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Serum-Mediated Suppression of Lymphocyte Transformation Responses in Coccidioidomycosis

REBECCA A. COX[1]* AND RICHARD M. POPE[2]†

Department of Research Immunology, San Antonio State Chest Hospital, San Antonio, Texas 78223,[1] and Department of Medicine, Division of Rheumatology, University of Texas Health Science Center, San Antonio, Texas 78284[2]

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Lymphocyte transformation (LT) responses to coccidioidin (CDN) and spherulin were suppressed in 11 (73%) of 15 patients with active coccidioidomycosis when their mononuclear cells were assayed in autologous serum as compared to serum from healthy, CDN skin test-positive subjects. Suppressed LT responses were specific for *Coccidioides immitis* antigens in 7 (64%) of the 11 patients. Immunoaffinity chromatography of patient sera with *Staphylococcus* protein A adsorbed the suppressor component(s) and thereby established that suppression was attributed to immunoglobulin G, either alone or complexed with antigen. The possibility that suppression was mediated by immune complexes was examined by adding complexes formed in vivo or in vitro to mononuclear cell cultures of healthy CDN-reactive persons before LT assays. Although complexes prepared in this manner were reactive in an enzyme-linked immunosorbent assay designed to detect *Coccidioides* antigen-specific immune complexes, no suppression of LT responses was observed. We conclude that serum-mediated suppression of LT responses in coccidioidomycosis is attributed to monomeric and not immune-complexed immunoglobulin G antibody.

The profile of cellular and humoral immune responses in patients with coccidioidomycosis is well established (4–10, 16, 21, 24, 27–29). Typically, patients with primary, noncomplicated infections manifest strong cellular immune reactivity to antigens of *Coccidioides immitis* and have low levels of serum antibodies. Conversely, patients with chronic or progressive disease have depressed T-lymphocyte reactivity to coccidioidal antigens and high levels of circulating antibodies, occurring alone and complexed with coccidioidal antigens.

The inverse correlation between cellular and humoral immune reactivities has led investigators to postulate that antibody or immune complexes may suppress T-lymphocyte reactivity in coccidioidomycosis (5, 16, 17, 34). Indirect support for this possibility is derived from the finding that sera of patients with active coccidioidomycosis suppress in vitro lymphocyte transformation (LT) responses to *C. immitis* antigens (5, 16, 21). Considering the immunosuppressive effects of immune complexes in other biologic systems (11, 15, 18, 22, 23, 25, 26, 31, 32), we sought to determine if *Coccidioides* antigen-specific immune complexes might be responsible for serum-mediated suppression of LT responses in patients with coccidioidomycosis.

MATERIALS AND METHODS

Study population. Fifteen patients with active coccidioidomycosis and 13 patients who had been in clinical remission for 1 year or longer were evaluated for LT responses. Fifteen healthy subjects who were skin test positive to coccidioidin (CDN) were recruited from the hospital staff.

LT assays. LT assays were performed as described previously (7), with optimal concentrations of CDN (lot TS6772, obtained from D. Pappagianis, University of California at Davis), spherulin (lot 4, Berkeley Biologicals, Berkeley, Calif.), and a culture filtrate preparation of *Candida albicans* (obtained from Marilyn Sutcliff, Veterans Administration Hospital, Nashville, Tenn.). Heparinized blood (20 ml; 20 U of heparin per ml) was obtained as a source of mononuclear cells, and an additional 10 ml of nonheparinized blood was obtained for serum. Mononuclear cells were isolated by centrifugation (450 × *g* for 40 min) on Ficoll-Hypaque as described by Boyum (3). The interface layers were collected and washed twice in Hanks balanced salt solution and once in tissue culture medium 199 (TC199; GIBCO Laboratories, Grand Island, N.Y.). Lymphocytes were enumerated by hemacytometer counts and suspended to a concentration of 2×10^6/ml of TC199 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 30% fresh autologous or heterologous serum. Portions (0.1 ml) of the cell suspensions were dispensed into quadruplicate microtiter wells (Microtest II; Falcon Products, Cockeysville, Md.) containing 0.1 ml of CDN (5 μg [dry weight]), spherulin (5 μg), *Candida* antigen (diluted 1:20), or, for a negative control, 0.1 ml of TC199 without antigen. The cultures were incubated for 5 days at 37°C under a 5% CO_2 humidified atmosphere. Phytohemagglutinin (10 μl of a 1:100 dilution; Difco Laboratories, Detroit, Mich.) was added to quadruplicate wells on day 2, i.e., 72 h before harvest. Cultures were pulse-labeled with 0.5 μCi of [^{3}H]thymidine (2 Ci/mmol; Amersham/Searle, Amersham, England) and harvested 5 h later on glass fiber disks with a MASH II harvester (Microbiological Associates, Bethesda, Md). The disks were transferred to scintillation vials containing 3 ml of a toluene-based scintillation fluid and counted for radioactivity.

