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Serum Regulation of In Vitro Lymphocyte Responses in Early Experimental Syphilis

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Sera from rabbits with early experimental syphilis were tested for their effect on in vitro lymphocyte transformation responses to related specific antigens (sonicated *T. pallidum*), unrelated specific antigens (sheep erythrocytes), and the T cell mitogen, concanavalin A. Results were compared with responses in preinfection sera and in sera from sham-infected rabbits. Titration experiments in which normal serum was used indicated that optimal lymphocyte responsiveness is obtained with a final serum concentration of 1%. Under these conditions, no differences in concanavalin A stimulation were observed in cultures with syphilitic sera. Responses to sonicated *T. pallidum* were inhibited, but only by 17 to 25% when compared with the response in preinfection sera. In cultures containing 10% serum, inhibition of lymphocyte proliferation to sonicated *T. pallidum* antigens was evident with sera from all syphilitic animals from day 10 (55% inhibition) through day 31 (80% inhibition) of infection. Responses to concanavalin A and sheep erythrocytes were significantly inhibited by day 10 sera; only 20% of the sera tested demonstrated substantial nonspecific inhibitory capacity. No differences were evident among sera from any of the sham-infected animals or among the preinfection sera from either group. Pooled serum with high inhibitory activity was fractionated by ammonium sulfate precipitation, DEAE ion exchange chromatography, and Sephadex G-200 gel filtration. Two separate inhibitors were identified: (i) a low-molecular-weight, ammonium sulfate-soluble, nonspecific inhibitory fraction containing albumin and alpha-globulins with the capacity to inhibit both antigen and mitogen responses and (ii) a high-molecular-weight, ammonium sulfate-precipitable, inhibitory fraction containing alpha-globulin and FTA-ABS-reactive immunoglobulin M which affected only the antigen-specific response to sonicated *T. pallidum*. Immunodiffusion failed to detect immunoglobulin or *T. pallidum* antigens in either fraction. DEAE-purified immunoglobulin G from immune serum was not inhibitory.

The presence of humoral factors which affect lymphocyte transformation in vitro has been suggested by numerous studies examining such pathological conditions as leprosy (36), multiple sclerosis (14), sarcoidosis (18), alcoholic cirrhosis (19), and chronic candidiasis (15). Other investigators have found that normal serum contains substances which may profoundly influence in vitro lymphocyte responses, including alpha-globulins (7–9), low-density lipoproteins (10), and others (6, 34). Furthermore, a variety of simple sugars may markedly inhibit antigen-specific lymphocyte proliferation (31). The relationship of these in vitro effects to immune responses in vivo remains unclear.

The impairment of lymphocyte responsiveness to mitogens or treponemal antigens in the presence of serum or plasma from syphilitic humans or experimentally infected rabbits has been described, but conflicting data have been reported. Levene et al. (22) found that plasma from 9 of 12 patients with secondary syphilis reduced the blast transformation response of normal peripheral blood lymphocytes (PBL) to phytohemagglutinin (PHA) by 40% compared with the response in normal human plasma. This effect was not exhibited by plasma from primary or latent syphilitics. Friedmann and Turk (16) found that plasma from patients with early syphilis caused 58 and 36% inhibition of the purified protein-derivative and PHA responses, respectively, as compared with control responses but that plasma from patients with latent or cardiovascular syphilis did not inhibit the responses.

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Thompson et al. (51) have also reported that plasma from some primary and many secondary cases slightly reduces the responses of lymphocytes to PHA and to allogeneic lymphocytes. These responses were, for the most part, still within the lower limits of the responses in normal plasma. When selected plasma were retested on normal PBL, inhibitory activity was still demonstrable.

Conflicting results have been observed by other investigators. From et al. (17) were unable to show any inhibition of the responses of normal PBL to PHA or pokeweed mitogen by plasma from nine patients with early syphilis, although they did report a minor transient decrease in concanavalin A (ConA) responsiveness. Musher and Schell (32) and Musher et al. (33) found no plasma-mediated inhibition of the responses of PBL from normal or syphilitic individuals to pokeweed mitogen, PHA, or streptolysin O.

The effect of serum factors on in vitro lymphocyte transformation in experimental syphilis is also unclear. Pavia et al. reported a 40% reduction in the response of PBL to ConA (40) and a 33 to 66% reduction in the response of sensitized lymphocytes to treponemal antigens (41) in the presence of sera from rabbits with early syphilis. Ware et al. (54) reported reduced transformation of normal PBL to ConA by 71% of sera from rabbits at the peak of orchitis (days 9 to 14). The effect was lost rapidly with titration and could be abrogated by increasing the ConA concentration. Adsorption of sera with *Treponema pallidum*, rabbit erythrocytes, or rabbit PBL did not remove the activity (55). Wicher and Wicher (56) found a variable inhibition of the PHA response of normal rabbit PBL cultured in sera from rabbits infected for 10 days to 6 months. Bey et al. (5) reported inhibition of ConA responses by sera from intratesticularly infected rabbits and found it to be associated with the presence of a mucopolysaccharide material. Finally, Maret et al. (28) were unable to show an effect of autologous serum on PHA or ConA responsiveness of splenic lymphocytes from syphilitic rabbits; however, specific responses to *T. pallidum* antigens were inhibited.

