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Antibody-dependent direct cytotoxicity of human lymphocytes

I. STUDIES ON PERIPHERAL BLOOD LYMPHOCYTES AND SERA OF PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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SUMMARY

Antibody-dependent direct cytotoxicity (ADDC) is generally believed to be unrelated to T-cell function in experimental animals. The role of ADDC in humans and its clinical usefulness was evaluated in patients with systemic lupus erythematosus (SLE) and normal controls. Peripheral blood lymphocytes from patients with active SLE were unable to lyse antibody-coated target cells *in vitro* to the same degree as lymphocytes from patients with inactive SLE and controls. Sera from patients with active SLE suppressed ADDC by lymphocytes derived from normal controls and this abnormality was not corrected by overnight incubation or by extensive washing of lymphocyte preparations. Although there was poor correlation between ADDC and the proportions of B cells and null cells in effector lymphocyte populations from SLE patients and controls, it is concluded that this assay provides another means of determining immune competence in man.

INTRODUCTION

Lymphoid cells from several species including mouse, rat, rabbit, guinea-pig, duck and man are able to mediate the destruction of antibody coated target cells (Zighelboim & Gale, 1974). Although evidence is now forthcoming that peripheral blood T lymphocytes as well as human thymus cells are ineffective in antibody-dependent direct cytotoxicity (ADDC) (Perlmann *et al.*, 1975) others have claimed that T cells, B cells and even macrophages are involved in this reaction (Fakhri & Hobbs, 1972; Moller & Svehag, 1972; Perlmann, Perlmann & Wigzell, 1972; Dennert & Lennox, 1972; Greenberg *et al.*, 1973).

We have previously reported increased numbers of lymphocytes lacking T and B surface markers, i.e. null cells, in the peripheral blood of patients with active systemic lupus erythematosus (SLE) (Scheinberg & Cathcart, 1974; Brenner, Scheinberg & Cathcart, 1975). In order to determine whether this null cell abnormality reflects B-cell dysfunction, we have evaluated ADDC activity in a series of patients with active and inactive SLE. Serological factors which might interfere with normal cytolysis of antibody-coated target cells have also been studied in SLE patients as well as normal controls.

MATERIALS AND METHODS

Media. All the experiments were performed using Eagle's Minimal Essential Medium supplemented with 10% foetal calf serum, antibiotics and essential amino acids (Microbiological Associates, MEM-FCS).

Effector cells (EC). Peripheral blood mononuclear cells were obtained by flotation sedimentation using a Ficoll–Hypaque gradient (Böyum, 1968). The cells were washed three times in MEM-FCS and made monocyte deficient using the carbonyl

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iron technique. The resulting cell population which contained 98% lymphocytes was 95% viable as judged by trypan blue exclusion and represented between 70% and 90% of the lymphocytes present in the whole blood. After the last wash cells were resuspended in MEM-FCS at a concentration of 2×10^6 cells/ml.

Antisera. Antisera to burro erythrocytes were prepared in guinea-pigs by subcutaneous injections at multiple sites with burro red cells mixed in Freund's complete adjuvant. The guinea-pigs were injected once and bled 3 weeks later. In all cases heat-inactivated antisera from a single batch were used.

Target cells (TC). Burro erythrocytes were obtained from Flow Laboratories, Rockville, Maryland, maintained in Alsever's solution and used within a week after they were bled.

ADDC assay. The method described by Van Boxel *et al.* (1972) for mouse spleen cells was adapted for human lymphocytes with minor modifications. Effector cells in 1 ml of MEM–FCS containing 2×10^6 cells were mixed with ^{51}Cr-labelled burro erythrocytes (0·5 ml, 1×10^5 red cells) and with 0·1 ml of a 1:4 dilution of heat-inactivated guinea-pig antisera to burro red cells. The final dilution was 1:68. Controls without added lymphocytes and others with normal guinea-pig antisera were included in the experiments. Unlabelled sheep erythrocytes (3×10^7, 0·1 ml) were added to all mixtures to prevent spontaneous lysis of the target cells. The reaction mixtures were incubated in duplicate tubes at 37°C in an atmosphere of 95% air and 5% CO_2 for 18 hr. Cold MEM was added to stop the reaction. The tubes were centrifuged at 200 *g* for 10 min. The supernatants were transferred to disposable test tubes and counted in a gamma counter. The degree of specific lysis was determined as follows:

$$\text{Percentage specific lysis}=\frac{\text{Experimental counts}-\text{background counts}}{\text{Total releasable counts}-\text{background counts}}\times 100.$$

Background counts represent counts released by target cells incubated with effector cells and normal guinea-pig sera and accounted for 10–20% of the total releasable counts. Total releasable counts represent the counts released by repeated freeze–thawing of target cells. This procedure released 80–90% of the total counts.