Adsorption of sera with protein A. Sera (1.5 ml diluted 1:3 in TC199) were applied to columns containing 1 g of *Staphylococcus* protein A coupled to Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.). After a 15-min incubation at room temperature, 3 ml of the effluent fraction was collected, filter sterilized, and added directly to mononuclear cell cultures. The immunosorbent columns were washed with physiologic saline, and the bound component(s) was eluted with 3 M $MgCl_2$ in Tris buffer or 0.1 M glycine hydrochloride. The

* Corresponding author.
† Present address: Department of Medicine, Northwestern University, Chicago, IL 60611.

TABLE 1. Augmentation of LT responses of patients by sera from healthy CDN-reactive donors[a]

Stimulant	LT response[b] in assays with:	
	Autologous sera (from patients)	Heterologous sera (from healthy subjects)
None	507 ± 135	697 ± 253
Phytohemagglutinin	34,641 ± 6,037	45,510 ± 6,375
CDN	6,391 ± 2,438	15,506 ± 4,609
Spherulin	5,548 ± 1,550	18,089 ± 4,957
Candida antigen	22,273 ± 4,847	38,136 ± 6,850

[a] Mononuclear cells from 15 patients with active coccidioidomycosis were assayed.
[b] Uptake of [^{3}H]thymidine (mean counts per minute ± standard error).

desorbed fraction was dialyzed against distilled water, lyophilized, and dissolved in 1.5 ml of normal human serum, which was then diluted 1:3 in TC199.

Isolation of immune complexes. Immune complexes were isolated from sera of patients by precipitation with 3% polyethylene glycol (PEG; 6 kilodaltons) as described previously (34). The precipitated complexes were washed by centrifugation in 3% PEG, dialyzed against distilled water, and lyophilized. Before assay, the PEG-precipitated complexes were dissolved in normal human serum.

In vitro formation of immune complexes. CDN (1 to 100 μg [dry weight]) was added to 2 ml of serum from a patient with coccidioidomycosis who had a complement-fixing antibody titer of 1:512 and was negative for immune complexes as tested by the enzyme-linked immunosorbent assay (ELISA) described below. After overnight incubation at 4°C, the complexes were isolated by precipitation with 3% PEG and dissolved in normal human serum.

Immune complex assay. *Coccidioides* antigen-specific immune complexes were detected by an ELISA. Thiocyanate-activated disks (Cordis Laboratories, Miami, Fla.) were coated with 200 ng of rabbit $F(ab')_2$ anti-human immunoglobulin G (IgG) (heavy chain specific) or with the $F(ab')_2$ fragment of normal rabbit IgG, prepared by pepsin digestion before chromatography on Sephadex-G200 (34). Nonreactive sites on the $F(ab')_2$-coated disks were blocked with 6% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20. Test samples (50 μl diluted 1:20 in bovine serum albumin–phosphate-buffered saline–Tween 20) were added and incubated at 37°C for 4 h. After three washes in phosphate-buffered saline–Tween 20, 1 ml of alkaline phosphatase-labeled rabbit anti-CDN, prepared as described below, was added, and the disks were incubated overnight at 37°C. Nonbound rabbit anti-CDN was removed by five washes with phosphate-buffered saline–Tween 20, and 1 ml of *p*-nitrophenyl phosphate (Sigma) diluted to 1 mg/ml in diethanolamine was added. The disks were incubated for 1 h at 37°C, and the enzymatic reactions were terminated by the addition of 0.5 ml of 3 N NaOH. Results were calculated as the difference between the A_{405} of disks coated with rabbit $F(ab')_2$ anti-human IgG and that of disks coated with normal rabbit $F(ab')_2$. Neither monomeric nor heat-aggregated IgG (Cohn fraction II heated at 63°C for 20 min) was positive by this assay, nor was the addition of CDN to normal human serum detected by this assay.

The anti-CDN used to detect immune-complexed antigen was isolated from the serum of a rabbit immunized at monthly intervals during a 1-year period with CDN (lot TS6772) administered in complete Freund adjuvant (Difco). The immunoglobulin fraction was obtained by precipitation of serum with an equal volume of saturated $(NH_4)_2SO_4$ and affinity purified by chromatography on a CDN-Sepharose 4B column (34). Adsorbed rabbit anti-CDN was eluted in 3 M $MgCl_2$ in Tris and dialyzed against distilled water and then saline. Affinity-purified rabbit anti-CDN was conjugated to alkaline phosphatase (Sigma) as described by Voller et al. (33).

Statistical analyses. Comparison of LT responses in autologous serum with those in heterologous serum and comparison of LT responses in the presence or absence of added immune complexes were done by Student's paired *t* test.

