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Suppression of Lymphocyte Response to Concanavalin A by Mucopolysaccharide Material from *Treponema pallidum*-Infected Rabbits

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The testicular fluid and serum from rabbits infected intratesticularly with *Treponema pallidum* inhibited the mitogenic response of normal rabbit peripheral blood lymphocytes to concanavalin A. Mucopolysaccharide material present in the testicular fluid and serum was associated with the lymphocyte-inhibitory activity. Degradation of the mucopolysaccharide material with hyaluronidase resulted in the loss of the inhibitory activity of testicular fluid and serum of *T. pallidum*-infected rabbits.

There is evidence that cellular immunity is impaired in congenital syphilis and during the early phase of venereal syphilis. Skin tests which are usually positive in the late secondary and latent phases of the disease are usually negative during primary and early secondary syphilis (10, 13). Spleens of infants dying from congenital syphilis are depleted of lymphocytes (12), as are the spleens of *Treponema pallidum*-infected neonatal rabbits (2). In patients with untreated secondary syphilis, a depletion of lymphocytes in the paracortical areas of the inguinal lymph nodes has been observed (12). Lymphocyte blastogenesis is also abnormal in the early phases of syphilis. Patients with primary and secondary syphilis manifest a reduced lymphocyte response to treponemal antigen and/or mitogens (7, 12, 15, 16, 31). In addition, a factor is present in the plasma of patients with secondary syphilis which interferes with the blastogenic response of normal human lymphocytes (11). Impaired lymphocyte function is also suggested by the observation that inhibition of leukocyte migration does not occur in the early phases of human syphilis (8).

Studies on experimental syphilis in rabbits have clearly established that the lymphocyte response to mitogens and *T. pallidum* is depressed during the first 3 to 4 weeks of the infection. In addition, a factor is present in the blood which inhibits the blastogenic response of lymphocytes from infected and uninfected rabbits (14, 17-19, 27-29).

In this report we describe the presence of mucopolysaccharide (MPS) material in the testes and blood of *T. pallidum*-infected rabbits which is associated with the suppression of the blastogenic response of normal rabbit lymphocytes to concanavalin A (ConA).

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MATERIALS AND METHODS

Animals. Healthy male Dutch Belt rabbits weighing 1.8 to 2.3 kg were used for passage of the treponemes and as a source of normal lymphocytes. These rabbits gave a negative response to the Rapid Plasma Reagin circle card test (Hynson, Erscott, and Dunning, Inc.). They were housed at 19 to 22°C and given antibiotic-free food and water ad libitum.

T. pallidum. The Nichols strain of *T. pallidum* was maintained by intratesticular passage. Approximately 10^7 to 3×10^7 treponemes were inoculated per testis. Daily intramuscular injections of cortisone acetate (Merck, Sharpe, & Dohme), 6 mg/kg of body weight, was initiated on the third day after testicular inoculation. After development of orchitis, usually 9 to 14 days, rabbits were killed by intracardiac injection of sodium pentobarbital. Testes were removed, rinsed in physiological saline, and placed into Eagle minimal essential medium with 4 mM $NaHCO_3$ buffered with 30 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid at pH 7.2. This medium was further supplemented with 10% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.), 4 mM glutathione, 1 mM cysteine, and 1 mM dithiothreitol. The testes were sliced and extracted aerobically for 20 to 30 min at 28°C, after which the suspension was centrifuged at $100 \times g$ for 7 min at 28°C. This sedimented the particulate matter, and the supernatant containing the organisms was decanted. A concentrated suspension of *T. pallidum* was obtained by centrifuging the supernatant at 20,000 $\times g$ for 30 min, and the resulting pellet was resuspended in a small volume of Eagle minimal essential medium. The number of treponemal cells was determined by use of a Petroff-Hausser counting chamber.