Analysis of surface characteristics of human T and B cells. The procedures used for T- and B-cell enumeration have been published in detail elsewhere (Scheinberg & Cathcart, 1974). In brief, cells were washed in MEM and brought to a concentration of 4×10^6 cells/ml. For T cells (E rosettes) 0·25 ml of cells plus equal volumes of a 0·5% suspension of washed sheep red cells (Colorado Serum Company) were added in 6×55 mm glass tubes. The tubes were incubated at 37°C for 15 min, centrifuged at 200 *g* for 5 min and incubated in an icewater bath overnight. The cells were then gently resuspended and the percentage of rosette-forming cells was determined microscopically. Two hundred lymphocytes were counted and only rosettes having three or more adherent erythrocytes were counted. B cells were enumerated by fluorescence microscopy (SIg) and by the proportion of rosettes formed between human lymphocytes and sheep red cells coated with amboceptor and mouse complement (EAC) as previously described. Surface immunoglobulin was detected using a direct fluoresceinated antibody technique. To 0·1 ml of cells ($1–2\times 10^6$) was added 0·1 ml of fluoresceinated goat anti-human polyvalent antisera (Meloy Laboratories, Springfield, Virginia) and the reaction mixture was incubated at 4°C for 30 min. Cells were washed with medium and resuspended in a glycerol–PBS buffer and viewed with a Zeiss Universal fluorescence microscope equipped with an UV light source and phase contrast. Routinely 200–300 cells were counted and the percentage of cells staining with fluoresceinated antisera was enumerated. EAC rosettes were detected by incubating 0·25 ml of cells with equal volumes of the EAC reagent (0·5% suspension) for 30 min at 37°C, and the percentage of rosette-forming cells determined microscopically. The EAC reagent was prepared by coating sheep red cells with sublytic concentration of 19S haemolysin (Cordis Laboratories, Miami Beach, Florida) and a 1/10 dilution of fresh mouse sera. Except for an occasional patient there was good agreement between the percentages of B cells detected in effector cell suspensions using both the SIg and EAC markers. The percentage of null cells was determined by subtracting the percentage of E rosettes and SIg-binding cells from 100.

Patient population. The study group consisted of ten patients with active and eight patients with inactive SLE. Twenty healthy adult volunteers served as normal controls. All patients in the study group satisfied diagnostic criteria formulated by the American Rheumatism Association. The criteria used for determination of the clinical activity of SLE patients has been published in full elsewhere (Scheinberg & Cathcart, 1974). Four patients were receiving prednisone in doses ranging from 10 to 30 mg/day; the remainder were receiving aspirin to tolerance and/or hydroxychloroquine 200 mg/day.

Statistical analyses were performed by Student's *t*-test and analysis of variance. Comparison was made between groups by Scheffe's test.

RESULTS

Effector cell function of human lymphocytes

Purified human lymphocytes were capable of mediating ADDC in the presence of specific antibody at EC:TC ratios ranging from 100:1 to 10:1. Serial determinations of ADDC performed on different occasions in two healthy volunteers gave reproducible results. Experiments using lymphocytes from twenty healthy individuals at fixed concentrations of antisera and 20:1 EC:TC ratios gave results ranging from 30 to 50% cytotoxic activity (Table 1).

