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Relation between lymphocytotoxic antibodies, anti-DNA antibodies and a common anti-DNA antibody idiotype PR4 in patients with systemic lupus erythematosus, their relatives and spouses

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SUMMARY

Forty-two patients with systemic lupus erythematosus (SLE), 65 of their healthy relatives and 20 spouses were studied for the presence of lymphocytotoxic antibodies (LCA), anti-lymphocyte antibodies (ALA), antibodies to DNA and a common idiotype (Id) PR4. Seventy-one per cent of the patients had positive levels of LCA, and in 34% the PR4 Id was detected; normal levels were found in their families. Anti-PR4, an anti-Id, failed to block the lymphocytotoxic activity in those nine patients who both carried the Id and had LCA. This indicates that the Id was not present on LCA. There was no correlation between anti-DNA antibodies and LCA, suggesting that different mechanisms are involved in their expression.

Keywords lymphocytotoxicity systemic lupus erythematosus cross-reactive idiotypes

INTRODUCTION

Lymphocytotoxic antibodies (LCA) have been identified in many patients with systemic lupus erythematosus (SLE). However, there are conflicting views as to whether the levels of these antibodies fluctuate with disease activity (Bresnihan, Grigor & Hughes, 1979; Bluestein & Zvaifler, 1979, Temesvari *et al.*, 1983). In addition, there are varying reports about the presence of LCA in the sera of first-degree relatives of these patients and those unrelated individuals who share the same environment, e.g. their spouses (de Horatius & Messner, 1975; Malave, Papa & Layrisse, 1976; Folomeeva *et al.*, 1979; Hazelton, 1984.)

Common anti-DNA antibody idiotypes (Ids) have been identified on LCA, and in one study (Shoenfeld *et al.*, 1985) the 16/16 Id was found on an antibody that both bound to DNA and had anti-lymphocyte activity. However, in another series this overlap was not found (Lydyard *et al.*, 1987).

We have undertaken a study to attempt to clarify these issues by using sensitive assays to determine the incidence of LCA in a group of 42 SLE patients, 65 of their first-degree relatives, 20 of their spouses and 29 healthy normal controls. In addition, we have determined whether any of these sera have anti-DNA antibodies to both double stranded (ds) and single stranded (ss) DNA of IgG and IgM isotype, or antibodies that carry another common Id, PR4. The PR4 Id was first identified on a human hybridoma-derived monoclonal antibody (Williams *et al.*, 1988) from a patient with leprosy. The parent antibody, PR4, was found to bind in a solid-phase assay to ss- and ds-DNA, poly-ADP-ribose, and the major *Mycobacterium leprae* determinant PGL-1 (Zumla *et al.*, 1988). Our data indicate that while raised levels of LCA are found in over 70% of the SLE patients, their relatives have normal levels. We also found no correlation between levels of Id, anti-DNA antibodies and LCA in the sera.

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MATERIALS AND METHODS

Patients and sera

We studied sera from 42 patients with SLE, each of whom met four or more of the revised criteria of the American Rheumatism Association for the classification of SLE (Tan *et al.* 1982), 65 of their first degree relatives and 20 of their spouses, none of whom had any history of autoimmune disease. Sera from 29 unrelated normal healthy subjects matched for age and sex were used as controls. The sera were stored in small aliquots at −20°C until tested. The clinical activity of the SLE patients was designated according to previously published criteria (Isenberg, Shoenfeld & Schwartz, 1984), recently validated in a comparative study with a more detailed, computer-based index (Symmonds *et al.*, 1988).