Lymphocyte preparation. Rabbit peripheral

blood lymphocytes were purified from blood obtained by cardiac puncture. Blood coagulation was prevented by the addition of 5 U of heparin (The Upjohn Co.) per ml of blood. This blood was diluted 1:2 in physiological saline, layered onto a Methocel-Hypaque gradient, and centrifuged at 650 × *g* for 30 min. Cells at the gradient interface were collected, washed twice in sterile physiological saline, and resuspended in RPMI 1640 medium supplemented with 100 μg of streptomycin and 100 U of penicillin per ml, 10% heat-inactivated normal rabbit serum, and 200 mM L-glutamine. The lymphocytes were 95 to 98% viable as determined by trypan blue exclusion (1).

Lymphocyte cultures. Lymphocytes were cultured in flat-bottomed tissue culture plates (Linbro) at a cell concentration of approximately 2×10^5 cells per 0.2 ml. Triplicate wells were used to monitor the effects of the various test materials. Cultures were incubated with the test substances in a humidified chamber of 95% air and 5% CO_2 for 72 h at 37°C. Then 10 μl of RPMI 1640 medium containing 0.5 μCi of [^{3}H]thymidine (specific activity, 10 Ci/mmol; ICN, Cleveland, Ohio) was added to each well, and the cultures were incubated for an additional 16 to 18 h. Lymphocytes were harvested (Ottohiller Co., Madison, Wis.) onto glass-fiber filter paper, and the radioactivity incorporated was determined in a liquid scintillation spectrometer.

Lymphocyte culture test substances. ConA, bovine hyaluronic acid, and crystalline type IV bovine testicular hyaluronidase (specific activity, 1,200 IU/mg) were obtained from Sigma Chemical Co. Crystalline *Streptomyces hyalurolyticus* hyaluronidase (specific activity, 2,000 IU/mg) was obtained from Miles Laboratories. The mucoid testicular fluid which accumulated between the tunicae of the testes was removed by needle aspiration at the time of maximal orchitis. Blood from infected and uninfected rabbits was obtained by cardiac puncture or from the median ear artery and was allowed to clot at room temperature for 3 h. The serum was harvested and inactivated at 56°C for 30 min. The serum was either analyzed that day or stored at −70°C until assayed. Normal rabbits received intratesticular injections of either 500 μg of lipopolysaccharide or 10^9 heat-inactivated (56°C, 30 min) *T. pallidum* cells per testis. Approximately 6 to 8 days postinjection, these testes were extracted, as were normal testes, in the same manner previously described for obtaining *T. pallidum*. The supernatant from the 10,000 × *g* centrifugation was used as the testes extract.

MPS content of the test substances was determined by a capillary turbidity microassay method which has a minimal sensitivity of approximately 8 μg of MPS per ml (9).

The testicular fluid and the syphilitic rabbit serum were treated with the hyaluronidase preparation (hyaluronidase-treated) or an equal volume of saline (saline-treated) for 24 h at 28°C.

Three to six rabbits were used for each experiment.

RESULTS

Normal peripheral blood lymphocytes obtained from four rabbits were used to establish the concentration of ConA which would result in maximal incorporation of [^{3}H]thymidine by these cells. Concentrations of 2.5, 5.0, 10.0, and 20 μg/ml were tested. A ConA concentration of 10 μg/ml provided a maximal lymphocyte response and was used in subsequent experiments.

The mitogenic activity of the testes fluid obtained from rabbits infected intratesticularly with 10^7 to 3×10^7 treponemes per testis was determined. This fluid was harvested from the rabbits at the time of maximal orchitis, which was 9 to 14 days postinfection. As can be seen in Table 1, the testicular fluid possessed a low level of mitogenic activity; however, it markedly impaired the response of normal lymphocytes to ConA. Lymphocytes incubated with 130 μg of testicular fluid MPS per ml had a 92% reduction in their response to ConA (Table 1). As the testicular fluid MPS concentration was increased to 520 μg/ml, the ConA response of the lymphocytes was reduced to that of the unstimulated control (Table 1). The capacity of testicular fluid derived from either cortisone-treated or untreated *T. pallidum*-infected rabbits to inhibit the blastogenic response of normal lymphocytes to ConA was found to be similar.