Despite these numerous studies, the mechanism of the inhibition of, the nature of the factor or factors responsible for, and the biological significance of inhibition of in vitro lymphocyte transformation mediated by syphilitic serum remain obscure. This report includes an investigation of the effect of serum obtained during syphilitic infection on specific and nonspecific T cell responses as well as a partial characterization of the nature of the lymphocyte inhibitory serum components from *T. pallidum*-infected rabbits.

(This study was presented in part at the Annual Meeting of the American Society for Microbiology [S. A. Baker-Zander, S. A. Lukehart, and S. Sell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, E13, p. 57].)

MATERIALS AND METHODS

Animals. Adult male New Zealand rabbits were obtained from Rancho de Conejo, Vista, Calif., or R & R Rabbitry, Stanwood, Wash. Each rabbit was tested for evidence of *Treponema paraluis-cuniculi* infection by the Venereal Disease Research Laboratory (VDRL) test or the fluorescent treponemal antibody-absorbed (FTA-ABS) test or both. Only those rabbits with negative serological tests as well as an absence of clinical signs of infection were included in this study. All rabbits were housed individually at 19 to 20°C and given antibiotic-free food and water.

Source of treponemes. *T. pallidum*, Nichols strain, was generously provided by James N. Miller, University of California, Los Angeles, and was passed intratesticularly in rabbits twice weekly. This source was found not to be contaminated with the passenger pleural effusion agent (13).

Experimental infection. Six VDRL-nonreactive rabbits were infected intratesticularly with 3×10^7 *T. pallidum* cells per testicle suspended in 1 ml of 10% normal rabbit serum in 0.14 M saline. Concurrently, four VDRL-nonreactive male rabbits were sham infected with serum-saline alone. Organisms for inoculation were extracted from the testes of a 10-day infected rabbit; gross tissue debris was removed by centrifugation at $280 \times g$ for 10 min, and the motile organisms in the supernatant were counted by dark-field microscopy. All six infected rabbits developed a palpable orchitis by day 10 of infection. Sham-infected animals showed no such signs. Rabbits were weighed on the day of infection and at regular intervals thereafter; no significant differences between the two groups were detected.

Serum collection. Whole blood (20 ml) was collected by cardiac puncture immediately before infection (day 0) and subsequently on days 10, 17, 24, and 31. Blood was allowed to clot in sterile glass tubes for 3 h at room temperature, and serum was collected after centrifugation at $500 \times g$ for 10 min. Sera were divided into aliquots and stored in sterile glass tubes at −20°C. Sera were heat inactivated at 56°C for 30 min before testing in the lymphocyte blastogenic assay.

Serum fractionation. Serum fractions were also prepared for testing in the lymphocyte transformation assay. Equal volumes of serum from two rabbits (infected for 17 days) were heat inactivated and pooled, and 20 ml was fractionated at room temperature with 50% saturated ammonium sulfate (30). The pellet (globulin) fraction was dissolved in 10 ml of saline. Both the globulin and albumin fractions were dialyzed repeatedly versus saline and then versus phosphate-buffered saline in the cold. The albumin fraction was concentrated to 20 ml with an Amicon unit (Amicon Corp., Lexington, Mass.) with a PM 30 filter.

The globulin fraction was adjusted to a volume of 20 ml, and 10 ml was separated by DEAE chromatography. Immunoglobulin G (IgG) was eluted first with

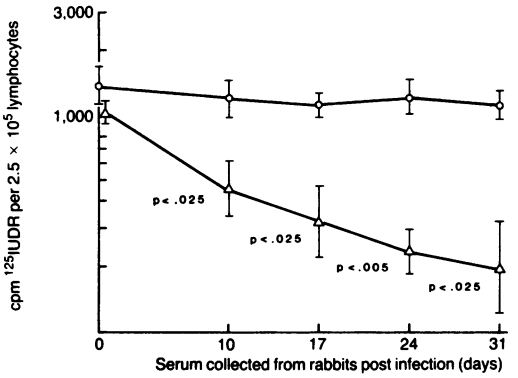

FIG. 1. Results from individual sera obtained sequentially from six *T. pallidum*-infected (△) and four sham-infected (○) rabbits and tested in triplicate cultures with splenic lymphocytes from an intratesticularly infected rabbit (30 days post-inoculation). The final serum concentration in culture was 10%. In vitro lymphocyte responses to *T. pallidum* antigens were determined, and background counts for unstimulated cultures were subtracted (≤400 cpm). The geometric mean and standard error for each group of sera are plotted.

