PATHOPHYSIOLOGY OF THE IMMUNE RESPONSE IN SYSTEMIC LUPUS ERYTHEMATOSUS: A NEW APPROACH

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I. INTRODUCTION

For the last 20 years, there has been a remarkable advance in the understanding of the pathogenesis and immunopathology of systemic lupus erythematosus (SLE). Research, both at the clinical and experimental levels, has focused on the exaggerated antibody-mediated immune responses and the apparent abnormalities in cell-mediated immunity. During the last few years we have studied over 100 cases of SLE in Venezuela from a prospective and interdisciplinary standpoint. The present report summarizes the results obtained from the analysis of antibody- and cell-mediated immune responses and our current view in terms of the physiopathology of SLE.

II. MATERIALS AND METHODS

A. Patient Population

Patients with a definite diagnosis of SLE (1), seen at the rheumatology unit of the University Hospital of the Central

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University Medical School were evaluated. Those with subcutaneous nodules, bone erosions, and lupuslike syndrome induced by drugs were excluded.

Active disease was defined by the presence of renal, hematological, vascular, or neurological involvement with a diminished serum hemolytic complement (CH_{50}). Renal biopsies were studied by light, electron, and immunofluorescence microscopy. Untreated patients in different stages of disease activity were selected for cell-mediated immunity (CMI) studies.

B. Immunological Studies

1. Antibody-Mediated Immune Responses

Antinuclear antibodies were studied by indirect immunofluorescence; anti-DNA activity was measured by radio immunoassay of the ammonium sulfate precipitate of patients' sera. Immune complexes, in patient serum and in lymphocyte culture fluid, were detected by the Raji cell method described by Theofilopoulos *et al.* (2). The presence of serum lymphocytotoxins was detected by microlymphocytotoxicity (NIH modification of Terasaki technique) against our HLA typed panel (40 normal cells representing 25 specificities for loci A and B), run at room temperature ($22^{\circ}C$). Deposits of IgG, IgA, IgM, C3, and fibrinogen were demonstrated in renal biopsies using fluoresceinated monospecific antisera.

2. The Complement System

This was evaluated in a serial fashion. Total hemolytic serum activity (CH_{50}) was measured as described by Kent and Fife (3). Activating pathways were determined by immunochemical measurements of serum levels of Clq, C4, C3, and Factor B using Mancini radial immunodifusion (4). Levels of Cl inhibitor were measured as well.

3. Cell-Mediated Immune Response

a. Delayed-type hypersensitivity skin tests (*DH*). Once the blood was drawn for the *in vitro* studies, skin tests were performed with Monilia, PPD, and SK-SD according to the WHO criteria (5).

b. Cell preparation. Lymphocytes were purified from heparinized peripheral blood using the Ficoll-Hypaque gradient method (6), counted in a Coulter counter (model Z.BI) and the concentration adjusted in medium (RPMI 1640) supplemented with 3% hepes (1 M, pH 8.1), gentamicin and 2% normal human serum [pooled frozen normal human (NHS) from 5-10 male blood donors, heat-inactivated].

c. SLE Sera. These were obtained from untreated patients before skin testing, aliquoted, and either kept frozen

for immune complex determination or heat inactivated for cultures.

d. Preculture. Cells were precultured in medium, 1×10^6 cells/ml at 37°C for 18 hours and preculture supernatants were concentrated by negative pressure dialysis.

e. Lymphocyte transformation test (LTT). Peripheral blood lymphocytes were cultured in triplicate by previously reported methods (7).

f. Leukocyte migration inhibition test (LIF). This was performed using the modified Bendixen and Soborg technique previously reported (8).

g. Mixed lymphocyte culture (MLC). Responding cells (5×10^4) and mitomycin-treated stimulating cells (1×10^5) were placed in microtiter culture plates (Falcon, U bottom) in triplicates. The medium consisted of RPMI 1640 supplemented with 3% Herpes, gentamicin, and 10% serum (either NHS or SLE serum). Plates were sealed with plastic sealers (Cooke, 7631) and incubated at 37°C. On day 5, 1 μC of ^3H thymidine (Amersham/Searle, specific activity 1 Ci/n*M*) was added to each well. 18 hours later cultures were harvested in a Mash II and counted in a Packard Tri-Carb (3330) scintillation counter. Results were expressed in counts per minute (cpm) relative response (RR) where $RR=(AB^m-AA^m)(AC^m-AA^m)$ C^m being a third party mitomycin-treated control. Allogenic response of fresh and precultured cells was analyzed on the same day under identical experimental conditions.