The testicular fluid was treated with bovine and *Streptomyces* hyaluronidase to ascertain whether an MPS component of the testicular fluid was responsible for suppressing the response to ConA. A concentration of testicular fluid MPS of 160 μg/ml suppressed the ConA-induced lymphocyte response to approximately that of the unstimulated control (Table 2). Treatment of the testicular fluid MPS with bovine hyaluronidase decreased its MPS content from 160 μg/ml to an undetectable level (less than 8 μg/ml). Coincidental with the degradation of the testicular fluid MPS was the loss of its capacity to inhibit the ConA-mediated lymphocyte blastogenesis (Table 2). Similar results were obtained when the *Streptomyces* hyaluron-

TABLE 1. *Effect of testes fluid from T. pallidum-infected rabbits on blastogenic response of normal rabbit peripheral blood lymphocytes*

Sample	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
Control	—	1.1 (0.8–1.5)
ConA (10 μg/ml)	—	223.7 (210.6–237.7)
Testes fluid	130	7.4 (6.7–8.1)
	520	7.2 (6.8–7.7)
ConA (10 μg/ml)	130	17.8 (15.7–20.3)
+ testes fluid	260	13.0 (12.4–13.7)
	520	1.2 (0.8–1.7)
	780	1.0 (0.9–1.3)

[a] Representative data from four rabbits. The 95% confidence limits are given in parentheses.

TABLE 2. *Effect of hyaluronidase treatment of testes fluid from T. pallidum-infected rabbits*

Sample	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
Control	—	1.1 (0.9–1.2)
ConA (10 μg/ml)	—	252.1 (169.1–375.1)
Saline-treated testes fluid	160	1.2 (1.0–1.4)
Saline-treated testes fluid + ConA (10 μg/ml)	160	1.8 (1.5–2.3)
Bovine hyaluronidase-treated testes fluid	160[b]	1.3 (0.9–1.9)
Bovine hyaluronidase-treated testes fluid + ConA (10 μg/ml)	160[b]	251.0 (236.1–266.8)

[a] Representative data from three rabbits. The 95% confidence levels are given in parentheses.

[b] After hyaluronidase treatment mucopolysaccharide was not detectable.

idase was substituted for the bovine hyaluronidase.

The mucoid testicular fluid that accumulates in the testes of *T. pallidum*-infected rabbits is unique. We were unable to induce the formation of testicular mucoid material by the intratesticular injection of starch, peptone, benzene, *Brucella abortus* strain 19, heat-killed *T. pallidum*, or lipopolysaccharide as a control. The following preparations were found to be devoid of lymphocyte-inhibitory activity: uninfected rabbit testes extract, bovine hyaluronic acid, lipopolysaccharide, and an extract from lipopolysaccharide-inflamed testes (Tables 3 and 4). Heat-killed *T. pallidum* caused a slight reduction (8 to 15%) in lymphocyte response to ConA, and the extract from testes injected with inactivated treponemes decreased the lymphocyte response by 7 to 31% (Table 5).

The data obtained with the testicular fluid from *T. pallidum*-infected rabbits indicate that it has lymphocyte-inhibitory activity that is associated with the presence of MPS material. Studies on experimental syphilis in rabbits have established that a factor is present in their blood during the infection which inhibits the blastogenic response of lymphocytes to mitogens and *T. pallidum* (14, 17–19, 28, 29). The possibility that this inhibitory factor in the blood of infected rabbits was associated with MPS material was examined. Serum was obtained from normal rabbits and from syphilitic rabbits at the time of maximal orchitis, which was usually 9 to 14 days after testicular infection. These sera were assayed for MPS content and lymphocyte-inhibitory activity. Serum from uninfected rabbits did not contain detectable MPS, nor did it possess lymphocyte-inhibitory activity. In contrast, the serum obtained from infected rabbits at the time of maximal orchitis contained MPS which varied

