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The immunomodulatory influence of serum factors and its relationship with clinical anergy

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The immune system is a stimulus response organic structure whose outstanding feature is its extraordinary specificity. Its physiologic expression (the immune response is a complex and highly sophisticated biologic process) is not only designed to provide the host with immunity but also to cooperate with other body systems in keeping appropriate conditions of self-antigenic homeostasis.

The immune response is genetically controlled and susceptible to regulatory influences generated in a milieu of cells, membrane receptors, and soluble factors, which guarantee effector mechanisms at both the fluid and the cellular level. The immune response is also susceptible to modulation, which will enhance, decrease, or inhibit its expression as defense or homeostatic mechanism. Thus immunomodulation is an ample concept, enclosing a wide spectrum of normal, pathologic, or induced stimuli with different types of end results. In fact, within the context of normal functioning of defense mechanisms, both regulation and modulation of the immune response may be looked at as merging concepts. During the past few years, as the results of an interdisciplinary approach, our center has focused on the modulatory influences of serum factors on cell reactivity, both in vivo and in vitro, as seen in several models. The basic framework of these investigations[1-8] includes: the simultaneous study of the various phases of cell–mediated immune responses (CMI), the exhaustive standarization of the selected methodology in normal controls, the systematic use of precultured cells, and the investigation of untreated patient populations.

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TABLE I. Allogeneic response (MLC) and cell-mediated lympholysis (CML) in patients with SLE and control patients

MLC (RPI)		CML (% of lysis)	
Patients (n = 12)	Controls (n = 15)	Patients (n = 12)	Controls (n = 15)
1.73	1.63	67	85
1.11	1.56	61	85
0.93	1.56	57	80
0.80	1.34	43	78
0.77	1.07	37	74
0.73	1.05	25	72
0.70	0.97	25	72
0.70	0.92	22	70
0.66	0.90	22	47
0.65	0.79	15	43
0.58	0.69	14	31
0.57	0.65	13	25
	0.64		17
	0.62		13
	0.54		7
$\bar{x}$ ± SD	1.00 ± 0.37	33.4 ± 19	53.3 ± 28
0.83 ± 0.21			
t = 1.24; $p > 0.1$		t = 2.07; $p < 0.025$	

MLC, mixed lymphocyte culture; RPI, relative proliferation index.

In this presentation we will analyze data obtained in three immunoclinical models: systemic lupus erythematosus (SLE), cancer, and infectious diseases. New insights into the definition and clinical implications of anergy will be considered as well.

IMMUNOCLINICAL MODELS

Systemic lupus erythematosus (SLE)

SLE has been associated with several immunopathologic abnormalities,[9-11] among them a depression of CMI responses, and more recently, immunoregulatory disturbances have been linked to the abnormal and exaggerated synthesis of multiple autoantibodies. We have investigated patients with SLE during both active and inactive stages. They were subjected to delayed type of cutaneous responses, using simultaneously three common recall antigens (streptokinase-strepto-

TABLE II. Modification of MLC by autologous SLE sera

SLE patient	Fresh responding cells			Precultured responding cells		
	NHS (cpm)	AS (cpm)	I (%)	NHS (cpm)	AS (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

MLC, mixed lymphocyte culture; NHS, pooled normal human serum; AS, autologous serum; I, inhibition.

TABLE III. MLC inhibition of autologous serum on precultured cells compared with fresh cells

Patients	Autologous sera inhibition %		Immune complex (μg/ml)*	
	Fresh cells	Precultured cells	Patients' serum	Precultured fluid
M. Z.	2	42	—	>1000
J. D.	18	57	12.5	80
O. B.	—	82	50	>1000
M. D. A.	33	47	—	15

MLC, mixed lymphocyte culture
*Immune complexes measured in sera and recovered from preculture fluids.

dornase, *Candida* and purified protein derivative). At the same time, peripheral blood mononuclear cells (PBL) were assayed in vitro to explore mitogenic or allogeneic lymphocyte responses and also their ability to generate cytotoxic T cells to the sensitizing blast. These studies were performed in the presence of either normal or autologous serum. In some experiments, search for immune complexes (IC) like material in the precultured fluid was carried out by the Raji cell method.[12] Cutaneous anergy was rarely seen (three out of 25 consecutive patients). In addition, the different phases of CMI in in vitro responses were intact (Table I). Autologous serum exerted a distinct inhibitory action on mitogenic or allogeneic responses (Table II), particularly on the precultured cells. Variable amounts of IC were detected in precultured fluids (Table III). These results and those obtained investigating SLE cell behavior in solid cultures, precluding direct cell-to-cell contact, prompted us to study cell commmunications between lymphocytes and accessory cells. A selective immunoregulatory disbalance was considered as the most likely mechanism for the exaggerated B cell function, commonly seen among patients with SLE.[2]