0.0175 M phosphate buffer, pH 6.8 (purity was determined by immunoelectrophoresis and precipitation with goat anti-whole rabbit serum antiserum). A second large peak was eluted with a continuous gradient of phosphate buffer from 0.0175 to 0.3 M. Each peak was dialyzed versus phosphate-buffered saline and concentrated to 10 ml by Amicon filtration.

The albumin and globulin fractions were further fractionated by Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) chromatography. Peaks were pooled and concentrated by Amicon filtration. Molecular-weight calibration was determined with blue dextran 2000 (Pharmacia Fine Chemicals), rabbit IgG (molecular weight, 150,000), and bovine serum albumin (molecular weight, 68,000; Sigma Chemical Co., St. Louis, Mo.). Similar separations were performed on pooled normal rabbit serum (NRS).

Selected fractions were tested by microzonal electrophoresis on cellulose acetate strips (Beckman Instruments, Inc., Fullerton, Calif.). Proteins were visualized by staining with Ponceau S dye. Fractions were also tested by immunoelectrophoresis and precipitation with goat anti-whole rabbit serum antiserum, goat anti-rabbit immunoglobulin antiserum (Cappel Laboratories, Inc., West Chester, Pa.), human anti-*T. pallidum* antiserum (Beckman Diagnostics), and pooled rabbit anti-*T. pallidum* antiserum. The FTA-ABS test was modified to test for IgM antibody by including an intermediate incubation with goat anti-rabbit IgM (Cappel Laboratories) and then incubation with fluorescein isothiocyanate-conjugated rabbit anti-goat antiserum (Cappel Laboratories). Carbohydrate content was determined by the phenol-sulfuric method (20), and protein was determined by the method of Lowry et al. (23).

Serological Tests. The VDRL slide flocculation and FTA-ABS tests were performed as described in the *Manual of Tests for Syphilis* (53) with some modification (26).

Lymphocyte Culture. Spleen cells were obtained from rabbits which had been infected with *T. pallidum* for 30 days (4×10^7 to 8×10^7/ml intratesticularly) or from rabbits which had been immunized with sheep erythrocytes (SRBC) 14 days previously (5 ml of 0.3% [vol/vol] SRBC injected intravenously). Spleens were removed aseptically, and the cells were prepared for culture as previously described (27).

Lymphocytes were cultured, pulsed, and harvested as previously described (27). The responsiveness assays were performed in a total volume of 0.2 ml, consisting of medium plus various concentrations (from 0.1 to 20%) of serum or serum fractions. Lymphocytes were tested in pooled NRS obtained from four VDRL-nonreactive rabbits or in sera from individual animals.

Mitogens. ConA (Miles Laboratories, Inc., Elkhart, Ind.) was stored frozen at 1 mg/ml in 1 M sterile saline and routinely used at 1 to 2 μg per microculture. Sonicated *T. pallidum* was prepared as previously described (27), and 10^7 sonicated organisms were added per microculture. SRBC were washed three times in balanced salt solution and diluted to 0.05% (vol/vol) in RPMI 1640 medium, and 10 μl was added per microculture. We have previously shown that lymphocyte transformation responses to ConA (47), SRBC (unpublished data), and *T. pallidum* antigens (27) are T cell functions.

Statistics. The geometric mean of three to six replicate cultures was calculated. After subtraction of counts in unstimulated cultures (200 to 400 cpm), the mean and standard error of each response for a given group of sera were recorded. Responses between groups were compared, using Student's *t* test.

Immune complex determinations. Serum was collect-

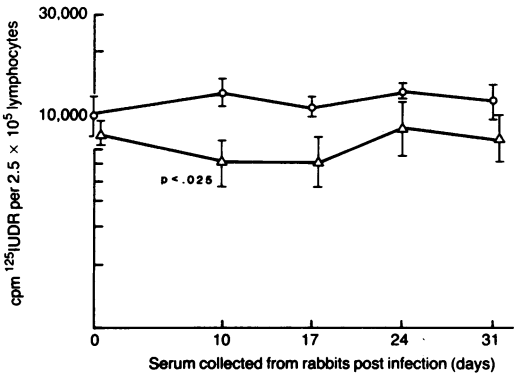

FIG. 2. Results from individual sequential sera obtained from sham-infected (○) and *T. pallidum*-infected (△) rabbits at the times specified and tested in triplicate as described in the legend to Fig. 1. In vitro lymphocyte responses to ConA were determined, and background counts for unstimulated cultures were subtracted (≤400 cpm). The geometric mean and standard error for each group of sera are plotted.