RESULTS

Effect of serum source on LT responses. Serum-mediated suppression of LT responses was assessed by comparing the blastogenic responses of cells from coccidioidomycosis patients in autologous serum and in serum from healthy CDN-reactive donors. The results obtained in assays of sera of 15 patients are shown in Table 1. Eleven (73%) of these patients showed twofold or greater decreases in LT responses to CDN and spherulin when assays were performed in autologous serum instead of in normal heterologous serum ($P < 0.01$ and $P < 0.005$ for CDN and spherulin, respectively). Suppression was antigen specific in most patients. That is, only 4 (27%) of the 11 patients showed a suppression of their response to *Candida* antigen in the presence of autologous serum.

Reciprocal assays were performed to determine whether sera from patients would suppress the LT responses of healthy CDN-reactive persons (Table 2). Sera from 7 (47%) of the 15 patients suppressed proliferation to coccidioidal antigens by twofold or more. Suppression was antigen specific with sera from three (43%) of the seven patients. The mononuclear cells of the same three patients exhibited antigen-specific suppression when their cells were cultured in autologous serum but not when they were cultured in heterologous sera from healthy subjects.

In contrast to the results obtained in assays of sera of patients with active coccidioidomycosis, LT responses of 13 patients in clinical remission were not augmented by the addition of sera from healthy CDN-reactive donors, nor did sera from patients with inactive coccidioidomycosis suppress the LT responses of healthy subjects (data not shown).

Adsorption of the suppressor factor(s) with protein A. To determine if suppression of LT responses could be attributed to IgG antibody, sera from three patients who exhibited antigen-specific suppression were applied to solid-phase immunoadsorbents containing protein A and then were assayed for suppressor activity with mononuclear cells of healthy CDN-reactive persons. The results (Table 3) estab-

TABLE 2. Suppression of LT responses of healthy CDN-reactive subjects by sera of patients with active coccidioidomycosis[a]

Stimulant	LT response[b] in assays with:	
	Autologous sera (from healthy subjects)	Heterologous sera (from patients)
None	1,731 ± 720	1,588 ± 409
Phytohemagglutinin	46,349 ± 6,460	41,241 ± 6,833
CDN	21,660 ± 3,047	13,304 ± 3,446
Spherulin	20,785 ± 3,199	12,782 ± 3,165
Candida antigen	32,176 ± 5,714	18,958 ± 4,120

[a] Mononuclear cells of 15 healthy CDN-reactive subjects were assayed.
[b] Uptake of [^{3}H]thymidine (mean counts per minute ± standard error).

TABLE 3. Effect of adsorption of sera with *Staphylococcus* protein A on LT responses of healthy CDN-reactive subjects

Serum source	LT response[a] to CDN in:	
	Unadsorbed serum	Adsorbed serum
Patient with active coccidioidomycosis		
1	9,354	18,799
2	9,786	26,951
3	16,268	35,474
Mean ± SEM	11,803 ± 2,236	27,075 ± 4,814
Healthy subject		
1	36,226	21,271
2	15,324	12,587
3	24,865	25,843
Mean ± SEM	25,472 ± 6,042	19,900 ± 3,888

[a] Uptake of [^{3}H]thymidine (counts per minute).

lish that LT responses to CDN were significantly increased in the presence of adsorbed sera from patients ($P < 0.0001$). The effect was antigen specific, i.e., LT responses to *Candida* antigen in adsorbed and unadsorbed sera were comparable (data not shown). In contrast to the results obtained with sera from patients, no significant difference was observed between the LT responses of healthy subjects in the presence of adsorbed or unadsorbed autologous serum (Table 3).

Attempts to demonstrate suppressor activity with the column eluate fraction from protein A immunoadsorbents were not consistently successful, i.e., the elute fraction proved toxic or, in some instances, stimulatory to lymphocyte cultures, possibly as a result of the desorption of protein A (19). In one experiment, however, the eluate fraction was recovered with 3 M $MgCl_2$ and shown to specifically suppress the LT responses of a healthy CDN-reactive donor to CDN and spherulin (Table 4).

Effect of in vivo- and in vitro-formed immune complexes on LT responses. The adsorption of serum suppressor activity with protein A was consistent with, but did not distinguish between, suppression of LT responses by monomeric or immune-complexed IgG antibody (20). Studies were undertaken, therefore, to isolate immune complexes formed in vivo and assess their effect(s) on the LT responses of healthy CDN-reactive persons.

The results obtained with immune complexes isolated from sera of patients by precipitation with 3% PEG are shown in Table 5. Although the PEG-precipitated complexes

TABLE 4. Effect of protein A eluate (desorbed) fraction on LT responses of a healthy CDN-reactive subject

Stimulant	LT response[a] in assays with:	
	Autologous serum	Autologous serum + eluate
None	299	260
Phytohemagglutinin	33,547	35,060
CDN	26,402	8,699
Spherulin	18,276	8,373
Candida antigen	13,827	13,767

[a] Uptake of [^{3}H]thymidine (mean counts per minute).