TABLE 3. *Response of normal rabbit peripheral blood lymphocytes to normal rabbit testes extract and bovine hyaluronic acid*

Sample	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
Control	—	0.9 (0.8–1.1)
ConA (10 μg/ml)	—	216.4 (206.9–226.1)
Normal rabbit testes extract	1.6	0.9 (0.8–1.1)
	3.2	0.8 (0.8–0.9)
Normal rabbit testes extract + ConA (10 μg/ml)	1.6	212.6 (204.3–221.2)
	3.2	236.4 (149.7–373.4)
	6.4	216.2 (204.6–228.5)
Control	—	1.4 (0.9–2.1)
ConA (10 μg/ml)	—	372.8 (351.9–394.5)
Bovine hyaluronic acid	50	0.8 (0.5–1.6)
	150	2.2 (1.8–2.8)
	250	2.3 (2.1–2.6)
Bovine hyaluronic acid + ConA (10 μg/ml)	50	304.5 (311.7–298.7)
	150	386.3 (446.0–333.7)
	250	345.7 (361.116–330.947)

[a] Representative data from three rabbits. The 95% confidence limits are given in parentheses.

TABLE 4. *Normal rabbit peripheral blood lymphocyte response to lipopolysaccharide and lipopolysaccharide-inflamed testes extract*

Sample	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
Control	—	1.4 (1.2–1.6)
ConA (10 μg/ml)	—	164.1 (138.1–195.0)
Lipopolysaccharide		
5 μg/ml	—	3.4 (2.6–4.3)
10 μg/ml	—	15.8 (9.3–26.8)
20 μg/ml	—	16.6 (6.8–40.8)
ConA (10 μg/ml) + lipopolysaccharide		
5 μg/ml	—	166.5 (147.6–187.9)
10 μg/ml	—	245.3 (191.2–314.5)
20 μg/ml	—	223.8 (200.9–260.4)
Control	—	0.9 (0.7–1.3)
ConA (10 μg/ml)	—	136.0 (121.2–155.3)
Extract of lipopolysaccharide-inflamed rabbit testes	1.6	1.2 (1.1–1.4)
	3.2	1.3 (1.3–1.5)
	4.8	1.6 (1.1–2.2)
Extract of lipopolysaccharide-inflamed rabbit testes + ConA (10 μg/ml)	1.6	230.2 (197.4–268.5)
	3.2	186.1 (184.1–188.2)
	4.8	160.1 (147.4–175.3)

[a] Representative data from three rabbits. The 95% confidence limits are given in parentheses.

from 8 to 64 μg/ml. This serum also possessed the lymphocyte-inhibitory activity. The addition of as little as 3.2 μg of syphilitic serum MPS per ml to the lymphocytes suppressed their response to ConA by 98% (Table 6). The treatment of this inhibitory serum wth hyaluronidase resulted in the degradation of the MPS to unde-

tectable levels. Coincident with the degradation of the MPS was the disappearance of the lymphocyte-inhibitory activity of the serum (Table 6).

It was necessary to analyze the lymphocyte-inhibitory activity in freshly harvested serum or serum which was stored at −70°C. If this serum was stored at 4 or −20°C, there was a decrease in its inhibitory activity.

The development of the lymphocyte-inhibitory activity in the serum of five *T. pallidum*-infected rabbits, as well as its MPS content and antibodies reactive in the Rapid Plasma Reagin circle card test, was monitored during a 67-day observation period. MPS and lymphocyte-inhibitory activity were detected as early as 3 days post-infection (Table 7). The highest concentration of MPS (64 μg/ml) was present in serum at the time of maximal orchitis. The MPS content of the serum then decreased to 8 μg/ml and remained at this concentration from day 15 to day 55. MPS could no longer be detected at day 67. A high level of lymphocyte-inhibitory activity (>90%) was present in serum from day 3