In this context, recently using tetradecanoyl phorbol acetate (a T cell mitogen) and highly purified human recombinant interleukin-2 in solid culture conditions, we have found in untreated patients with SLE several cell communication defects, probably simultaneously compromising interleukin-1 and 2 secretion and the lymphocyte response to such molecules, as previously shown by Alcocer-Varela et al.[10] and Linker-Israeli et al.[11] in liquid cultures.

Cancer

In regard to malignant tumors, we have investigated three kinds of human cancer: patients with stage III lung cancer, those with early or advanced gastric tumors, or long-term survivors from gastric or colorectal cancer.[3-6]

While studying the allogeneic responses in both lung and gastric cancer, besides finding an inhibitory action of autologous serum factors in an otherwise intact cell reponse to alloantigen, a second modulatory influence from autologous serum was evident (Table IV); in five out of 18 patients with lung cancer, enhancement rather than inhibition of allogeneic response was observed. This bimodal property of autologous serum seems particularly prevalent among patients with cancer since we also found it in gastric and colorectal carcinoma.[4,5] More recently we compared autologous and allogeneic mixed lymphocyte

TABLE IV. Influence of autologous serum on MLC response in lung cancer

Cases	NHS	AS	Inhibition (%)	Enhancement (%)
A	1.24	0.36	70.97	—
B	0.93	<0.01	100.00	—
C	0.85	<0.01	100.00	—
D	0.87	0.35	59.78	—
E	1.16	1.38	—	15.95
F	1.70	0.54	68.24	—
G	3.90	0.60	84.62	—
H	1.88	2.64	—	28.79
I	0.83	0.07	91.57	—
J	0.80	0.13	83.75	—
K	0.91	0.09	90.20	—
L	0.75	0.20	73.34	—
M	1.16	0.57	50.87	—
N	0.89	1.05	—	17.97
O	1.17	1.07	8.55	—
P	0.82	0.14	17.35	—
Q	0.79	0.93	—	18.53
R	0.76	0.95	—	25.00

MLC, mixed lymphocyte culture; NHS, pooled normal human serum; AS, autologous serum; RPI, relative proliferation index (normal values ≥ 0.66).

reactions in patients with gastric or colorectal adenocarcinoma[6]; it was noted that autologous serum factors were able to induce both inhibition or enhancement in the autologous mixed lymphocyte reaction, although the difference was not statistically significant when compared with the response to both normal human serum and alloantigens (Table V).

Finally, in long-term survivors from gastric or colorectal malignant tumors, not only was cell proliferation to both mitogens and alloantigens preserved but autologous serum exerted a nonsignificant bimodal action.[5] Thus in human cancer, both the recognitive and proliferating capacities of precultured PBL may be unaltered as in the case of lung cancer or long-term survivors from gastrointestinal tumors. Furthermore, those patients with advanced gastric cancer in whom an initial depression of in vitro cell reactivity was found during the pretreatment phase may recover their capacity to respond to mitogens or alloantigens after treatment.[4] In any event, serum factors seem to be of utmost importance in the state of immune competence of the tumor-bearing host.

Infectious diseases

Alterations in the immune response of patients with infectious diseases have been the subject of numerous reports. In fact the description of both "reactive" or "anergic" forms of disease is commonly found among various infectious diseases, particularly those due to fungus or parasites.[13-15] Furthermore, it is well known that viral infections may induce a reversible depression of cell reactivity in vivo and in vitro[16]; we have investigated the immune response of patients with both paracoccidioidomycosis (PCM)[7] and onchocerciasis.[8]

PCM is an endemic fungal disease of the third world. We studied eight active, nontreated patients with PCM and compared them with six patients with inactive disease treated with sulphonamides and with negative cultures. The investigations allowed us to study simultaneously in vivo and in vitro cell reactivity to both mitogens and specific antigens (including PCM antigen) as well as levels of circulating immune complexes and specific antibodies to PCM antigen. As can be seen in Table VI, only two out of eight patients with active disease had in vivo and in vitro anergic responses; furthermore, all inactive patients with PCM showed adequate cell reactivity to both nonspecific and specific stimuli.