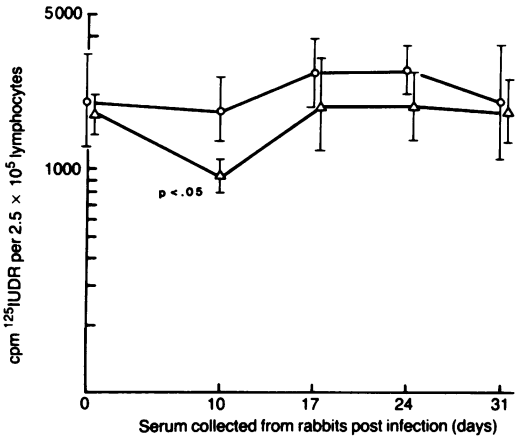

FIG. 3. Results from individual sera from five *T. pallidum*-infected (△) and four sham-infected (○) rabbits tested in quadruplicate with lymphocytes from a normal rabbit which had been immunized with SRBC intravenously 14 days before sacrifice. In vitro lymphocyte responses to SRBC antigens were determined. The final concentration of serum in culture was 10%. Background counts for unstimulated cultures were subtracted (≤350 cpm). The geometric mean and standard error for each group of sera are plotted.

ed from 25 adult male rabbits before intratesticular infection with 8×10^7 to 10×10^7 *T. pallidum* cells. Five animals per group were bled and sacrificed at 10, 17, 24, 31, and 60 days after infection. Serum was collected for immune complex determinations, and splenic lymphocytes were cultured in the lymphocyte responsiveness assay in the presence of 10% NRS or 10% autologous serum as previously described. Circulating immune complexes (CICs) were determined by the Raji cell radioimmune assay of Theofilopoulos et al. (50). Normal rabbit IgG was heat aggregated at 63°C for 30 min and used as the control (AGG). The amount of complexes in each serum tested was expressed as micrograms of AGG equivalent per milliliter of serum. The limit of sensitivity of this test was 10 μg of AGG per ml of serum.

RESULTS

Effect of 10% serum from syphilitic rabbits infected with *T. pallidum* on in vitro lymphocyte responses. Serum samples from rabbits infected intratesticularly with *T. pallidum* and from sham-infected rabbits were tested at 10% concentration in culture for their effect on the in vitro blastogenic response of splenic lymphocytes to sonicated *T. pallidum*, ConA, and SRBC. Inhibition of in vitro blast responses to *T. pallidum* antigens (Fig. 1) was evident when all sera taken on days 10 through 31 of infection (55 to 80% inhibition) were used, as compared with responses seen in preinfection sera. No differences were found among any of the sera from sham-infected rabbits or among the baseline sera from either group.

A slight depression of ConA responsiveness was observed when cells were cultured in sera acquired on days 10 and 17 of infection (an approximate 25% reduction as compared with the response in preinfection sera [Fig. 2]). Only 20% of the sera tested inhibited the response by 50% or greater. Again, the responses to ConA in sera from sham-infected rabbits remained uniform throughout the test period.

Sera obtained from syphilitic rabbits on day 10 of infection reduced the response of sensitized spleen cells to SRBC by 53% as compared with preinfection sera (Fig. 3). No differences in responses were observed in sera from days 17 through 31 of infection.

Variability of responses in NRS. The initial experiments for this study were performed by supplementing cultures with 10% serum as described previously (1, 26, 27). The in vitro system was further characterized to address the following two pertinent points: (i) the variability among responses in normal sera and (ii) the optimum serum concentration for rabbit splenic lymphocyte responses in vitro. Lymphocytes from a single donor rabbit were stimulated with treponemal antigens or ConA in the presence of 13 different putatively normal sera (VDRL- and FTA-ABS-nonreactive rabbits). The variability of lymphocyte responsiveness in cultures supplemented with 10% NRS is shown in Table 1. The range of the peak response of lymphocytes to sonicated *T. pallidum* varied from 4,654 to 15,287 cpm. ConA responses varied from 10,470

TABLE 1. Variability of in vitro lymphocyte responsiveness in different individual NRS

Rabbit no.	[^{125}I]UDR uptake (cpm)[a]:	
	Sonicated *T. pallidum*	ConA
1	7,371	19,376
2	6,876	15,042
3	8,564	10,470
4	15,037	22,960
5	7,270	23,567
6	13,628	26,134
7	15,287	23,211
8	4,654	22,222
9	9,298	21,847
10	12,359	24,993
11	6,647	24,779
12	7,536	22,988
13	14,582	33,207
Pool[b]	6,506	18,271

[a] Background values have been subtracted (≤350 cpm), and the geometric mean of four replicate cultures is shown.