TABLE 1. ADDC, T, B, null cells and lymphocyte count (LC) in peripheral blood from patients with active and inactive SLE and controls

	Case no.	Age	Sex	Percentage ^{51}Cr release	T	B	NC	LC
Active SLE	S1	17	M	24	50	7	43	1400
	S2	57	F	11	n.d.*	n.d.	n.d.	850
	S3	18	F	18	38	20	42	1260
	S4	60	F	18	47	6	47	1200
	S5	36	F	12	45	6	49	1000
	S6	58	M	19	40	13	47	1350
	S7	18	F	4	42	16	42	1480
	S8	40	F	18	35	18	47	1180
	S9	26	F	29	51	9	40	640
	S10	50	F	19	20	13	67	600
Mean ± s.d.				17 ± 7	41 ± 10	12 ± 5	47 ± 8	1096 ± 311
Inactive SLE	S11	40	F	46	65	27	7	2000
	S12	50	F	50	52	29	23	2400
	S13	36	F	45	65	15	29	2000
	S14	50	F	45	66	7	27	2380
	S15	18	M	34	65	20	15	1380
	S16	32	F	51	70	20	10	2610
	S17	32	F	49	56	15	29	1520
	S18	44	F	31	n.d.	n.d.	n.d.	1008
Mean ± s.d.				44 ± 8	63 ± 6	18 ± 6	18 ± 8	1912 ± 562
Controls†		20–55		37 ± 6	63 ± 4	18 ± 4	19 ± 4	2582 ± 687

* n.d. = Not determined.
† Fourteen male and six female.

TABLE 2. Effect of SLE sera on ADDC by normal peripheral blood lymphocytes

	Case no.	Sex	Age	Percentage suppression*	ANA titre†
Active SLE	S1	M	17	48	1/2048
	S2	F	57	55	1/1024
	S3	F	18	60	1/512
	S5	F	36	92	1/512
	S6	M	58	72	1/1024
Inactive SLE	S13	F	36	2	1/4
	S15	M	18	0	1/4
	S16	F	32	5	1/4

* The percentage suppression represents the decrease in specific lysis induced by treatment of effector cells with lupus serum (0·2 ml) in comparison with the decrease induced with pooled human AB sera (0·2 ml).

† Anti-nuclear antibody titre performed by indirect immunofluorescence using rat liver as a nuclei substrate.

Effector cell function in SLE

Patients with active SLE consistently showed reduced cytotoxic activity when compared to inactive SLE patients and normal controls (Table 1) ($P<0{\cdot}01$). Five out of ten patients with active SLE had ADDC assays performed two or three times and yielded identical results. Attempts to improve ADDC by incubating SLE lymphocytes overnight and washing the effector cells extensively before testing (data not shown) failed to correct this abnormality. Sera from patients with active SLE who showed reduced ADDC were able to suppress the cytotoxic activity of normal lymphocytes against antibody-coated target cells while sera from patients with inactive SLE (who also showed normal ADDC) failed to do so

TABLE 3. Effect of overnight incubation of SLE sera on ADDC by normal peripheral blood lymphocytes*†

	Immediately tested		Overnight incubation	
	Percentage ^{51}Cr release	Percentage suppression	Percentage ^{51}Cr release	Percentage suppression
Media	32	0	25	0
AB sera	31	0	26	0
N4	9	70	14	44
N5	25	21	17	32
N6	4	88	9	64
N7	1	99	3	88

* Freshly isolated lymphocytes were tested immediately or after overnight incubation in the presence of AB or SLE sera.

† Viability in the cell suspensions cultured overnight ranged between 85% and 90% dye exclusion.

TABLE 4. ADDC by peripheral blood lymphocytes of patients receiving high dose steroid treatment

Case no.	Percentage ^{51}Cr release	Prednisone (mg%)	Clinical condition
P1	50	20	Relapsing polychondritis
P2	33	20	Bronchial asthma
P3	32	60	Pemphigus vulgaris
P4	40	30	Multiple sclerosis

(Table 2). Overnight incubation of freshly isolated normal lymphocytes with SLE sera followed by washing and retesting for ADDC gave less inhibition than cells reacted with SLE sera and tested immediately but ADDC was still reduced when compared to cells incubated with AB sera (Table 3).

Since some of the patients included in this study were receiving prednisone, we also tested the possibility that reduced ADDC in some patients with active SLE might be a steroid effect. Patients receiving high dose steroids for other medical reasons gave normal results when tested in a similar manner (Table 4). Finally, no correlations could be made between the degree of ^{51}Cr released from target cells and the percentage of T cells, B cells (EAC, SIg) or null cells contained in the effector cell populations of normals and patients with inactive and active SLE.