h. EAC Rosettes. 0.025 ml of a cell suspension (25×10^4) was mixed with 0.025 ml of sheep red blood cells (SRBC) sensitized with amboceptor and mouse serum, in 0.025 ml of heat-inactivated fetal calf serum (FCS). Mixture was centrifuged at 900 rpm for 5 minutes, and kept at room temperature for 1 hour. Rosette-forming cells (with three or more SRBCs) were counted by acridine orange staining. The influence of either SLE or NHS serum on the number of rosettes was investigated by incubating 1×10^6 cells in 1 ml of the test serum. After incubation, cells were spun down, supernatant discarded, and pellet resuspended in medium. The rosette assay was set up either immediately, after one or two washes, or after 18 hours preculture.

i. Lymphocyte-mediated cytotoxicity (LMC). Cytotoxic T lymphocytes, sensitized *in vitro* by MLC, were investigated as previously described and the results expressed by percentage of ^{51}Cr release (9) with both control and SLE patients.

III. RESULTS

A. Antibody-Mediated Immune Response

Antinuclear antibodies were present in 100% of the cases and lymphocytotoxins in 97 of the evaluated patients (100 cases).

1. The Complement System

There were two main groups of features of complement activation in our SLE population: group A, characterized by prolonged hypocomplementemia (a minimun of 6 months), and group B, who started either with low levels and returned to normal in an average of 4-6 weeks or were normal from the beginning. When the activating pathways were analyzed (Table I), it was noticed that those with prolonged hypocomplementemia frequently showed activation of the system simultaneously by both classic and alternative pathways, while those of group B showed mainly classical activation. Interestingly enough, in both groups there were certain patients with depressed serum hemolytic activity and still normal immunochemical levels.

TABLE I
SLE Complement-Activating Pathways, Analysis in 63 Cases

Pathway	Group A (44) Prolonged↓ CH_{50}	Group B (19) ↓ CH_{50} →(N)
Classical	7 (16%)	10 (58%)
Alternative	3 (7%)	0
Both	27 (61%)	4 (16%)
↓ CH_{50} → (N) Immunodiffusion values	7 (16%)	5 (26%)

2. Relationship between Circulating Immune Complexes, Anti-DNA Activity, and Serum Complement

We have found several patterns within the SLE complex with regard to the relationship between clinical disease and serum levels of circulating immune complexes, anti-DNA activity, and total hemolytic complement (10). As an illustration, in Table II we depict what we have described as the malignant form of SLE (11).

TABLE II
Patient L. N. Illustrates the Malignant Form of SLE

		*		*	*
Active-severe					
Clinical disease					
	Remission				
	>250				
CH_{50} u/ml	150				
150-250	100				
	0				
		•		•	•
	>109	•		•	•
Anti-DNA u/ml	20				
0-20					
	0				
	1000	•		•	•
IC	100				
0-25 ug/ml					
	0				
	S			•	•
Therapy		Untreated			
	IS	(3 months)		•	•
LN		4.1.75	† Onset of Rx	4.6.75	27.7.75 + DEAD

L.N. was a patient with severe multisystemic disease who developed, in a short period, all the complications of SLE and died in about 7 months; serial measurements disclosed very high levels of circulating immune complexes, anti-DNA activity, and prolonged hypocomplementemia. The "silent" phase of a particular systemic involvement in the presence of severe disease is another remarkable feature of SLE. Another patient, D.B. (Table III), with persistent activity, developed all the complications of SLE except overt renal disease (she did have "silent" nephropathy with dense subendothelial deposits by electron microscopy and granular distribution if IgG, IgA, IgM, and C3 since August 1974, without evidence of clinical renal disease).

TABLE III[a]
Patient D.B. with Severe SLE and Absence of Clinical Nephropathy

Clinical* disease	Active-severe	*	*	*	*	*
	Remission					
CH_{50} u/ml 150-250	>250					
	150					
	0	•	•	•	•	•
Anti-DNA u/ml 0 - 20	>104		•	•	•	•
	20					
	0					
I.C. ug/ml 0-24	1000		•	•	•	•
	100					
	0					
Therapy	S	•	•	•	•	•
	IS			•	•	
	D.B.	6.11.73	13.8.74	13.11.75	8.11.76	8.7.77

[a]*Silent nephropathy.*

She has continuously shown very high levels of circulating immune complexes and anti-DNA activity in the presence of prolonged hypocomplementemia.

B. Cell-Mediated Immune Responses

1. In a previous report from this laboratory (7) we have shown that the response to a given antigen varied from test to test, in both SLE and control subjects, ranging from being anergic in one test to being definitely reactive in others. Only three SLE patients (13%) had total anergy by DH, LTT, and LIF when performed simultaneously. The MLC results are expressed as relative response and shown on Table IV. A significant response was always obtained in both control and SLE patients, although in the latter group this could only be shown significantly after preculture.

To further study the function of the effector phase of CMI in SLE, we carried out experiments of *in vitro* generation of cytotoxic effector cells in three SLE patients. As shown in Table V, two out of three SLE patients were able to generate and induce the appearance of cytotoxic T cells, which could be demonstrated against normal and SLE target cells.