[b] Pool consists of 13 sera tested.

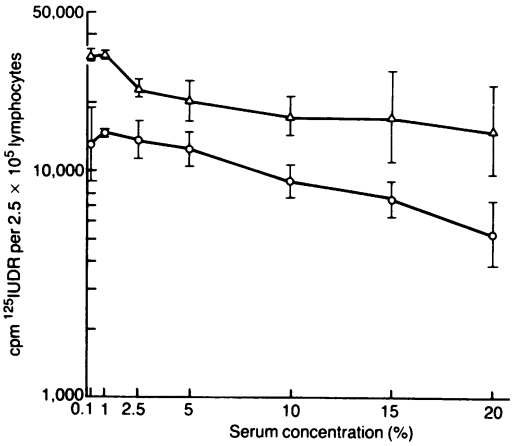

FIG. 4. Splenic lymphocyte responses to treponemal antigens (○) and ConA (△) determined in various concentrations of pooled NRS. Background counts from unstimulated cultures were subtracted (≤250 cpm). The geometric mean and standard error for each concentration of NRS are plotted.

to 33,207 cpm. When these sera were pooled and tested, the response to sonicated *T. pallidum* and ConA was 6,506 and 18,271 cpm, respectively. These data serve to illustrate the inherent variability observed when testing lymphocyte responsiveness in high (10%) serum concentrations.

Determination of optimal serum concentrations for in vitro lymphocyte responses. To determine the effect of serum concentration on in vitro responses, splenic lymphocyte responses to sonicated *T. pallidum* antigens or ConA were assessed in various concentrations of pooled NRS. Cultures containing 1% serum demonstrated maximal lymphocyte proliferation (Fig. 4). The responses to sonicated *T. pallidum* and ConA in 10% serum were 63 and 54%, respectively, of the responses in 1% serum. It is also important to note that the variability of results was reduced in 1% serum (as indicated by the magnitude of the standard errors).

Effect of 1% sera from syphilitic rabbits on lymphocyte proliferation. The battery of sera from *T. pallidum*-infected rabbits was retested at 1% serum concentration in culture. The results of lymphocyte stimulation with treponemal antigens or ConA are shown in Fig. 5A and B. Responses to sonicated *T. pallidum* were slightly reduced on days 10 through 31 (17 to 25%) as compared with sera collected before infection (Fig. 5A). ConA responses (Fig. 5B) were virtually equivalent in all sera tested and were actually slightly higher in sera collected on day 31 of infection. As previously mentioned, the variability of responses in 1% serum was greatly reduced from that seen in 10% serum.

Effect of serum fractions on lymphocyte proliferation. An attempt was made to isolate the inhibitory factor in sera from rabbits infected with *T. pallidum*. The activity of serum fractions, as well as that of the pooled whole serum from which they were derived, was tested in the lymphocyte responsiveness assay with splenic lymphocytes from rabbits 30 days postinfection. The results determined in immune rabbit serum (IRS) fractions were compared with those determined in the appropriate NRS control fractions (Table 2). Two separate experiments are represented in Table 2. Background counts from unstimulated cultures were subtracted (≤300 cpm). Whole IRS inhibited the response to sonicated *T. pallidum* (antigen) and ConA (mitogen) by approximately 80 and 83%, respectively. Likewise, the albumin fraction of IRS inhibited the antigen and mitogen responses approximately 89 and 63%, respectively. However, the whole globulin fraction and DEAE-fraction 2 of globulin from IRS inhibited the antigen response approximately 90% while only slightly affecting the mitogen response. DEAE-purified IgG from IRS showed virtually no effect on antigen or mitogen responses.

The inhibitory effects shown were not due simply to an absence of necessary growth factors, because all cultures were supplemented with 1% whole NRS. Trypan blue exclusion

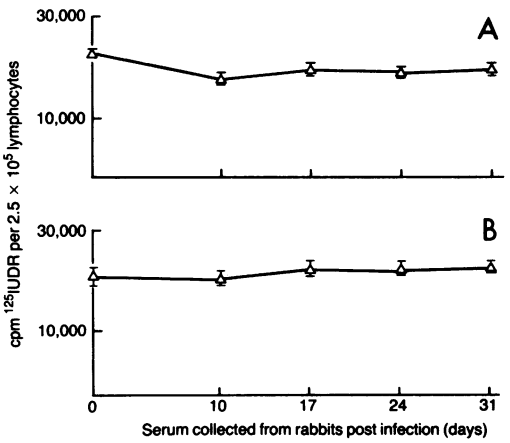

FIG. 5. Individual sera from six *T. pallidum*-infected rabbits tested in triplicate cultures with splenic lymphocytes from an intratesticularly infected rabbit (30 days post-inoculation). In vitro lymphocyte responses to *T. pallidum* antigens (A) and ConA (B) were determined. The final concentration of serum in cultures was 1%. Background counts for unstimulated cultures were subtracted (≤300 cpm). The geometric mean and standard error for each group of sera are plotted.