Lymphocytotoxicity assays

Conventional assay. Tonsils were obtained by routine tonsillectomy operations and were kindly provided by the Royal Ear, Nose and Throat Hospital, London. Cell suspensions were

prepared by gentle teasing of the tissue and centrifugation over Ficoll-Hypaque to remove dead cells. Triplicate microwells containing 1×10^4 cells in 25 μl of RPMI + 10% fetal calf serum (FCS) were incubated with 25 μl of test serum at a final dilution of 1:8 for 60 min at 37°C. Control wells containing cells only were included on each plate. The cells were washed once, and 50 μl of rabbit serum pre-absorbed with tonsil cells were added at a 1:5 final dilution (as a source of complement) and incubated for a further 30 min at 37°C. The cells were pelleted and resuspended in 5 μl of a mixture of acridine orange and ethidium bromide (Lee, Singh & Taylor, 1975). Percentages of dead cells were scored blind under a microscope using u.v. optics.

Cell ELISA. Macrophage-depleted peripheral blood lymphocytes (PBL) (2×10^5/well) were added to poly-L-lysine-coated, 96-well plates and incubated for 1 h at room temperature. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol. After additional blocking with 5% normal rabbit serum, test sera diluted 1:50 and a known positive sample were added and incubated overnight at 4°C. The plates were washed with RPMI + 2% FCS, and binding was detected using peroxidase-conjugated F(ab')$_2$ rabbit anti-human IgM or IgG.

Anti-DNA antibodies

Antibodies to native ds- and ss-DNA were measured by ELISA; 96-well, polystyrene plates (Immulon II, Dynatech, Plochingen, FRG) were precoated with 50 μl of 50 μg/ml poly-L-lysine (Sigma Chemical Co., St Louis, MO) and incubated for 60 min at 37°C. The plates were then washed with phosphate-buffered saline (PBS) and coated overnight at 4°C with 50 μl per well of ds-DNA (10 μl/ml) or ss-DNA (5 μl/ml). The ds-DNA was prepared by treating calf thymus DNA with S1 nuclease (Sigma) for 45 min at 37°C. The ss-DNA was prepared by boiling calf thymus DNA for 10 min and cooling on ice for 15 min, and was stored at 4°C until needed. After washing three times with PBS the plates were then blocked with 100 μl/well of 100 μg/ml poly-L-glutamate (Sigma) followed by 2% casein (BDH, Poole, UK) in PBS to prevent non-specific binding. After further washing with PBS-0·1% Tween (PBS-T), 50 μl of test serum diluted 1:200 in PBS-T were added in duplicate and incubated for 1 h at 37°C. A known high-positive serum in quadruplicate plus sera from six healthy individuals was included on each plate. The plates were washed three times with PBS-T, and 50 μl of goat anti-human immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in PBS-T were added to each well and incubated for 60 min at 37°C. After further washing (six times with PBS-T) 50 μl/well of alkaline phosphatase substrate (Sigma) in bicarbonate buffer were added and incubated for 1 h at 37°C. The plates were read on a Dynatech 580 ELISA reader at 405 nm and all results expressed as a percentage of the known positive control.

As an additional control, a selection of positive samples was titrated out at dilutions of 1:200 to 1:25 600 both on plates that were coated with antigen and plates treated with poly-L-lysine alone, to account for any non-specific 'background' binding.

Detection of PR4 Id

The presence of PR4 Id was measured by indirect ELISA, a modification of that previously described (Williams *et al.*, 1988). Briefly, affinity-purified rabbit anti-idiotype PR4 (4 μg/ml) (prepared as described by Lockniskar *et al.*, 1988) in bicarbonate buffer (pH 9·6) was coated onto 96-well microtitre plates (Nunc, Roskilde, Denmark) overnight at 4°C. Control wells were coated with normal rabbit serum (NRS). After washing three times with bicarbonate buffer and blocking with 2% bovine serum albumin (BSA) in bicarbonate buffer for 1 h at 37°C, 100 μl duplicates of the test sera diluted 1:640 in PBS–T/1% BSA were added. A standard curve was prepared as described and the assay was completed as before.

Inhibition of lymphocytotoxicity with anti-PR4 Id (α-PR4Id).

In order to determine the relation between PR4 Id and LCA we attempted to block lymphocytotoxicity by the addition of α-PR4Id, using a modification of a previously described method (Lydyard *et al.*, 1987). α-PR4Id at 1:20 dilution was incubated with patient serum at 1:4 final dilution for 30 min at 37°C and then overnight at 4°C. The sera/α-PR4 mixture was added to tonsil cells, and cytotoxicity was determined as before. A parallel, control experiment was performed using NRS at an equivalent dilution.

