehyde was fixative. Increase in the concentration of fixative resulted in reduced fluorescent staining, indicating damage of antigenic determinants. *b* Brownish autofluorescence observed when glutaraldehyde was used.

The results are shown in Table 1. A formaldehyde concentration of 0.1% gave the best results for preservation of antigenic determinants. Cells fixed with this concentration were resistant to repeated washings. In contrast, 0.01% formaldehyde preserved antigenic determinants but did not fix the cell membrane effectively for repeated washings. Glutaraldehyde gave less fluorescence than formaldehyde, indicating damage of the antigenic determinants.

Effective citric acid buffer pH. The most effective citric acid buffer pH for elution of cell-bound antibody was 3.0 (Table 2). Before elution, immunofluorescent staining of absorbing cells was strongly positive indicating heavy binding of X-ALS. After elution, surface staining was negative when citric buffer under pH 4 0 was used. The best recovery was obtained with a citric acid buffer of pH 3.0.

TABLE 2. ANTIBODY ELUTION EFFICIENCY OF CITRIC ACID BUFFER WITH CHANGES IN pH

Citric acid buffer (pH)	Titre of eluate[a]	Immunoflurescent staining of absorbing cells[b]	
		Before elution	After elution
4.5	× 4	++	+
4.0	×16	++	−
3.5	×32	++	−
3.0	×64	++	−
2.5	× 8	++	−

a Elution continued for 15 min with gentle agitation at room temperature. *b* BALB/c mouse thymocytes were used as absorbents and indirect membrane immunofluorescent technique was used to detect cell-bound antibody before and after elution. The pH 3.0 citric buffer removed almost all cell-bound antibodies without loss of lymphocytotoxic activities.

Characterization of eluates from tonsil cells and thymocytes. Cell bound protein with antilymphocytic activity was eluted at 37°C. A description of these eluates is presented in Table 3. The protein concentration of the parent serum was 81 mg/ml. Its lymphocytotoxic titre was 1 : 4. ANF was observed as a diffuse staining pattern. Eluate (Ton-e) obtained from formaldehyde-fixed tonsil cells contained 32 mg/ml of protein. Its cytotoxicity had increased to 1 : 24 compared with the same protein concentration of the parent serum. This eluate, however, was shown to be contaminated with ANF (diffuse staining). To eliminate ANF activity, Ton-e was further absorbed and eluted using viable tonsil cells in the same fashion. The resultant eluate, Ton-E, had not lost its lymphocytotoxic activity but had lost its ANF activity. Other eluates (Thy-e from fixed thymocytes; Thy-E from unfixed viable thymocytes) showed the same tendency as Ton-e and Ton-E (Table 3). The cytotoxic activity of Thy-E was somewhat

TABLE 3. CHARACTERIZATION OF ELUATES FROM TONSIL CELLS AND THYMOCYTES

	Eluted at	Protein conc. (mg/ml)	Cytotoxicity	ANF
Parent serum	/	81	4(+)	++
Ton-e[a]	37°C	32	24(+)	++
Ton-E[b]	37°C	/	24(+)	−
Thy-e[a]	37°C	20	16(+)	++
Thy-E[b]	37°C	/	12(±)	−
Ton-e & Thy-e at pH 3.0		Undetectable	—	−

a Eluates from fixed cells. *b* Eluates from viable cells. To eliminate ANF, Ton-e and Thy-e were further absorbed with viable absorbent cells and then eluted at 37°C. Ton-E contains 3.7 mg/ml of IgG and Thy-E contains 3.3mg/ml of IgG. The protein concentration of IgM and IgA in eluates was undetectable by radial immunodiffusion.

less than that of Ton-E. Eluates with low citric acid buffer pH failed to show any antilymphocytotoxic activity. Both eluates contained IgG, IgM, and IgA antibodies but not IgD or IgE. C1q, C3a and C4 were not detected.

Cytotoxic activity of ALA. Table 4 shows the cytotoxic activity of each immunoglobulin at different temperatures. When Ton-E was absorbed with anti-immunoglobulin as shown in Table 4, anti-IgM caused complete loss of lymphocytotoxic activity. In contrast, anti-IgG failed to have such an effect.

