Lymphocyte destruction by antibody-dependent cellular cytotoxicity mediated in vitro by antibodies in serum from patients with systemic lupus erythematosus

MAREN L. MAHOWALD AND AGUSTIN P. DALMASSO

From the Departments of Medicine and of Laboratory Medicine and Pathology, University of Minnesota Medical School and the Minneapolis VA Medical Center, Minneapolis, USA

SUMMARY Sera from patients with systemic lupus erythematosus (SLE) were tested for the presence of IgG antilymphocyte antibodies that are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) of peripheral blood lymphocytes (PBL) from normal donors. The effector cells employed were PBL autologous with the target PBL. Positive ADCC responses were obtained with serum from 5 SLE patients with severe active disease. It is possible that ADCC is a mechanism by which IgG antilymphocyte antibodies in patients with SLE may mediate in-vivo lymphocytolysis.

Antilymphocyte antibodies (ALA) occur spontaneously as autoantibodies in many patients with rheumatic diseases and are most frequently found in patients with systemic lupus erythematosus (SLE).^{1–6} They may also appear after alloimmunisation by multiple pregnancies, multiple blood transfusions, and organ transplantation and with certain infections.^{3, 7–9} Although certain properties of these antibodies have been established, their pathogenetic significance remains unclear.

ALA are usually detected by complement lysis in a microcytotoxicity test,³ indirect immunofluorescence,^{10–12} inhibition of certain immune functions such as response to allogeneic stimulation and phytohaemagglutinin,^{13, 14} inhibition of generation of suppressor cell activity by concanavalin A,^{15–17} and by binding of ¹²⁵I-protein A.¹⁸ The microcytotoxicity test depends on antibody binding at low temperature followed by complement activation to damage the target cell. Most of these antibodies are IgM with increased reactivity at cold temperatures. The antibodies which interfere with lymphocyte functions are found in both the IgG and IgM classes and react optimally at 37°C. Immunofluorescent studies have also demonstrated both IgG and IgM ALA. A higher incidence of warm reactive IgG ALA was found by preventing capping and shedding of antibody, as previously described.⁶ ¹²⁵I-labelled protein A detects

ALA in patients with active SLE by combining to the Fc portion of lymphocyte-bound IgG.¹⁸

One potential mechanism for lymphocyte damage produced by ALA which has not been extensively studied is antibody-dependent cellular cytotoxicity (ADCC). ADCC is a cytolytic process in which target cells generally coated with an IgG antibody are lysed by nonimmune killer cells. The Fab fragment of the antibody reacts specifically with the target cell and the Fc fragment attaches to the Fc receptor of the effector killer cell.^{19, 20} Since this mechanism reacts optimally at 37°C, it could be operative in vivo. Therefore, since IgG is the principal antibody which mediates ADCC, and SLE patients have IgG ALA, we propose that this mechanism might produce self-destruction of peripheral blood lymphocytes in vivo.

Materials and methods

PREPARATION OF PERIPHERAL BLOOD LYMPHOCYTES (PBL)

Heparinised human venous blood was diluted 1:2 with phosphate buffered saline (PBS), layered over Ficoll-Hypaque, and centrifuged at 600 g for 40 minutes at room temperature.²¹ The interface layer of PBL was removed and washed twice with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (medium). For target cells 20 × 10⁶ PBL were mixed with 150 μCi Na⁵¹CrO₄ (New England Nuclear, NEZ 030, 200–500 Ci/g) at 37°C for one hour and washed twice with medium. The remaining PBL were kept at room

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Correspondence to Dr Maren L. Mahowald, Rheumatology Section, Minneapolis VA Medical Center, 54th Street & 48th Avenue S, Minneapolis, MN 55417, USA.

temperature until used as effector cells. Aggregated IgG was prepared according to a published procedure²² and used at 5–10 mg/ml for incubation with effector cells (*vide infra*).