Table 1. Number of samples with positive levels of lymphocytotoxic antibodies, antibodies to DNA and expressing the PR4 idiotype

	anti-DNA antibodies								Lymphocytotoxic antibodies							
	ssIgM		ssIgG		dsIgM		dsIgG		Classical assay		ELISA					
											IgM		IgG		PR4	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Patients *n*=42	19	45	20	49	5	12	8	19	30	71	18	43	6	14	14	34
Relatives *n*=65	15	23	3	5	15	23	1	2	8	12	6	9	5	8	2	3
Spouses *n*=20		0		0		0		0	3	15	8	40	8	40		0
Controls *n*=29		0		0		0		0	1	3		0		0	1	3

RESULTS

Lymphocytotoxicity assays

All results with the conventional assay were expressed as a percentage of killed cells above the value of the blank wells (normally 5–20% killing.) Reactions > mean + 2 s.d. of a group of 29 normal healthy controls were taken as positive (Table 1). Of the SLE patients, 71% were positive compared with 12% of their first degree relatives, 15% of their spouses and 3% of the healthy controls. Comparing the results by χ^2 with Yates correction for small numbers, there is no significant difference between the numbers of relatives who had raised levels and the healthy controls. In contrast, the number of patients ($P < 0.001$) and spouses ($P < 0.05$) with high levels are significantly different. It was notable, however, that the positive relatives were confined to four families, one of which included a positive spouse.

Table 2. Percentage killing in serial samples from SLE patients compared with disease activity

Patient no.	Date of sample	Killing (%)	Disease activity
1	09.11.1978	0	2
	02.05.1982	1·3	2
	11.09.1983	11·0	3
	25.02.1988	21·1	2
2	18.06.1979	0	2
	11.03.1981	5·0	1
	22.10.1987	7·9	3
	26.11.1987	4·4	3
3	04.10.1984	0	2
	13.06.1985	0	2
	10.06.1987	0	2
4	02.03.1985	0	4
	21.03.1985	4·8	4
	20.02.1986	11·4	3
	06.08.1987	0	3
5	03.12.1984	0·4	4
	14.10.1985	0·4	2
	09.01.1986	0	2
	03.12.1987	0·8	2
6	12.10.1978	29·5	2
	23.08.1979	0	3
	03.01.1980	9·5	2
	06.11.1980	23·7	2
7	20.03.1980	5·8	2
	10.02.1983	29·1	2
	20.01.1987	14·5	2
	03.12.1987	14·7	3
8	26.06.1982	16·0	3
	10.05.1984	7·0	4
	03.10.1985	12·6	4
	16.07.1987	16·7	2
9	18.07.1985	15·5	3
	01.08.1985	11·0	3
	20.06.1986	4·5	2
	26.11.1987	13·6	2
10	17.05.1984	34·4	4
	07.03.1985	13·2	3
	26.09.1985	20.4	2
	10.03.1988	13·4	1

Upper limit of normal = 5%

Disease activity 1 = inactive 2 = mildly active 3 = moderately active 4 = severely active.

Table 3. Relatives and spouses with positive levels of LCA, bled on two occasions to investigate fluctuations in levels

	Sample 1	Sample 2
Relative	Killing (%)	Killing (%)
Sister*	7·5	6·9
Spouse*	14·8	5·4
Brother†	10·4	7·3
Brother†	7·0	3·8
Mother†	10·5	11·0
Spouse	8·8	9·5
Spouse	7·1	4·1

* Of patient no. 2 in Table 2.
† Of patient no. 10 in Table 2.

Three further serum samples from each of 10 patients taken over the past 10 years were tested for LCA and fluctuation of antibody level was seen in some patients. However, this fluctuation did not follow the pattern of disease activity in these patients (Table 2). A further serum sample was obtained and tested from seven of the relatives and spouses who had had positive levels of LCA and of these, five still had positive values with the other two falling just below the upper limit of normal (Table 3).