TABLE 4. LYMPHOCYTOTOXIC ACTIVITIES OF ANTILYMPHOCYTE ANTIBODY (ALA)

ALA	Absorbed with	Cytotoxicity 15°C	Cytotoxicity 37°C
Ton-E	unabsorbed	++	±
〃	anti-IgG	++	−
〃	anti-IgM	−	−
〃	anti-IgA	±	±
〃	anti-IgM+IgG	−	/
〃	anti-IgG+IgA	+	/
〃	anti-IgM+IgA	−	/
〃	anti-IgG+IgM+IgA	−	/
SLE sera		++	−
SLE sera+B cells		+	−
SLE-IgG[a]		+	−
SLE-IgG+B cells[b]		+	±
SLE-IgA[a]		−	−

a IgG and IgA fractions of sera from three patients with ALA separated by DEAE-cellulose chromatography. The IgG fraction from only one case tested showed lymphocytotoxicity.

b B cells were fractionated by removing SRBC-rosetting T cell by density gradient.

The single case of three SLE-IgG fractions separated by DEAE-column chromatograhy, however, showed lymphocytotoxic activity, suggesting that the IgG might play a role by destroying lymphocytes in restricted cases. IgA was not cytotoxic.

TABLE 5. REDUCED CYTOTOXIC ACTIVITY BY REPEATED ABSORPTIONS WITH EITHER TONSIL CELLS OR THYMOCYTES

Absorbed with	Cytotoxicity
None	++
Tonsil cells[a]	−
Thymocytes[a]	+

a Sera absorbed two times with an equal volume of cell at 37°C for 60 min, followed by 4°C for 120 min.

The ALA target lymphocytes were also studied. B-cells were fractionated by eliminating the SRBC rosetting T-cells on density gradient, resulting in more than 80% purity. SLE sera with positive lymphocytotoxic activity were cytotoxic to B-cells as well as to T-cells. One case of fractionated IgG also showed cytotoxicity against B-cell. This lymphocytotoxic activity occurred at lower temperatures (15°C) but not at 37°C.

To define the T- and B-cell specificities of SLE-ALA, sera with lymphocytotoxic activity were extensively absorbed either with tonsil cells or thymocytes. Tonsil cell absorption removed completely the cytotoxicity whereas thymocyte absorption only reduced their activity somewhat. These data suggest the presence of activity directed at non-T-cells.

DISCUSSION

Absorption with tonsil cells removes most of the lymphocytotoxic activity from SLE sera. With this as the basic idea for the present experiments, highly purified ALA from SLE sera was obtained from the surface of tonsil cells or thymocytes by immunoabsorption and elution. ALA was easily eluted by warming at 37°C.

Bluestein *et al.* (10) have reported that absorbed lymphocytotoxic antibodies were eluted from brain tissue absorbents at 37°C. The cytotoxicity of the brain eluates was blocked by antibodies to human IgM but not by anti-IgG. Furthermore, the unabsorbed SLE sera, brain-absorbed sera and brain eluate were equally cytotoxic to T- and B-cells fractionated from normal human peripheral blood lymphocytes. In the present study, therefore, the nature of SLE sera and the eluate from lymphocyte surface have been examined. SLE sera retained residual lymphocytotoxicity that could not be removed by additional thymocyte

absorptions. In contrast, tonsil cells (rich in B-cells) could eliminate the lymphocytotoxic activity. Most lymphocytotoxic antibody, therefore, is directed at antigenic determinants on both T- and B-cells. This was confirmed by cytotoxic activity against B-cells fractionated from normal human peripheral blood. Failure of the removal of lymphocytotoxic activity by absorption with thymocytes may indicate differences in the antigenicity of thymocytes and PBL.

Lymphocytotoxic antibodies in SLE sera and the naturally occurring thymocytotoxic autoantibody (NTA) of NZB mice share several characteristics. Both have increased binding affinity in the cold and both are directed at antigenic determinants found on brain cells and lymphocytes. NTA in NZB mice is specific for T-cells. This seems to be somewhat different from ALA in SLE. As studies using purified T- and B-cell populations from normal individuals have shown, lymophocytotoxicity in SLE serum is effective against B- and T-cells.