PREPARATION OF SERUM

Blood was allowed to clot at room temperature and the serum was separated by centrifugation, incubated at 56°C for one hour to inactivate complement, and stored in aliquots at –40°C until used. To obtain the IgG fraction, serum was subjected to diethylaminoethyl (DEAE) cellulose chromatography with 0.01 M sodium phosphate buffer, pH 7.3. To prepare the IgM fraction serum was chromatographed on Sephadex G-200 (Pharmacia) employing 0.2 M Tris HCl–0.5 M NaCl buffer, pH 8.0. On analysis by double immunodiffusion the IgG fraction contained no IgM or IgA, and the IgM fraction was free of IgG and IgA. Rabbit antihuman lymphocyte antiserum (RaALA) was produced by immunisation with 4–6 weekly intravenous injections of about 10⁸ PBL from different normal donors.

ADCC ASSAY

This assay was based on methods used to detect ALA in serum from multiply transfused patients.²³ Labeled target cells (1 × 10⁴) were placed in 12 × 75 polystyrene tubes (Falcon 2054) and incubated with dilutions of patient and control serum for 30 minutes at 37°C and 30 minutes at 20°C to permit antibody coating. The cells were then washed with 20 volumes of medium to remove excess serum which could contain immune complexes or antieffector cell ALA.²⁴ Effector cells were added to give target-effector cell ratios of 1:50 to 1:100 in a final volume of 0.2 ml. The reaction mixture was placed on a slowly rocking platform in a humidified incubator with 5% CO₂ for 1.5 hours at 37°C. After addition of 0.8 ml of ice-cold PBS the tubes were centrifuged at 700 g for 10 minutes, and 0.5 ml of the supernatant fluid was removed. The radioactivity in the supernatant fluid and in the sedimented cells was measured in a gamma counter. Samples were prepared in triplicate and the data reported as the mean percentage lysis (± standard deviation), which was calculated as follows:

$$\% \text{ lysis} = \frac{\text{counts in supernatant fluid}}{\text{total counts}} \times 100.$$

Total counts comprised the sum of 'pellet' and 'supernatant' counts. 'Spontaneous release' of ⁵¹Cr from target cells was determined by measuring the counts in the supernatant fluid from a mixture of effector and target cells without antibody. Release of ⁵¹Cr from target cells alone, and target cells with antibody, was monitored in each experiment. Specific lysis (SL) was calculated by subtracting the

mean percentage spontaneous release from the mean percentage experimental release in tubes with antibody-coated target cells and effector cells. The low levels of spontaneous ⁵¹Cr release demonstrated the antibody dependence of this assay. The cellular dependency of the assay was demonstrated by lack of lysis in tubes containing antibody-coated ⁵¹Cr labeled target cells but no effector cells. Each human serum (1/10 dilution) was tested with targets and effectors from 3 to 5 normal donors with different HLA phenotypes.

As has been recently reported with the chicken erythrocyte-ADCC assay,²⁵ this PBL-ADCC assay with human and rabbit ALA is readily reproducible from day to day with the same PBL donor but is quite variable from donor to donor. ADCC specific lysis of human PBL targets from many different normal donors in the presence of RaALA (1/640 dilution) ranged from 8% to 37% (mean 19.5%). Therefore, in order to compare the results from testing an individual patient's serum in different experiments we converted specific lysis to a cytotoxicity index (CI²⁶). The CI expresses the mean specific lysis produced by the patient's serum relative to the positive serum control with RaALA for that experiment. CI was calculated according to the following formula:

$$CI = \frac{\text{specific lysis with patient serum}}{\text{specific lysis with RaALA}} \times 100.$$

Results

CHARACTERISTICS OF THE ADCC ASSAY

The time course of this assay is illustrated in Fig. 1. ADCC lysis is detectable within 30 min and increases rapidly over the next hour. From 2.5 to 20 hours lysis roughly parallels the spontaneous release. Therefore the time of incubation that was selected for all studies was 1.5 hours.

In preliminary experiments different effector-to-target cell ratios (E/T) were tested. ADCC specific lysis increased linearly with increasing E/T ratios of 1:1, 10:1, 20:1, and 50:1 (data not given). Increasing the E/T ratio from 50:1 to 100:1 produced only a 2–4% increase in ADCC-SL. Therefore we used an effector cell/target cell ratio of 50:1 when testing patient sera for ALA.