Correlation between the standard lymphocytotoxicity assay and the cell ELISA for ALA is shown in Fig. 1. Greater correlation is seen with IgM ($r = 0.45$) than with IgG ($r = -0.1$), but overall the correlation was relatively poor.

Anti-DNA ELISAs

All the samples were tested for IgM and IgG antibodies to both ds- and ss-DNA. Results were expressed as a percentage of a known positive control and values > mean + s.d. of a group of 25 normal controls were taken as positive. Of the 65 relatives tested, 24 had anti-DNA antibodies (Table 1), but only one of these had positive levels of ALA.

Twenty-one of the patients with positive levels of LCA also had antibodies to DNA, but there was poor correlation between the titre of the antibodies to these two antigens (ds IgM, $r = -0.2$; ds IgG, $r = -0.4$; ss IgM, $r = 0.3$; ss IgG, $r = 0.25$).

PR4Id ELISA

All results were expressed as μg equivalence of PR4Id/ml and values > mean + s.d. of a group of 15 normal controls were taken as positive. Fourteen of the SLE patients (34%) were found to carry the Id, as did two of the relatives and one of the normal controls. Of the 14 patients carrying the Id, nine had LCA, but χ^2 analysis with Yates correction showed that this was not statistically significantly different from those patients with LCA but without PR4Id expression. Sera of relatives and controls which carried the Id did not have LCA or anti-DNA activity.

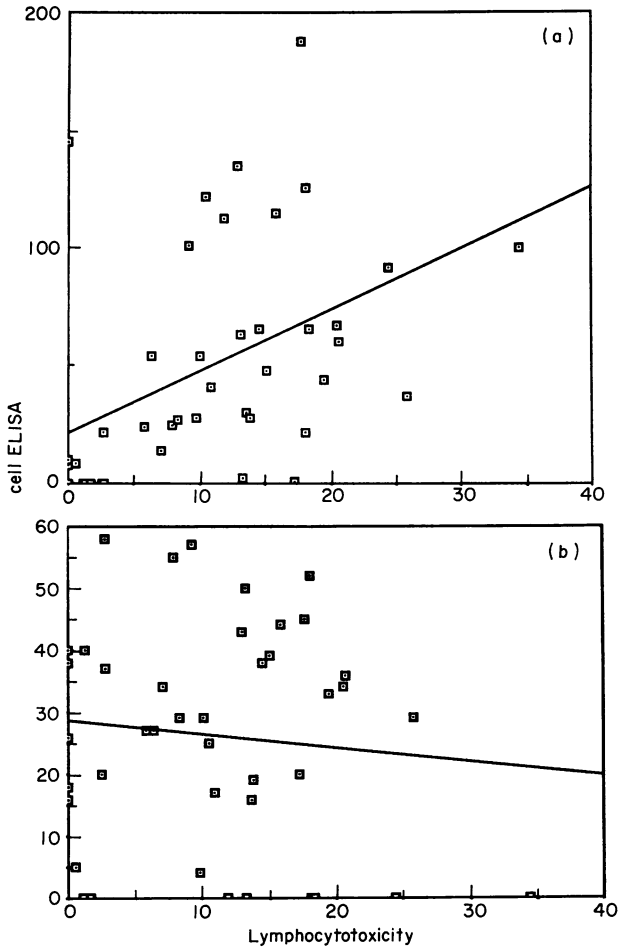

Fig. 1. (a) Correlation between IgM cell ELISA and lymphocytotoxicity assay; $r = 0{\cdot}45$; (b) correlation between IgG cell ELISA and lymphocytotoxicity assay, $r = -0{\cdot}1$

All nine patients who carried the Id and had LCA were tested to determine whether α-PR4Id would inhibit lymphocytotoxicity. However, no inhibition was found (results not shown).