It is generally agreed that NTA is an IgM antibody. In SLE, however, anti-lymphocytotoxic antibody seems to be heterogeneous. Winfield *et al.* (6) have reported that sera from patients with SLE frequently contained IgM and IgG antibodies for lymphocyte surface determinants. The IgM antibodies exhibited broad reactivity with B- and T-lymphocytes. IgG antibodies, however, reacted with PBL from different individuals and appeared to be more specific for B-cells. The eluate in our experiments contained IgG, IgM and IgA antibodies. Absorption with anti-immunoglobulins revealed that most of the lymphocytotoxic activity was on the IgM antibodies. It was hardly detected for IgG and IgA. It is possible, therefore, that nonspecific immunoglobulins were carried over in our elution experiment. This possibility, however, is negated by the fact that loss of ANF occurred in the eluate. Furthermore, the IgG antibodies were relatively non-cytotoxic in conventional complement dependent microcytotoxicity assays. Among IgG antibodies fractionated from three patients with SLE, however, IgG antibody from one patient showed cytotoxicity to PBL and B-cells fractionated from PBL. IgG antibodies, therefore, seem to occur less frequently in the sera of patients with SLE. This agrees with the findings of Winfield *et al.* (6). This group also reported that IgG antibodies were often warm-reactive, binding to lymphocytes equally well at 4°C and at 37°C; moreover, they exhibited relatively restricted antigenic specificities compared to IgM antibodies. IgA antibodies which moderately eluted from lymphocyte absorbents did not show any antilymphocytotoxic activity at 37°C or at 15°C. The reason for this remains unclear.

For the absorption and elution techniques, cells used as the absorbent must be resistant to repeated washings. This was obtained by fixing the cells with a low concentration of formaldehyde (9). This did not affect the antigenic determinants on the cell surface. These fixed cells, however, were not effective in

eliminating antinuclear antibody, probably because of increased cell membrane permeability. Almost complete removal of ANF was obtained using viable lymphocytes as the absorbent. The ALA activities in SLE as described herein seem to be more heterogeneous than the NTA in NZB mice. A further investigation, therefore, is expected to clarify the differences of both antibodies. This might solve the pathogenesis of SLE.

REFERENCES

1. Mittal, K. K., Rossen, R. D., Sharp, J. T., Lidsky, M. D. and Butler, W. T.: Lymphocyte cytotoxic antibodies in systemic lupus erythematosus. *Nature (Lond.)* **225**, 1255-1256, 1970.
2. Terasaki, P. I. and McCleland, J. D.: Microdroplet assay of human serum cytotoxins. *Nature* **204**, 998, 1964.
3. Winfield, J. B., Wincheser, R. J., Wernet, P., Fu, S. M. and Kunkel, H. G.: Nature of cold reactive antibodies to lymphocyte surface determinants in systemic lupus erythematosus. *Arthritis Rheum.* **18**, 1-8, 1975.
4. Michlmayr, G, Pathouli, C., Huber, C. H. and Huber, H.: Antibodies for T lymphocytes in systemic lupus erythematosus. *Clin. Exp. Immunol.* **24**, 18, 1976.
5. Searles, R. P., Husby, G. and Messner, R. P.: Immunoglobulin class of antilymphocyte antibodies in systemic lupus erythematosus patients and their families. *Acta Pathol. Microbiol. Scand.* (C) **85**, 463-468, 1977.
6. Winfield, J. B., Lobo, P. I. and Singer, A.: Significance of anti-lymphocyte antibodies in systemic lupus erythematosus. *Arthritis Rheum.* **21**, s219-s221, 1978.
7. Levey, R. H. and Medawar, P. B.: Nature and mode of action of antilymphocytic antiserum. *Proc. Natl. Acad. Sci. (U. S. A.)* **56**, 1130-1135, 1966a.
8. McConahey, P. J. and Dixon, F. J.: A method of trace iodination of proteins for immunoglobulin, *Int. Arch. Allergy*, **29**, 158-193, 1966.
9. Yamana, S., Read, R. S. D., Davis, D. J. and Nairn, R. C.: Antilymphocyte antibody purified by immunoabsorption and elution. *Clin. Exp. Immunol.* **16**, 367-374, 1974.
10. Bluestein, H. D. and Zvaifler, N. J.: Brain reactive lymphocytotoxic antibody in the serum of patients with systemic lupus erythematosus. *J. Clin. Invest.* **57**, 509, 1976.