TABLE 2. In vitro inhibition of lymphocyte responsiveness in fractions of pooled serum from syphilitic and normal rabbits

Serum fraction and expt. no.	[^{125}I]UDR uptake (cpm) in indicated serum:					
	Sonicated *T. pallidum*			ConA		
	NRS	IRS[a]	% Inhibition[b]	NRS	IRS	% Inhibition
Whole serum (10%)[c]						
1	3,615	557	85	33,805	7,447	78
2	5,182	1,320	75	14,638	1,838	87
Albumin (10% + 1% NRS)						
1	11,202	1,859	83	46,219	17,767	62
2	11,749	590	95	17,454	6,458	63
Globulin[c] (10% + 1% NRS)						
1	13,708	1,511	89	29,371	23,583	20
2	17,339	771	96	23,480	15,368	35
DEAE fraction 1 (IgG)[c] (10% + 1% NRS)						
1	16,154	10,012	28	36,745	40,918	−11
2	32,439	28,344	13	38,710	40,430	−4
DEAE fraction 2[c] (10% + 1% NRS)						
1	16,224	1,584	90	29,924	25,943	23
2	19,936	3,639	82	27,253	16,651	39

[a] Pooled serum from two rabbits which had been infected with *T. pallidum* for 17 days.

[b] The percent inhibition for each experiment was determined as follows: [(counts per minute of NRS control sera or fraction) − (counts per minute of IRS sera or fraction)]/(counts per minute of NRS control sera or fraction).

[c] Denotes retention of FTA-ABS reactivity in IRS fractions.

counts of lymphocytes cultured for 72 h were performed, and the viability of cells in all serum fractions was comparable, indicating no cytotoxic effect by the fractions.

Finally, the IRS albumin and globulin fractions were passed over Sephadex G-200, and the peaks (as determined by optical density at 280 nm) were pooled and concentrated. Testing in the lymphocyte responsiveness assay indicated that the inhibitory activity in the albumin fraction resided in the low-molecular-weight (30,000 to 100,000) peak, whereas the activity in the globulin fraction was found in the high-molecular-weight (≥600,000) peak (data not shown). The low-molecular-weight fraction contained albumin and alpha-2 and beta-proteins as determined by microzonal electrophoresis. Three bands were present after immunoelectrophoresis and precipitation with goat anti-whole rabbit serum antiserum and are consistent with albumin and alpha- and beta-proteins. No immunoglobulin was detected by precipitation with anti-rabbit immunoglobulin antiserum, nor were *T. pallidum* antigens evident by precipitation with rabbit or human anti-*T.pallidum* antiserum. The fraction was nonreactive in the FTA-ABS test and contained 8.5 mg of protein and 150 μg of carbohydrate per ml.

The high-molecular-weight fraction contained alpha-2 proteins as detected by microzonal electrophoresis. Two bands precipitated in immunoelectrophoresis and development with anti-whole rabbit serum antiserum, consistent with alpha-proteins. No immunoglobulin was detected by precipitation with anti-rabbit immunoglobulin antiserum, nor were *T. pallidum* antigens detected, as previously described. The fraction, however, retained FTA-ABS reactivity when tested in a modified assay that detected specific IgM antibody. It contained 1.3 mg of protein and 640 μg of carbohydrate per ml.

Immune complex determination. Twenty-five rabbits infected intratesticularly with *T. pallidum* were divided into groups of five rabbits each. Groups were bled and sacrificed at varying times after infection, from 10 days to 2 months. Serum specimens were collected for CIC determinations, and splenic lymphocytes were cul-

TABLE 3. Comparison of CIC with lymphocyte responses in syphilitic rabbits

Time after infection	CIC (μg of AGG equivalent per ml)		Lymphocyte response (cpm) to sonicated *T. pallidum* in:		Increased CIC[b]	Decreased cellular responses[b,c]
	Preinfection	Postinfection	NRS	AUTO[a]		
10 days	0	0	3,359	4,290	3/5	2/3
	145	110	3,755	953		
	1,200	71,600	926	698		
	130	540	144[d]	60		
	0	60	37[d]	0		
17 days	0	3	7,080	5,929	3/5	4/5
	0	130	447	9		
	0	94	5,613	674		
	0	0	3,692	1,849		
	0	290	645	145		
24 days	71,600	71,600	717	162	2/4	5/5
	0	0	1,787	0		
	ND[e]	94	5,198	9,265		
	0	440	1,897	351		
	0	160	562	0		
1 mo	0	0	5,137	1,257	1/5	4/5
	0	0	7,403	104		
	0	0	13,750	8,187		
	240	780	11,756	32,442		
	0	0	12,654	6,533		
2 mo	0	0	5,806	566	1/5	4/5
	0	0	7,454	1,012		
	0	0	13,809	1,894		
	0	0	15,589	7,234		
	0	110	9,158	10,190		