DISCUSSION

We have demonstrated that human lymphocytes exert cytotoxic activity against antibody-coated burro red cells in a manner similar to that described by Perlmann & Perlmann (1970) for chicken red cells. ADDC of burro erythrocytes by mouse spleen cell suspensions has been shown by Van Boxel *et al.* (1972) to be reduced following the removal of EAC-binding lymphocytes or by the elimination of cells carrying large amounts of surface immunoglobulins. These studies indicated that the principal cell population participating in ADDC is composed of B lymphocytes and Zighelboim & Gale (1974) arrived at a similar conclusion for human lymphocytes mediating ADDC against EL-4 cells. On the other hand, although we and others have removed monocytes from our effector cell suspensions by iron treatment, we cannot completely exclude the possibility that a non-phagocytic monocyte is involved in our assay as recently proposed by Faulk, Greene & Faulk (1974). Despite these limitations, we now consider it valid to compare cytotoxic activity in different individuals and disease states provided standard conditions (same antisera, same EC:TC ratio, etc.) are maintained throughout the study.

Although healthy volunteers manifested a considerable range of variation in our assay it was possible to classify them as normal with some degree of confidence. By contrast, patients with active SLE had reduced ADDC activity for antibody-coated red cells and this abnormality was not corrected by overnight incubation, a procedure which has been shown by Winchester *et al.* (1974) to remove adherent autoantibodies from both T and B lymphocytes. Despite the recent report by Basten, Miller & Abraham (1975) showing that antibodies against H-2 determinants nonspecifically block the Fc receptor, we therefore conclude that anti-lymphocyte antibodies on the surface of effector cells are not solely responsible for the ADDC abnormalities noted above. Indeed, the data are more consistent with an alternative explanation, i.e. that the number of killer cells may actually be decreased in active SLE as a result of complement-mediated cytolysis. A similar mechanism has been implicated in the genesis of haemolytic anaemia which occurs in some patients with this disorder. Since some of our patients with uncontrolled SLE were receiving prednisone, it was noteworthy that steroid therapy did not alter ADDC in patients with other pathologic conditions including asthma, relapsing polychondritis, pemphigus vulgaris and multiple sclerosis.

Studies from several laboratories now suggest that surface receptors for immune complexes also play a significant role in ADDC. We have therefore considered the possibility that reduced cytotoxic activity of SLE lymphocytes might be due to blocking of these receptors by DNA–anti-DNA soluble immune complexes, particularly since sera from patients with reduced ADDC activity are capable of suppressing the cytotoxic activity of normal lymphocytes even after overnight incubation. Other factors may also be involved, however, since Dickler (1974) was unable to obtain a linear correlation between the concentration of immune complexes and suppression of human ADDC. Studies are now under way to determine more precisely the size and binding properties of immune complexes in the sera of SLE patients with reduced ADDC and to purify their killer cells in discontinuous gradients.

The inverse relationship between ADDC activity and the percentage of cells lacking B- and T-cell markers in patients with active SLE was at first somewhat surprising since MacDermott, Chess & Schlossman (1975) had shown that null cells in normal individuals have potent ADDC activity. On the other hand, we have recently shown that there is a marked reduction in the same null cell population after *in vitro* incubation with thymus extract (thymosin). This latter finding is consistent with the notion that the majority of null cells in active SLE are probably immature T cells rather than B cells (Scheinberg, Goldstein & Cathcart, 1975) and therefore not responsible for antibody-mediated cytotoxicity.

After this investigation was completed a similar study was reported by Schneider *et al.* (1975). These investigators also noted reduced ADDC in patients with active SLE although they were unable to show that sera from the same patients were capable of interfering with ADDC by normal lymphocytes. Discrepancies between the two studies may be due to differences in the content of patient populations or technical variations since only four patients in the present series were receiving steroids and since we routinely remove phagocytic cells from effector populations by carbonyl iron treatment. Recent reports have also demonstrated deficiencies in ADDC in patients with Waldenstrom's macroglobulinaemia,

acute lymphoblastic leukaemia, and various immunodeficiency syndromes (Campbell *et al.*, 1972, 1973; Rachelefsky *et al.*,1975) and we and others have shown that sera from patients with rheumatoid arthritis may inhibit ADDC by normal lymphocytes (Scheinberg & Cathcart, 1976). We conclude from these findings that the ADDC assay will become a clinically useful tool to assess immune function in an increasing number of disease states.

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