2. Serum factors

Experiments were designed to explore the modifications produced by SLE serum factors on the reactivity of SLE and normal subjects to allogenic cells. Inhibition, and less frequently stimulation, was observed in both SLE and controls, as a result of culturing in autologous or allogeneic SLE sera. Inhibitory effect was more striking on precultured patients cells (Table VI) suggesting that factors from the patient's own serum might be adsorbed on the lymphocyte membranes and shed when precultured.

3. Nature of serum factors

In order to investigate the possible nature of serum factors, complement-fixing antilymphocyte antibodies were measured in 12 SLE patients and were detected in both active and inactive patients. These did not correlate with the results of lymphocyte responsiveness.

We then proceeded to further investigate the shedding of serum factors from lymphocyte membranes. Preculture fluids from SLE cells were concentrated and variable amounts of immune complexes (IC) were detected by the Raji cell assay. Table VII shows these results where, for example, patient M.Z. exhibits a significant inhibition by autologous serum on her precultured cells (over 1000 µg/ml of immune complexes were detected in preculture fluid).

TABLE IV
One-Way Mixed Lymphocyte Culture[a]

Controls				SLE Patients			
Number	Relative response	Number	Relative response	Number	Relative response	Number	Relative response
1	65	13	138	1	123	11	134
2	105	14	118	2	132	12	98
3	145	15	178	3	53	13	74
4	153	16	153	4	43	14	89
5	95	17	85	5	53	15	46
6	68	18	129	6	79	16	85
7	88	19	72	7	120	17	65
8	57	20	84	8	100	18	118
9	60	21	55	9	133	19	127
10	113	22	77	10	161		
11	174	23	65				
12	165	24	116				
$\bar{x}$ = 107	δ = 38			$\bar{x}$ = 98.36	δ = 32		

[a]*Responding cells: controls and SLE patients. Stimulating cells: third-party mitomycin-treated normal cells in all experiments.*

TABLE V
Lymphocyte-Mediated Cytotoxicity (LMC) after *in vitro* Sensitization of SLE[a]

	LMC	
MLC X 5d[a]	Target ^{51}Cr	^{51}Cr release (%)
1 (Cm)	1	7
	C	0
2 (Cm)	2	1
	C	25
3 (Cm)	2	1
	4	0
	C	17

[a]*Responding cells: 1,2,3,4; C third-party control; Cm mitomycin-treated cells.*

TABLE VI
Modification of MLC by Autologous SLE Sera[a]

	Fresh responding cells			Precultured responding cells		
SLE patient	NHS (cpm)	A.S. (cpm)	I (%)	NHS (cpm)	A.S. (cpm)	I(%)
1	90,100	73,000	19	49,739	7,395	85
2	96,700	69,000	29	56,017	5,126	91
3	67,748	66,634	2	102,358	59,002	42
4	56,775	38,587	32	62,466	38,146	39
5	48,599	32,500	33	44,964	23,693	47
6	63,400	73,300	15	43,803	6,382	86

[a]*NHS: pooled normal human serum; AS: autologous serum; I: inhibition.*

TABLE VII
MLC Inhibition (%) by Autologous Serum on Precultured Cells Compared to Fresh Cells[a]

	Autologous sera inhibition (%)		Immune complex (µg/ml)	
Patients	Fresh cells	Precultured cells	Patients serum	Preculture fluid
M.Z.	2	42	-	>1,000
J.D.	18	57	12.5	80
O.B.	-	82	50	>1,000
M.D.A.	33	47	-	15

[a]*Immune complexes measured in sera and recovered from preculture fluids.*

From these findings we assumed that the nature of these serum factors is at least partly related to immune complexes. To further explore this hypothesis, the influence of heat- and non-heat-inactivated inhibitory sera on EAC rosettes was examined. EAC rosettes were done with either fresh cells or after incubation in 0.5 ml of SLE sera or NHS. We were able to show marked and statistically significant inhibition of EAC rosettes when incubation was carried out with noninactivated SLE sera. This phenomenon was less striking with inactivated sera and absent with NHS. Full recovery to original values (fresh cells) was observed when treated cells were precultured or even just washed once or twice, suggesting a weak interaction between the immune complexes and membrane receptors.

IV. DISCUSSION

Since the discovery by Hargrave *et al.* of the LE cell in 1948 (11), there has been a remarkable advance in the understanding of the pathophysiology of SLE. Two main avenues of research have been stressed: one concerns the abnormalities of antibody-mediated immune responses, illustrated by a myriad of autoantibodies and especially antinucleic acid antibodies, which may lead to the development of an immune complex disease.