In Fig. 1 the evolution of a patient with multisystemic "anergic form" PCM is depicted (patient A. M.). The specific response to PCM antigen (both in vivo and in vitro) was negative and very high levels of circulating immune complexes and specific antibodies to PCM antigen were present. After 18 months of treatment with sulphonamides and amphotericin B,

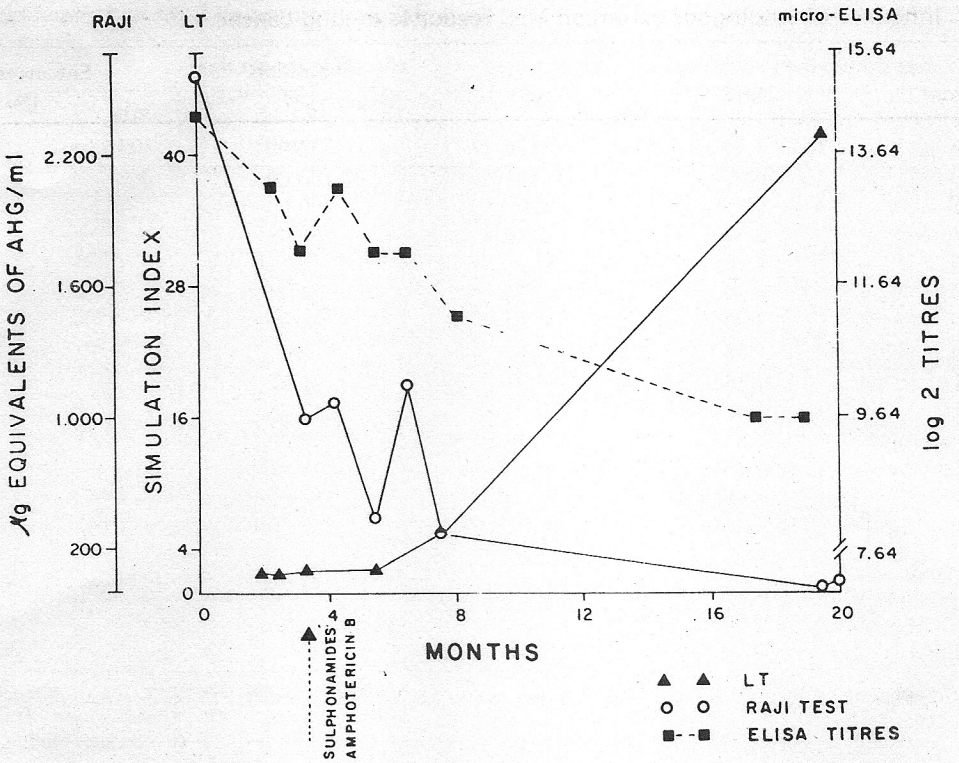

FIG. 1. Evolution of cellular and humoral immunity during treatment of a patient with PCM (A.M.) with disease-related immunodepression.

TABLE V. Autologous mixed lymphocyte reaction in gastric and colorectal adenocarcinoma

Cancer type	Disease stage	cpm	
		NHS	AS
Gastric adenocarcinoma	II	43440	19227
	III	10267	<1000
	III	3095	17504
	IV	<1000	7325
	IV	<1000	<1000
	IV	1981	2926
	IV	<1000	<1000
	IV	13255	19399
	IV	6631	2097
	IV	1599	1504
Colorectal adenocarcinoma	I	2718	1306
	II	1000	<1000
	II	43618	23037
	II	5660	6360
	II	<1000	<1000
	II	3896	6228
	III	<1000	<1000
	III	18287	32148
	III	2480	<1000
	III	5950	6867

NHS, pooled normal human serum; AS, autologous serum.

lymphocyte transformation to PCM antigen gradually became positive along with a decrease in levels of both circulating immune complexes and specific antibodies. Clinical improvement was dramatic. In Table VII we show that the proliferative capacity of the patient's lymphocytes depends to a substantial degree on the conditions of the culture; after preincubation for 18 hours the responses approached those of normal individuals when stimulated in the presence of either autologous serum or homologous sera. The Raji test detected 37.5 μg Eq of aggregated human globulin/ml in the preculture supernatant.