[a] AUTO, Autologous serum.
[b] Number of animals showing characteristic/total number of animals tested.
[c] A 25% or greater inhibition of response in autologous serum when compared with response in NRS.
[d] Lymphocytes not significantly responsive to *T. pallidum* antigens.
[e] ND, Not done; serum unavailable for assay.

tured in the lymphocyte responsiveness assay in the presence of 10% NRS or autologous serum. Lymphocyte responses to sonicated *T. pallidum* and micrograms of AGG equivalent per milliliter of serum collected before infection and at the time of sacrifice are shown in Table 3. In vitro inhibition of lymphocyte responses in the presence of autologous serum occurred in 80 to 100% of the animals tested from 17 days to 2 months after infection. In contrast, measurable CICs were detected in three of five animals at days 10 and 17 and declined to only one of five animals at 1 and 2 months after infection.

DISCUSSION

Sera from rabbits infected with *T. pallidum* inhibited lymphocyte responsiveness to treponemal antigens. This antigen-specific inhibitory activity was first apparent on day 10 and increased to 80% inhibition by days 24 and 31 of infection. Both ConA (mitogen) and SRBC (unrelated antigen) lymphocyte responses remained essentially unaffected by syphilitic sera, with one exception: a slight transient diminution of all responses tested occurred with sera from rabbits with active orchitis. All sera sequentially drawn from a total of 10 rabbits with early experimental syphilis (preliminary experiments and experiments reported herein) reduced the response to treponemal antigens by 50% or greater, whereas only 20% of the sera reduced ConA responses.

The demonstration of this inhibitory activity required serum culture concentrations (10%) which were suboptimal and promoted greater variability among the responses. When sera were tested at optimal 1% culture concentrations, no nonspecific inhibitory effect on ConA responses was detected, and only slight (17 to 25%) specific inhibition of lymphocyte prolifera-

tion induced by *T. pallidum* antigens was demonstrated. Previous studies reporting inhibition of mitogen responses in experimental syphilis used high (10 to 26.6%) serum concentrations to demonstrate an effect (5, 40, 41, 54–56).

There are at least two immunoregulatory factors present or elevated or both in serum obtained during early syphilitic infection: a low-molecular-weight, nonspecific factor and a high-molecular-weight, antigen-specific factor. No effect by purified IgG from inhibitory sera (which had retained its antibody activity as measured in the FTA-ABS test) could be detected.

The low-molecular-weight, nonspecific factor bore a resemblance to one or several serum agents previously reported. Muchmore et al. (31) showed that certain simple sugars markedly inhibit the capacity of human PBL to respond in various antigen-specific systems; PHA responses, however, remain unaffected, and ConA was not tested. The nonspecific factor present in syphilitic sera is found on days 10 through 17, corresponding to the period of peak orchitis and resolution. The clearance of treponemes from the primary site could result in large quantities of circulating treponemal antigen at this time. Indeed, Fischer et al. (15) have shown that an ammonium sulfate-soluble polysaccharide antigen of *Candida albicans* is present in the sera of some individuals with chronic mucocutaneous candidiasis and is responsible for inhibiting *Candida*-induced lymphocyte proliferation in vitro. However, this factor failed to inhibit responses induced by either an unrelated antigen or ConA. Nevertheless, we were unable to demonstrate the presence of treponemal antigens in similar fractions of syphilitic rabbit serum by either immunoprecipitation in agar or sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting techniques.

Bey et al. (5) have shown the presence of mucopolysaccharide in sera from intratesticularly infected rabbits. These sera inhibited responses to ConA; the inhibition was abolished after treatment with hyaluronidase. In contrast to our results, this inhibitory activity was observed through day 35 of infection, at which time responses rapidly returned to control values despite the persistence of comparable levels of serum-borne mucopolysaccharide. Antigen responses were not tested. Although the authors cited their observations as evidence for immunosuppression during syphilis, it is possible that the mucopolysaccharide contains mannose residues which could bind to ConA (43), thus effectively blocking the binding of mitogen to lymphocyte receptor sites. Baseman et al. (2) have reported that *T. pallidum* binds to ConA. Glycosaminoglycans in sera from infected rabbits have been shown to react with active binding sites on ConA and effectively block its capacity to stimulate lymphocytes (3). Indeed, when sera from rabbits in the *T. pallidum*-infected group were tested for carbohydrate, the content in sera from day 10 of infection was significantly elevated (by 20%) over preinfection sera. Thus, circulating treponemal antigen or elevated carbohydrate content in sera from rabbits with orchitis might serve to effectively block in vitro ConA stimulation by this mechanism.