TABLE 5. *Effect of heat-killed T. pallidum and extract of testes inflamed by heat-killed T. pallidum on normal peripheral blood lymphocytes*

Sample	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
Control	—	1.0 (0.9–1.1)
ConA (10 μg/ml)	—	124.3 (113.9–135.0)
Heat-killed *T. pallidum*		
10^7 cells/ml	0.02	1.9 (1.8–2.1)
10^8 cells/ml	0.16	1.3 (1.2–1.5)
10^9 cells/ml	1.6	1.1 (0.9–1.4)
ConA (10 μg/ml) + heat-killed *T. pallidum*		
10^7 cells/ml	0.02	105.7 (103.5–107.9)
10^8 cells/ml	0.16	114.5 (99.9–132.4)
10^9 cells/ml	1.6	107.7 (88.3–131.4)
Control	—	0.8 (0.7–1.0)
ConA (10 μg/ml)	—	337.3 (243.8–466.4)
Extract of rabbit testes injected with heat-killed *T. pallidum*	1.6	0.6 (0.5–0.6)
	3.2	0.6 (0.3–0.9)
	6.4	0.6 (0.4–1.0)
Control	—	1.0 (0.8–1.3)
ConA (10 μg/ml)	—	295.4 (281.9–308.9)
Extract of rabbit testes injected with heat-killed *T. pallidum* + ConA (10 μg/ml)	1.6	276.0 (236.0–332.2)
	3.2	219.2 (191.4–251.0)
	6.4	204.7 (180.2–232.6)

[a] Representative data from three rabbits. The 95% confidence levels are given in parentheses.

TABLE 7. *Appearance of lymphocyte-inhibitory factor in serum of T. pallidum-infected rabbits*

Sample[a]	Mucopolysaccharide (μg/ml of serum)	Mean cpm/0.2 ml of culture × 10^{3b}	RPR[c] titer
None		0.6 (0.5–0.7)	
ConA		209.3 (195.8–223.6)	
Preinfection rabbit serum + ConA	<8	215.5 (205.0–225.5)	0
Postinfection rabbit serum + ConA			
Day 3	8	5.6 (4.2–7.4)	0
Day 9	64	3.3 (2.0–5.3)	2
Day 15	8	7.8 (6.3–9.7)	8
Day 21	8	8.4 (6.8–10.4)	8
Day 28	8	1.3 (1.0–1.7)	16
Day 35	8	1.4 (1.0–1.8)	32
Day 42	8	161.8 (132.7–196.3)	16
Day 55	8	212.2 (209.6–214.4)	16
Day 67	<8	217.9 (194.9–243.3)	4

[a] ConA, 10 μg/ml; rabbit serum, 100 μl/ml.
[b] Representative data from five rabbits. The 95% confidence limits are given in parentheses.
[c] Rapid Plasma Reagin.

TABLE 6. *Effect of hyaluronidase treatment on lymphocyte-inhibitory factor present in serum from T. pallidum-infected rabbits*

Sample	Serum (μg/ml)	Mucopolysaccharide (μg/ml)	Mean cpm/0.2 ml of culture × 10^{3a}
None		—	1.1 (1.0–1.2)
ConA (10 μg/ml)		—	279.4 (272.3–286.2)
Preinfection serum	100		1.3 (0.8–4.3)
Preinfection serum + ConA (10 μg/ml)	100		283.0 (276.5–291.0)
Saline-treated serum from infected rabbits	50	3.2	1.5 (0.3–5.2)
	100	6.4	1.6 (0.7–3.6)
Saline-treated serum from infected rabbits + ConA (10 μg/ml)	50	3.2	6.6 (2.3–15.6)
	100	6.4	5.2 (3.6–7.5)
Hyaluronidase-treated serum from infected rabbits	50	3.2[b]	4.6 (3.8–5.2)
	100	6.4[b]	5.5 (4.1–7.2)
Hyaluronidase-treated serum from infected rabbits + ConA (10 μg/ml)	50	3.2[b]	255.0 (238.4–264.8)
	100	6.4[b]	246.7 (232.0–260.3)