Onchocerciasis is the other infectious disease studied; the disease is highly prevalent among Amerindians of the high Orinoco basin in the Venezuelan Amazonian territory. Among other sequelae, the disease caused by *Onchocerca volvulus* may lead to irreversible blindness. Nineteen patients with untreated active disease underwent exploration.

PBL reactivity to both phytohemagglutinin and alloantigens in the presence of normal or autologous serum were tested; in addition, cell coculture experiments were carried out in which allogeneic response of a control system was tested in the presence of 10 different patient cells and compared with the response when a third-party control cell was cocultured in the system. The results showed: inhibition or enhance-

TABLE VI. Clinical and immunologic status of patients with active (group 1) or inactive (group 2) PCM

Subjects	Age (yr)/sex	Cutaneous reactivity			Lymphocyte transformation			Antibodies micro-Elisa (log_2 titres)	Raji cell (μg/ml)
		PPD	Ca	Pbs	PHA (SI)	Ca (SI)	Pb (SI)		
Group 1									
I. B.	47/M	0	10	0	40.0	1.5	1.0	10.64	118.75
V. B.	54/M	0	0	0	3.0	1.0	1.0	11.64	675
L. E.	51/F	13	12	11	48.0	7.0	27.5	9.64	30
F. I.	35/M	0	9	10	60.0	2.0	10.0	8.64	6.2
A. M.	16/M	0	0	0	11.8	1.0	1.0	13.64	1000
J. M.	27/M	0	0	8	28.0	2.2	6.3	10.64	6.2
R. P.	38/M	0	5	0	40.0	1.8	1.2	12.64	6.2
J. S.	51/M	8	12	10	35.0	8.2	6.7	13.64	75
Group 2									
A. A.	65/M	7	8	5	70.0	13.7	18.0	7.64	37.8
W. N.	10/M	13	10	0	76.5	7.6	15.1	7.64	6.2
A. P.	57/M	16	0	14	67.7	6.1	28.0	8.64	19.5
C. P.	51/M	8	6	10	70.0	3.7	25.0	ND	39.8
M. R.	41/M	13	20	12	113.0	13.5	9.4	8.64	6.2
R. T.	24/M	15	7	15	117.0	3.4	43.0	8.64	6.2

PPD, *Mycobacterium tuberculosis* purified protein derivative; Ca, candidin; Pbs, paracoccidioidin; PHA, phytohemagglutinin; Pb, nonviable particulate *Paracoccidioides brasiliensis* antigen; ELISA, enzyme–linked immunosorbent assay; SI, stimulation index; Raji, circulating immune complex method; ND, not done.

ment of autologous serum on phytohemagglutinin responses (not shown) or inhibition on allogeneic reaction (Table VIII). In addition, it was striking to find (Table IX) that patients' cocultured cells were able to induce either inhibition or enhancement greater than the cutoff limit established in our laboratory (20%) over the response of normal PBL to allogeneic stimulus.

In addition, immune complexlike material was detected in preculture fluid from 14 different patients.

Thus in onchocerciasis, we were able to identify the simultaneous presence of modulatory serum and cell factors; both serum and cell factors may modulate cell function by inhibiting or enhancing its proliferating capabilities.

SYSTEMIC ANERGY: A REAPPRAISAL

As the knowledge of the various modulatory mechanisms of the immune response evolves, new insights on the pathophysiology of both T and B cell reactivity raises the need to review not only the concepts involved but the clinical management of the host defense reactions.

Anergy, its causes, and relevance are some of the immunologic phenomena that have prompted a reappraisal and a new approach at the clinical level.