Elevated serum alpha-globulin levels may also occur at this time of infection. Alpha-globulin purified from normal human serum has been shown to possess immunoregulatory activity for both mitogen-induced and antigen-induced blastogenesis (7–9). A similar component has been isolated from normal mouse serum (35, 37). The mechanism of this inhibition is unclear; it appears, however, not to be alpha-globulin binding of lectin (57) but rather reversible inhibition of lymphocyte surface receptors in vitro (29). Electrophoretic analysis of the low-molecular-weight fraction indicated that alpha-globulins remained as one component.

The high-molecular-weight, antigen-specific inhibitory serum component is more difficult to assess. Its effect is potent and long-lived. High-molecular-weight alpha-2 macroglobulin (6) as well as low-density lipoprotein (10) isolated from normal human serum have been reported to inhibit lectin responses; effects on antigen-specific systems were not reported. DeJong et al. (11) described the presence of a high-molecular-weight, cold-reacting autolymphocytotoxin (presumably IgM) in sera from patients with syphilis (particularly secondary disease). The authors speculated that, at physiological temperatures, these molecules, although no longer cytotoxic, might influence the immune reactivity of lymphocytes. We were unable to detect any cytotoxic activity by fractions from syphilitic rabbit serum in our cultures. Residual anti-*T. pallidum* activity in this fraction (detected by FTA-ABS IgM) might affect responses of *T. pallidum*-sensitized lymphocytes while having no effect on responses to mitogens or unrelated antigens.

The presence of CICs with regulatory potential provides another possible identity of the high-molecular-weight component. Soluble complexes of antigen in antibody excess have been shown to inhibit lymphocyte responses to specific antigens, whereas precipitated complexes enhance responses (38, 49). CICs have been shown in humans with syphilis (12, 48) and in rabbits with disseminated experimental syphilis (4). It is possible that, in the waning days of syphilitic orchitis, a condition exists in which antigen is released into the circulation to form soluble complexes at a time when FTA-ABS

antibody titers are still on the rise. This condition may serve to regulate T cell reactivity and control unnecessary cellular proliferation as the lesion resolves. The CIC data presented herein do not tend to support this supposition, however. Complex levels were elevated in the majority of rabbits per group at days 10 and 17 of infection but rapidly declined thereafter, whereas inhibition of the blastogenic response to *T. pallidum* antigens in the presence of these sera persisted. The specific components of these complexes were not determined. The data indicate that it is unlikely that detectable immune complexes are solely responsible for the regulation of the specific response to *T. pallidum* antigens, although the possible effect of minute, undetectable levels of CICs on these responses cannot be ruled out.

In conclusion, it is important to stress the pitfalls inherent in interpreting in vitro data and determining the relevance it has, if any, to in vivo biological and clinical events. The presence of serum inhibitors has been used by some investigators (5, 22, 41, 42, 51, 55) to promote a role for immunosuppression in early syphilis. This hypothesis purportedly explains the persistence of the infecting organisms during latency and the subsequent progression to tertiary disease. All rabbits used in this study, however, displayed an uncomplicated course of early syphilitic infection, with resolution of orchitis by day 31. The presence or absence of nonspecific serum inhibitory activity in individual rabbits had no overt influence on the clinical course of the disease. If serum inhibitory factors did play an important role in the course of infection with *T. pallidum*, passive transfer of serum from previously infected rabbits to newly infected rabbits would be expected to enhance the infection. Such an exacerbation of disease has not been reported. Our previous studies have shown that during early experimental syphilis, a vigorous cellular immune response occurs (27, 46), accompanied by an influx of T cells to the site of infection (25). These sensitized lymphocytes are capable of releasing soluble mediators which activate macrophages (24). The electron microscopic demonstration of treponemes within macrophages in vivo in rabbits has been reported (21, 39), and destruction of *T. pallidum* within phagocytic vessicles appears to be the mechanism of clearance of organisms from sites of infection (45). These events are accompanied by long-lasting immunity (1, 52) to symptomatic reinfection.

Since the presence or absence of in vitro inhibitory factors in serum in individual animals did not overtly affect the clinical course in these animals, an important role for generalized immunosuppression, as hypothesized by others, may not be appropriate. An alternate explanation for the presence of these "suppressive" serum factors would seem to be the induction of normal immunoregulatory factors during infection. These homeostatic mechanisms appear to come into play at the resolution sequence of the active infection in experimental syphilis and may serve to control a fully normal immune response. The effector stage of the immune response to *T. pallidum* apparently turns off when most of the organisms have been eliminated from the primary site of infection, but a few organisms survive this otherwise prompt and effective immune response.

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