[a] Representative data from six rabbits. The 95% confidence levels are given in parentheses.
[b] After hyaluronidase treatment mucopolysaccharide could not be detected.

through day 35. By day 42 the level of serum inhibitory activity was markedly decreased (26%) and was undetectable on days 55 and 67 postinfection (Table 7). The rabbit serum became reactive with the Rapid Plasma Reagin card test at day 9 and remained reactive through day 67. Serum samples obtained on days 3, 9, and 35 were also treated with hyaluronidase. This treatment resulted in the coincident disappearance of serum MPS and lymphocyte-inhibitory activity.

DISCUSSION

Lymphocyte function is transiently impaired in the early phase of human syphilis as well as in experimental rabbit syphilis (8, 11, 12, 14, 17-19, 27-29). Associated with this depressed lymphocyte responsiveness is the presence of an inhibitory factor in the blood of syphilitic humans (11, 12) and rabbits (14, 17-19, 27-29). The factor(s) present in blood which is responsible for the lymphocyte hyporeactivity has not been identified.

A characteristic of *T. pallidum* infections is the presence of mucoid MPS material in the syphilitic lesions. This MPS material is believed to be hyaluronic acid or chondroitin sulfate (24), and the amount of MPS material which accumulates appears to be related to the virulence of the infecting treponeme (26). The origin of the MPS material has not been elucidated. It has been proposed to be either of treponemal origin (24) or of host origin (20). Studies on experimental syphilis in the rabbit by Scott and Dammin (21-23) also suggested that MPS accumulates in the tissues during infection and that this MPS material is probably hyaluronic acid or chondroitin sulfate based upon its reaction with hyaluronidase. A recent study on the interaction of *T. pallidum* with cultured mammalian cells reported the accumulation of amorphous material on the surface of tissue cells to which *T. pallidum* had attached, and the amount of amorphous material present was directly related to the number of attached treponemes (6). Only a minimal amount of this material was present on the surface of the tissue cells in the absence of attached treponemes. This material appeared to be similar to hyaluronic acid or chondroitin sulfate based on its reaction with wheat germ agglutinin and soy bean agglutinin (5). Also, treatment of tissue cells with hyaluronidase caused a decrease in the amount of amorphous material present (6). The authors suggested that this amorphous material that accumulates in vitro at the surface of cultured cells may be similar to the mucoid material that accumulates in vivo during syphilitic infection. Amorphous MPS material has also been observed to be associated with the surface of *T. pallidum* (3, 4, 32).

The results presented in this report suggest that MPS material present in *T. pallidum*-infected rabbits may be the inhibitory factor responsible for the suppression of rabbit peripheral blood lymphocyte activity. Testicular fluid from *T. pallidum*-infected rabbits was found to interfere with the blastogenic activity of normal rabbit peripheral blood lymphocytes. Degradation of the MPS material in the testicular fluid by hyaluronidase resulted in the loss of its lymphocyte-inhibitory activity. In addition, MPS material was detected in the serum of *T. pallidum*-infected rabbits, and its presence closely correlated with the lymphocyte-inhibitory activity of the serum. Moreover, hyaluronidase treatment of the serum resulted in the degradation of the MPS material and the concurrent loss of the lymphocyte-inhibitory activity of the serum.

Circulating immune complexes are present in secondary syphilis, and they are believed to play a significant role in the immunopathogenesis of syphilitic nephropathy (24). Since immune complexes are known to modulate the activation of T and B lymphocytes (30), the possibility exists that the MPS material in *T. pallidum*-infected rabbits may be in the form of immune complexes.

The transient suppression of lymphocyte function during the early stages of human syphilis may play an important role in the widespread dissemination of the spirochetes in this disease. In addition, it may be related to the prolonged infectious phase of syphilis (31).

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