Anergy is defined as "the state of an organism that has lost the ability to specifically react to an antigen to which it was previously sensitized." Clinically,

TABLE VII. Influence of different treatments on the in vitro proliferative response of AM lymphocytes

Treatment	Stimulation index	
	PHA	*P. brasiliensis*
Stimulated directly	3.92	1.0
Washed and stimulated in		
Autologous serum	24.00	1.0
Homologous serum	30.15	1.7
Washed and incubated 18 hr in medium containing homologous serum and stimulated in		
Autologous serum	93.00	ND
Fetal calf serum	100.77	ND
Homologous serum	151.00	5.8

AM, patient with PCM; PHA, phytohemagglutinin; ND, not done.

anergy is commonly related to the inability to react to a battery of recall skin antigens, since cellular responses are more conveniently studied in the skin (cutaneous anergy). However, we believe that anergy should be viewed and approached in a different way: not as a localized state but as a systemic state of unresponsiveness to a recall antigen. Furthermore, the lack of response and the state of systemic anergy should be related to impairment of T cell function, since other immune compartments, particularly B cell

TABLE VIII. Allogeneic response in onchocerciasis

Patients	NHS*	AS*	% I
1	0.72	0.76	
2	2.24	1.26	44
3	1.19	1.14	
4	1.01	0.79	22
5	0.87	0.76	13
6	1.10	0.95	14
7	0.90	0.65	28
8	1.34	0.94	30
9	1.02	0.57	44
10	1.25	0.39	69
11	0.39	0.36	
12	0.54	0.30	45
13	0.61	0.07	89
14	0.28	0.30	
15	0.32	0.13	59
16	0.61	0.39	36
17	0.50	0.36	28
18	0.52	0.30	58
$\bar{x}$	0.86	0.58	33

NHS, pooled normal human serum; AS, autologous serum; %I, percent inhibition by AS over NHS.
*Relative proliferation index in normal individuals $\geq$ 0.66.

TABLE IX. Cell-mediated modulation on allogenejc response in onchocerciasis

A + Bm + Cm	cpm 50.186	%
A + Bm + P N° 10m	20.377	59*
A + Bm + P N° 5m	24.597	51*
A + Bm + P N° 9m	36.975	26*
A + Bm + P N° 8m	62.447	24†
A + Bm + P N° 7m	75.310	50†
A + Bm + P N° 6m	89.488	78†
A + Bm + P N° 1m	108.665	116†
A + Bm + P N° 2m	110.259	120†

A, B, C, control cells; mitomycin treated; P N°, patients' cells.
*Percentage of inhibition.
†Percentage of enhancement.

function, keep going in the "anergic phase" of chronic or infectious diseases. Therefore we believe that anergy should be more properly defined as "the state of an organism where T cells have lost their ability to specifically react to an antigen to which it was previously sensitized."

The other critical aspect involving systemic anergy is whether it should be regarded as a primary process or a secondary effect; within this context, since a previous contact to an antigen is required, congenital T cell immunodeficiencies should be excluded as true causes of systemic anergy.

TABLE X. Clinical classification of systemic anergy

Primary
Aging
Lymphomas
Chronic lymphocytic leukemias
Secondary
Chronic diseases
Cancer
Uremia
Sarcoidosis
Infectious diseases
Viral
Bacterial
Mycotic
Parasitic
Induced
Surgery
Burns
Malnutrition
Immunosuppression

To establish the clinical diagnosis of systemic anergy and its probable mechanism, integrated and simultaneous in vivo and in vitro cell-mediated studies should be performed.

As we have shown in the data presented above, serum factors may alter cell reactivity, modifying significantly in vitro studies.

The protocol designed, when a given patient is being evaluated, requires not only exhaustive standardization but the assurance that the various phases of CMI reactions have been tested. In addition, uniform clinical criteria and the need to investigate the patient before any treatment is initiated are essential to reach a proper diagnosis of primary or secondary systemic anergy.

As shown in the selected models, serum factors may inhibit or enhance in vitro cell responses. For instance, the inhibitory action of serum factors may explain at least in part the apparent loss of T cell reactivity to a recall antigen, as was found in patients with PCM; the shedding process as the result of preculturing contributes to demonstrate that memory to specific antigen was intact. Similarly, even in advanced cases of human cancer, the proper handling of in vitro methods may elicit preserved responses to mitogen or alloantigens.

The analysis of the nature of immunomodulatory

serum factors is beyond the scope of this presentation; in the presence of the current evidence, research should be orientated to reexamine the so-called "anergic forms" of chronic or infectious diseases.

We propose a new classification of systemic anergy (Table X) that covers both primary and secondary conditions in which a T cell memory and effector responses may be impaired.

Finally, in addition to the clinical and diagnostic implications of systemic anergy, it seems appropriate to suggest that once an anergy status has been diagnosed, new therapeutic strategies should be tried to alter its course and consequences.

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