TABLE 5. Effect of PEG-precipitated immune complexes from sera of patients on the LT responses of a healthy CDN-reactive subject

Amt (μg) of complexes added	Reactivity (A_{405}) complexes in ELISA	LT response to CDN (cpm)
0	0.068	21,622
10	0.423	19,136
25	0.729	19,302

were reactive in the ELISA for *Coccidioides* antigen-specific complexes, no suppression of LT responses to CDN was observed. To rule out the possibility that the complexes may have lost suppressor activity during dialysis or lyophilization of the PEG precipitates, immune complexes were dissolved in normal human serum immediately after precipitation with PEG. The results were consistently negative for suppressor activity (data not shown).

Studies were next conducted to determine whether immune complexes formed in vitro by the addition of CDN to patient serum containing antibody (complement-fixing titer of 1:512) but negative for complexes by ELISA and by the C1q-binding assay (34) would suppress LT responses to CDN. The results obtained with complexes prepared in this manner and precipitated by 3% PEG are shown in Table 6. Although the PEG-precipitated material was highly reactive in the ELISA, addition of the complexes to mononuclear cell cultures was without effect on LT responses to CDN.

The preceding results, taken together, argue against immune-complex-mediated suppression of LT responses but do not preclude the possibility that immunosuppressive complexes may have been present but not precipitated by 3% PEG. To examine this possibility, incremental doses of CDN ranging from 0.01 to 20 μg were added to patient sera containing antibodies but not immune complexes. The in vitro-formed complexes were added directly to mononuclear cell cultures without previous precipitation with PEG. In no instance was suppressor activity demonstrable (data not shown). The addition of higher doses of CDN (i.e., >20 μg) proved toxic to antigen-stimulated cultures and could not be evaluated for suppressor activity.

DISCUSSION

Numerous studies have established that cellular immunity is crucial to host defense in coccidioidomycosis (1, 2, 5). Elucidation of mechanisms that might suppress T-lymphocyte reactivity to *C. immitis* is, therefore, of primary importance. The present study was undertaken to determine whether suppression of LT responses by sera from patients with coccidioidomycosis might be ascribed to immune complexes. The results established that the LT responses of patients with active coccidioidomycosis, to *C. immitis* antigens were significantly suppressed when these cells were

TABLE 6. Effect of in vitro-formed immune complexes on the LT responses of a healthy CDN-reactive subject

Amt (μg) of CDN added to serum from patient	Reactivity (A_{405}) of PEG-precipitated complexes in ELISA	LT response to CDN (cpm)
0	0.004	30,924
1	0.003	35,984
10	0.147	28,637
100	1.089	36,635

assayed in autologous serum and that, conversely, sera of patients suppressed the LT responses of cells from healthy CDN-reactive subjects. The specificity of serum-mediated suppression in some patients, together with the adsorption of the suppressor factor(s) by *Staphylococcus* protein A, provided evidence that suppression was due to anti-*Coccidioides* IgG, either alone or complexed with *C. immitis* antigen(s). Although significant levels of immune complexes, compromised of coccidioidal antigen(s) and anti-*Coccidioides* IgG, were demonstrable in sera that suppressed LT responses, immune complex levels did not correlate with suppressor activity (data not shown). Moreover, immune complexes isolated from sera of patients or formed in vitro by the addition of CDN to patient sera failed to suppress LT responses to *C. immitis* antigens. These results argue against the likelihood that serum-mediated suppression is caused by immune complexes. An alternative possibility is that suppression is attributed to anti-*Coccidioides* IgG, in which case the question must be raised as to whether anti-*Coccidioides* IgG directly suppresses T-lymphocyte proliferation or merely neutralizes the coccidioidal component that stimulates LT responses. One other mechanism that would be consistent with the results is suppression of LT responses by an anti-idiotypic antibody, e.g., by its interaction with a T-lymphocyte population bearing idiotypic determinants (12–14, 30). Studies to examine this possibility have not been performed.

Our inability to demonstrate immune-complex-mediated suppression of LT responses does not preclude the possibility that immune complexes are immunosuppressive in patients with coccidioidomycosis. In a recent investigation, we reported that intravenous injection of normal BALB/c mice with sera from syngeneic donors infected with *C. immitis* suppresses the response of recipients to immunization with CDN (6). Analyses of the suppressor sera by modifications of the ELISA used in the study presented here established the presence of *C. immitis* antigen-specific immune complexes and low or nondetectable levels of free antibody. Further investigations with this murine model should help elucidate an immunosuppressive role(s), if any, of immune complexes in cases of coccidioidomycosis.

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