The other is concerned with alterations of cell-mediated immune responses, either at the effector level or at the level of immune regulation. Certainly the findings in the NZB model (considered by many investigators as the animal model for human SLE) have greatly influenced these two lines of research.

During the last 4 1/2 years, we have carried out a prospective interdisciplinary study on our SLE population to obtain further knowledge of its physiopathology and natural history (7,10,13). We shall focus our discussion on the following aspects: First, if the evaluation of antibody-mediated immune responses is performed in a serial fashion, a more precise and integrated immunopathological view can be obtained as to the participation of immune complexes and complement in the tissue injury of SLE. Sequentially, the association of high levels of circulating immune complexes, high concentration of anti-DNA antibodies, depressed total hemolytic complement, the appearance of "silent phases" of renal disease, and its possible evolution to clinical nephropathy, further support the hypothesis that SLE is indeed an immune-complex-mediated disease.

The presence of prolonged hypocomplementemia in a SLE patient with the simultaneous utilization of both classical and alternative pathways is an ominous sign, since it correlated with severe clinical disease; further, we have described the malignant form of SLE (11), characterized by rapidly progressing systemic involvement, within a pattern of persistent activity, associated invariably not only with prolonged hypocomplementemia but with high levels of circulating immune complexes and anti-DNA activity. This polar clinical form of SLE is refractory to therapy and has a mean survival of 2 years. On the other end of the spectrum we find patients with a benign course, characterized by prolonged periods of clinical remission and absence of immunological abnormalities and who are usually untreated. Obviously, within the context that SLE, contrary to the NZB mouse, is a very heterogeneous human disease, a wide range of variations concerning immune complexes and the host should be anticipated.

All this accumulated experience, both at the clinical and experimental levels, has allowed the development of the concept of clinical subsets (14) within the lupus complex. We would not only support this notion, but definitely suggest it be extended to areas related to the types of immune responses and to the different genetically determined patterns that may be affecting the development of the clinical subsets.

Second, results on SLE CMI responses are contradictory. While some authors have reported varying degrees of anergy (15,16) others have failed to reproduce such findings (17,18). Our experiments were programmed to simultaneously explore the lymphocyte-mediated responses *in vivo* and *in vitro*. Furthermore, the *in vitro* assays were chosen so as to cover the anti-

gen recognition phase, a subsequent clonal expansion, immunological memory, lymphokine production, and the final cytotoxic effector phase. The varying optimal dose of mitogens from subject to subject and the influence of serum factors made standardization of immunological assays difficult.

Based on our findings, we want to stress the difficulties in studying CMI by isolated *in vitro* tests, since reactivity by a given test does not necessarily correlate with reactivity by other CMI assay (19). In the particular case of SLE, our results suggest that both *in vitro* and *in vivo*, CMI responses are primarily intact. Our data are in disagreement with those obtained in the NZB/W mouse (15,16,20) in which a general depression of CMI was observed.

It has recently been appreciated that serum factors may alter cell reactivity *in vitro*. Data from other labs (21,16), as well as from our own, suggest that the different phases of lymphocyte responses may be inhibited or, less frequently, stimulated by these serum factors. The reports on the nature of these factors have included antilymphocyte antibodies with T cell specifities (22), immune regulatory circulating IgG (23), or else immune complexes (7). Within this context, one might have to consider the role played by drugs, food, or infections. Our own experiments suggest a significant role played by immune complexes in modifying CMI *in vitro* responses. Using the Raji cell method we were able to show immune complexes in the preculture fluid of SLE lymphocytes. It seems probable that the process of shedding makes the cell not only able to fully respond to allogenic cells, but their susceptibility to inhibition by serum factors is greatly enhanced. Inhibitory sera block the formation of EAC rosettes, suggesting immune complex interaction with complement and/or Fc receptors on the surface of the cells. However, the role of antilymphocyte antibodies in these assay systems needs further investigation.

From all the available data, it seems appropriate to propose several points in terms of the physiopathology of SLE:

(1) The normal CMI response in SLE suggest that the pathogensis of this disease might not be related to primary abnormalities of lymphocyte reactivity and makes the existence of a generalized defect in thymic function and/or at the level of immune regulation unlikely.

(2) The evidence of an exaggerated antibody-mediated immune responses, expressed by an immune-complex-mediated disease, by the presence of a great number of autoantibodies and by those antibody systems that secondarily may alter lymphocyte reactivity suggests that a selective defect to immune regulation of antibody synthesis may be a central event in the production of clinical lupus.

(3) Since antibody synthesis is actively regulated by

suppressor cells, we might speculate that this selective defect found in SLE may reside in one of the subpopulations of suppressor cells that control antibody synthesis.

(4) Once the appropriate methodology to separate lymphocyte subpopulations is fully developed and standardized, it would be possible to explore the nature and extent of this proposed selective defect at the regulatory level and its susceptibility to environmental factors.

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