CONTROLS

A positive control with RaALA was performed for each experiment. A preliminary study with this antibody showed that it reacted with PBL from all 15 normal donors tested. In a total of 55 experiments RaALA produced ADCC specific lysis of 19.5% ± 7.5% (mean ± SD) with a range of 8–37%. The negative serum control for each experiment was a

Fig. 1 Time course of the ADCC Assay. Results at different time points represent values from 3 experiments performed on the same day with PBL from different donors. Experimental release ($\circ$) of ^{51}Cr from tubes containing targets, 1/320 RaALA, and effectors. Spontaneous release (Δ) of ^{51}Cr from tubes containing targets, medium, and effectors. ADCC specific lysis ($\bullet$) is experimental release minus spontaneous release. Vertical lines represent one standard error of the mean. Note parallel increase in experimental and spontaneous release after 2.5 h incubation so that calculated ADCC-SL does not increase with extended periods of incubation.

1/10 dilution of pooled normal human serum (NHS) (Gibco, lot 200-6252). In 55 determinations specific lysis for NHS was $0.7\% \pm 0.9\%$ (range of 0 to 2.4%), and the cytotoxicity index was $5.2 \pm 7.5\%$. When patient sera were tested, a CI of 21% or greater (mean of negative controls + 2 SD) was designated as positive for ALA mediating ADCC.

Before sera from patients with SLE were studied, it was important to be certain that this ADCC assay could be mediated by human ALA. Therefore an experiment was performed with serum from a patient who received more than 50 blood transfusions because of chronic anaemia. This serum was positive in ADCC to a 1/640 dilution. The ADCC reaction was mediated by the IgG but not by the IgM fraction. When this serum was reacted with PBL from several donors, ADCC-CI ranged from 26 to 117%. These studies showed that the ADCC assay was mediated by human ALA. Comparative titrations of human and rabbit ALA showed that this human ALA usually induced less target cell lysis than the rabbit ALA (Fig. 2).

An effector cell negative control was included in all experiments. To verify that ADCC was the operative

mechanism causing lysis of antibody-coated target lymphocytes, effector cell Fc receptors were blocked by incubating effector cells with 5–10 mg/ml heat-aggregated IgG²² for one hour at room temperature before mixing with the target cells. In 22 determinations this treatment of effector cells inhibited ADCC of RaALA coated target cells by a mean of 75%. A similar degree of inhibition also occurred with human ALA-mediated ADCC.

PATIENT STUDIES

Ninety-four serum samples from 51 patients with LE were tested for ALA in the ADCC assay with PBL from 4–6 normal donors with different HLA phenotypes. Thirteen samples were positive with CI ranging from 22 to 115% (Table 1). The positive sera were obtained from 5 female LE patients who had severe multisystem disease and were being treated with prednisolone and/or cytotoxic therapy. As indicated in Table 1, sera 2, 4, and 5 were positive with PBL from all donors tested, and sera 1 and 3 induced ADCC of PBL from 3 of 6 and 3 of 4 donors respectively. The mean ($\pm$ SD) ADCC-CI for all SLE sera was $12.8\% \pm 1.9\%$. The mean ADCC-CI for positive sera was $57\% \pm 7.6$. For the remaining negative sera the mean CI was $6.3\% \pm 0.8$, which is similar to the CI of 5.5% obtained with NHS, the negative serum control. Four of the positive LE sera and the RaALA were separated into IgG and IgM fractions. These fractions were tested in the ADCC Assay

Fig. 2 Comparative titrations of rabbit antilymphocyte antibody (RaALA) and human antilymphocyte antibody (HuALA) in the ADCC Assay. Peripheral blood lymphocytes from a normal donor were used as target and effector cells (see 'Materials and methods' for further details). Note that RaALA produced higher percentage lysis than the HuALA. Incubation time was 90 min at 37°C, with effector/target ratio of 50/1.