DISCUSSION

The results of this study confirm the high incidence of LCA in the sera of patients with SLE and agrees with results of Bresnihan *et al.* (1979). Although the levels of ALA fluctuated, this did not appear to coincide with disease activity. De Horatius & Messner (1975) found that LCA levels were greater than normal in 58% of the relatives of SLE patients, whereas our results show that the numbers of first-degree relatives with raised LCA were no different from the normal controls ($P > 0{\cdot}01$). Although we found the number of spouses with positive levels of LCA to be statistically significantly increased compared with the normal controls, we only found 15% to have positive levels, compared with the 50% reported by de Horatius & Messner (1975). Hazelton (1984) also failed to find high levels of ALA in the families of SLE patients. He suggested that this may be due to the clinical state of the patient at the time of the study.

The assay conditions used to detect the presence of LCA can also effect the results obtained. The poor correlation between the more conventional assay and the IgG cell ELISA strongly support this. Peake *et al.* (1988) have recently studied the temperature dependence and binding characteristics of LCA and reported that lymphocytotoxic activity resides predominantly in the IgM fraction and is maximal at 15°C. Previous studies have mostly used the method of Terasaki & McClelland (1964), in which incubations are performed at 15°C. Previous studies have mostly used the method of Terasaki & McClelland (1964), in which incubations are performed at 15°C; we chose to assay the sera at 37°C to give more physiologically relevant results (Lydyard *et al.*, 1987). At 37°C, cytotoxicity is predominantly directed against CD8-positive cells, whereas at 15°C killing of CD4-positive lymphocytes is greater (Peake *et al.*, 1988). This may account for our finding normal levels of LCA in the relatives. However, in our study those relatives who had ALA belonged to just six families out of the 42 studied, with one family having all four members affected. This implies that there may be a genetic component in the expression of these antibodies in some families.

Thirty-seven percent of the first-degree relatives, compared with only 10% of the spouses, had antibodies to DNA. Isenberg *et al.* (1985) studied 147 first-degree relatives of lupus patients and found 22% to have anti-DNA antibodies. Given that most of these individuals were not living in the same environment as the patients, this indicates that genetic rather than environmental factors have a greater influence on the production of anti-DNA antibodies. The mechanisms giving rise to the induction of anti-lymphocyte and anti-DNA antibodies are likely to be different, as only one relative had high levels of both antibodies in his serum, and in the patients there was no correlation between the levels of the two antibodies. However, a close association cannot be completely excluded as even a single amino acid substitution may have profound effects on antigen binding (Diamond & Scharff, 1984). Lehman *et al.* (1982) reported similar results with no relation between anti-lymphocyte and anti-nuclear antibodies in the relatives of children with SLE.

SLE patients also have high titres of other autoantibodies to antigen such as cardiolipin, poly-ADP-ribose and antibodies to external antigens, e.g. the *Klebsiella* polysaccharide K-30. Although these antibodies vary in respect of their antigen-binding profiles, they may share a common Id. For example, Kaburaki & Stollar (1987) demonstrated that the common Id 16/6 was present on antibodies binding DNA, Ro- and SM-RNP. Furthermore, Shoenfeld *et al.* (1985) showed that the monoclonal anti-DNA antibody carrying the 16/6 Id also had lymphocytotoxic activity. In contrast, Lydyard *et al.* (1987) found no clear correlation between sera possessing 16/6 Id, anti-DNA antibodies, and LCA. We have shown that 14 (34%) of the SLE patients tested express another common Id, PR4; however, inhibition studies using the α-PR4Id showed no molecular relation between LCA and PR4Id. Cross-inhibition experiments detailed elsewhere (Lockniskar *et al.*, 1988) have demonstrated partial overlap between the 16/6 Id and the PR4Id. They are not identical, but both have been identified as common anti-DNA antibody Ids, detectable in the serum of many lupus patients. Similarly, anti-DNA antibodies and LCA are frequently found

in these patients. However, if anti-DNA antibodies and LCA are linked, it seems unlikely that this is through the mechanism of anti-DNA antibody Id sharing, at least of 16/6 or PR4.

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