Table 1 Antibody-dependent cellular cytotoxicity induced by serum from patients with SLE

Normal donor of target and effector cells	Serum from SLE patient number									
	1		2		3		4		5	
	%SL*	CI†	%SL	CI	%SL	CI	%SL	CI	%SL	CI
A	4.3	38	6.5	49	8.8	84			5.3	51
B	14.0	52	10.3	45	8.4	44	18.4	92	12.3	64
C	1.1	8	7.4	56			15.3	115	12.8	96
D	7.0	15	6.9	28	8.1	33	16.9	68	5.7	21
E					2.1	19	7.2	49		
F	3.4	22					20.5	103		
G	2.5	13								

*SL, Specific lysis=experimental percentage lysis minus spontaneous percentage lysis.

†CI, cytotoxicity index = $\frac{\text{SL with patient ALA}}{\text{SL with rabbit ALA}} \times 100$.

(Table 2). The ADCC activity was present in the IgG but not the IgM fractions. The ADCC activity mediated by whole serum and the IgG fractions was inhibited 50–75% by the pretreatment of effector cells with 6 mg/ml heat-aggregated IgG. Four of the 94 SLE serum samples tested appeared to be toxic to the ^{51}Cr -release. Pretreatment of effectors with aggregates did not inhibit ^{51}Cr -release from the target cells during the ADCC reaction, showing that these toxic sera produced false positive results.

Table 2 ADCC specific lysis with IgG and IgM fractions of LE ALA and inhibition by aggregated IgG

Serum	ADCC SL*	Agg ADCC SL†
RaALA whole	18.7	2.9
IgG‡	26.1	6.4
IgM	2.9	0.8
LE ₁ whole	7.3	3.7
IgG	6.4	3.5
IgM	0	0
LE ₂ whole	12.0	2.1
IgG	5.7	0.7
IgM	1.3	1.1
LE ₃ whole	7.1	0.9
IgG	6.3	1.4
IgM	1.1	0
LE ₄ whole	12.3	ND§
IgG	7.9	2.2
IgM	0.5	1.1

*ADCC SL=percentage specific lysis (experimental—spontaneous release) in ADCC assay.

†Agg ADCC SL=percentage lysis in ADCC assay in which effectors were preincubated with 5 mg/ml heat-aggregated IgG for 1 h, 37°C, before mixing with antibody coated targets. Spontaneous release was less than 4%. Time of incubation was 90 min at 37°C. Effector/target ratio: 50/1.

‡IgG and IgM fractions were prepared as described in 'Materials and methods.'

§ND=not done.

Discussion

The occurrence of ALA and lymphocytopenia has been described in patients with SLE.⁶ In these studies ALA were detected primarily by complement-dependent cytotoxicity assays, and it has been assumed that this is also the in-vivo mechanism of lymphocytolysis. Kumagai *et al.*²⁷ have recently reported that IgG ALA from 8 patients with SLE mediated ADCC against T lymphocytes. The results of our study confirm that IgG ALA can mediate lymphocytolysis by ADCC. The ADCC mediating ALA is a warm-reactive IgG antibody, which suggests that ADCC might be an in-vivo lymphocytolytic mechanism in patients who produce these antibodies. The ALA reactive in this assay were obtained from 5 patients who had severe multisystem disease and required prednisone or cytotoxic therapy. By using several target cell donors to test the sera we observed that the reactivity was not restricted to an individual HLA phenotype but occurred with PBL from unrelated donors of different HLA phenotypes. Therefore it is unlikely that ALA from these patients were the result of pregnancy-induced alloimmunisation. This ADCC assay has several features not present in other methods commonly used to study human ALA. The complement-dependent microcytotoxicity assay detects primarily IgM ALA and not IgG ALA because of a limited capacity of low concentrations of IgG to activate complement.²⁸ In spite of the relatively low level of specific lysis produced by ALA-mediated ADCC, the consistently low levels of spontaneous ^{51}Cr release in the absence of ALA, together with the ability to inhibit the reaction with aggregated IgG, permit use of this assay to study lymphocytolysis mediated by human IgG ALA.²⁹

ADCC with rabbit ALA consistently produced greater lysis of PBL target cells than the human

ALA. This relatively low level of lysis was also found in other studies of ADCC with human ALA.^{7 8 20 23} The rabbit antiserum may have a higher concentration of immune ALA or may contain naturally occurring additional ALA^{22 28} which could augment the ADCC. These naturally occurring rabbit ALA are thought to play an important role in enhancing the complement-dependent microcytotoxicity tests used to detect cold reactive human IgM ALA.³⁰

ADCC inducing ALA from patients with LE may react with only a PBL subpopulation, whereas all PBL appear to react with rabbit ALA. Other investigators have reported restricted specificity of SLE ALA for certain lymphocyte subpopulations.^{4 6} For example, it has been shown that some SLE ALA react with a subset of suppressor T cells.¹⁵⁻¹⁷ The mechanism by which these ALA coated cells are eliminated or their function impaired has not been clearly defined. It has also been reported that after incubation with sera from a patient with SLE there is loss of T cells in one of the fractions obtained by discontinuous Ficoll gradient centrifugation.³¹ The loss of these cells was minimised by the addition of aggregated IgG, suggesting that autologous ADCC, produced by ALA, might have been responsible for the decrease in the T cell subpopulation. Our study supports this suggestion and directly demonstrates PBL cytotoxicity by ADCC induced by sera from some patients with SLE.

Another possible explanation for the low levels of specific lysis of PBL in our study is that the IgG ALA might be of low avidity or might be capped and shed from the PBL target cell surface during the ADCC reaction. This could result in decreased target cell lysis or autoinhibition by the shed immune complexes reacting with Fc receptors of the effector cells. It is also possible that the standard ADCC assay may not detect all the lymphocyte damage that is produced. This has been shown for ADCC of tumour target cells by mouse spleen effector cells.³² Perhaps other methods to detect cell damage, such as incubation of the presumably damaged cells in hypotonic medium, might increase the sensitivity of the ADCC assay.³²

In the present study the frequency of ADCC mediating ALA in a random collection of stored LE sera was low, only 10%. Therefore ADCC is not useful for screening LE sera for IgG ALA. However, the 5 patients with ADCC mediating IgG ALA had severe multisystem disease. It was impossible to determine the exact state of clinical activity of the disease in all the patients by retrospective review of the charts. It may be that these warm-reactive ALA appear in serum during exacerbation of SLE disease activity. The actual incidence or frequency of ADCC mediating ALA will have to be determined by a prospective study of consecutive SLE patients and

correlation with parameters of disease activity.

It remains to be established whether the SLE ALA can produce lymphocytopenia by the mechanism of ADCC in patients with SLE. This possibility is supported by recent evidence that ADCC can be demonstrated with plasma, effector cells, and target cells from patients with SLE.²⁷ During exacerbation of SLE, ADCC in a completely autologous system may be difficult to demonstrate, because when the antibody is present in serum, the susceptible PBL population may have been eliminated by ADCC. PBL from patients with active LE may no longer contain the subpopulation of lymphocytes reactive with the ADCC inducing ALA. It may be necessary to obtain serum during active disease and then react it with PBL obtained at a time when the disease is quiescent. The ALA titre may increase in plasma during periods of increased disease activity. Alternatively, they may be present only in patients with severe disease. Longitudinal studies of patients with SLE are being done to determine the actual incidence of ADCC inducing ALA and correlation with disease activity. It also remains to be determined which subsets of lymphocytes are susceptible to ALA in ADCC.

In vitro the ADCC assay system utilises target cell lysis as the measured end point. It is not clear whether this immune mechanism is cytolytic in vivo. It is possible that the interaction of target cell, antibody, and effector cell may have nonlytic functional effects on the target cell which are not detected in the in-vitro cytolytic assay. In vivo the interaction of ALA, lymphocytes, and effector cell may damage and/or alter the functional capacity of the target cell rather than produce lysis. If this were the case, it would then be anticipated that the heterogeneous aspects of ALA specificity apply also to their functional effects. These possibilities await further study.

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