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Aberrant Secondary Antibody Responses to Sheep Erythrocytes in Rabbits with Experimental Syphilis

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Rabbits infected with *Treponema pallidum* have strikingly depressed in vivo immunoglobulin G responses to sheep erythrocytes. To gain further insight into the nature of this suppression, the immune responses of splenic and peripheral blood lymphocytes from infected rabbits to sheep erythrocytes were studied in vitro. Spleen cells from rabbits that had been sensitized with sheep erythrocytes during active syphilis had greatly decreased immunoglobulin M and G responses after in vitro incubation with sheep erythrocytes, when compared to the results obtained with cells from sensitized uninfected animals. Suppressor cells could be demonstrated in peripheral blood lymphocytes of control rabbits 6 months after sensitization with sheep erythrocytes; these cells could be removed by nylon wool filtration. When primary sensitization with sheep erythrocytes was carried out during active syphilis, these suppressor cells were not detectable in peripheral blood lymphocytes 6 to 9 months later. These findings provide further evidence that induction of immune responses may be abnormal early in treponemal infection and may help to explain the failure of the host to produce antibodies which eradicate the organism during the first 2 to 3 months of infection.

The appearance of treponemal and nontreponemal antibodies together with marked plasma cell proliferation in the lymphoid tissues during syphilitic infection might seem to indicate that B-lymphocyte function is normal or enhanced. However, disseminated (secondary) syphilis both in human subjects and in rabbits is characterized by a paradoxical situation in which large numbers of *Treponema pallidum* persist in lesions despite a vigorous humoral immune response. A similar paradox has been noted in other infections in which aberrations in immune regulation are thought to contribute to the ability of the infecting organisms to survive despite strong humoral immune responses (6, 10, 17, 30).

In an earlier study (2), we used the Jerne plaque technique to show that syphilitic infection in rabbits caused dysfunction of the primary in vivo humoral response to sheep erythrocytes (SRBC), a heterologous T-dependent immunogen. After a transient suppression, immunoglobulin M (IgM) plaque-forming cells (PFC) in rabbit spleens were increased for up to 7 weeks after intravenous (i.v.) challenge with *T. pallidum*. The IgG plaque response to SRBC was depressed throughout clinically active syphilis, reaching levels $\leq$10% of uninfected controls at the height of the infection. The current investigation extends these observations by examining the effect of syphilitic infection on secondary in vivo and in vitro responses to SRBC in rabbits.

MATERIALS AND METHODS

Animals. Outbred New Zealand white male rabbits weighing 2 to 3 kg were housed in individual cages at an ambient temperature of 18°C. Rabbits with a positive VDRL reaction were excluded because of the possibility that this may have resulted from a subclinical infection with *T. cuniculi*.

Microorganisms and infections. Maintenance of the Nichols strain of *T. pallidum* via intratesticular passage in cortisone acetate-treated rabbits has been described previously (3). Virulent spirochetes for infection were prepared as follows (15). Infected testes were removed aseptically, opened longitudinally, snipped along the edges, and placed in a sterile beaker with 10 ml of prewarmed minimal essential medium (MEM; GIBCO, Grand Island, N.Y.). Treponemes were extracted by agitating the beaker on a rotary shaker for 30 min at room temperature. After centrifugation at 754 $\times$ *g* for 10 min and passage of the supernatant fluid through a 0.8-μm filter (Nucleopore Corp., Pleasanton, Calif.) to remove cellular debris, the number of treponemes was determined by darkfield microscopy. Rabbits were inoculated with 4×10^7 organisms i.v. in ear veins within 1 h of obtaining syphilitic testes. These animals were then shaved three to four times weekly throughout the experi-

ments, and the progression of infection was monitored by daily examination.

Sensitization with SRBC. To assess the immune response to SRBC, rabbits were sensitized i.v. at various times after treponemal infection with 2×10^9 SRBC. Uninfected animals which served as controls received supernatant fluids from testes containing 4×10^7 heat-killed (60°C for 2 h) *T. pallidum*. Unsensitized rabbits were used to determine background levels of antibody-secreting cells. In vivo PFC assays were performed, and cell cultures were set up 7 days after primary or secondary sensitization.

Preparation of splenic lymphocytes and peripheral blood lymphocytes (PBL) for assays and cultures. Single-cell suspensions of splenic lymphocytes were obtained as previously described (2). Lymphocytes were separated from peripheral blood by using 3% pigskin gelatin (22). In certain experiments, 20 ml of heparinized blood was added to a 10-ml syringe containing 3 g of nylon wool and incubated at 37°C for 30 min (8). The suspension was expressed, and the wool was washed twice with 10 ml of warm MEM. Whole blood or nylon wool-treated blood was mixed with an equal volume of 3% gelatin, and the red cells were allowed to settle out for 1 h at 37°C. Lymphocyte-rich supernatant fluids were centrifuged, and pellets were washed twice in MEM. Cells were counted in a model ZF Coulter cell counter (Coulter Electronics, Hialeah, Fla.) and pelleted on glass microscope slides (23), using a centrifuge (Shandon Elliot Corp, Sewickley, Pa.) for differential counts.

Conditions for cell culture (25) were modified as described by Luzzati and LaFleur (14). Lymphocytes were suspended in RPMI 1640 with HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; GIBCO), containing 10% heat-inactivated fetal calf serum (Rehatuin lot no. R60804 or R61808; Reheis Chemical Co., Chicago, Ill.), 100 U of penicillin, 100 μg of streptomycin, and 250 μg of amphotericin per ml. Portions (1 ml each) of 1×10^6 to 4×10^6 cells were distributed in culture tubes (Falcon Plastics, type 2003), and antigen was added in the form of 50 μl of a 2% suspension of SRBC. In certain experiments, autologous PBL were admixed with splenic lymphocytes for culture. Tubes were incubated upright in a humidified, 5% CO_2 incubator until the time of assay, usually day 4 or 5 of culture.

Detection of immunoglobulin-bearing cells. Splenic lymphocytes or PBL bearing surface membrane immunoglobulin were counted by direct immunofluorescence (29), using fluorescein isothiocyanate conjugated goat anti-rabbit immunoglobulin (Microbiological Associates, Inc., Bethesda, Md.). At least 200 nucleated cells per slide were examined. B lymphocytes were recognized by a uniform rim of fluorescence encompassing ≥1/2 the cell circumference.

Hemolytic plaque assay. Spleen cells secreting antibody specific for SRBC were enumerated by using the Jerne plaque assay (11), as described previously (2). Direct and indirect PFC in vivo assays on freshly obtained spleen cells were performed in quadruplicate at two 10-fold dilutions. In vitro assays on cultured cells were performed by pooling duplicate cultures, washing the cells once in MEM after NH_4Cl treatment, resuspending in MEM, and then performing direct and indirect tests in duplicate at several dilutions.

RESULTS

In 12 separate experiments, rabbits received 2×10^9 SRBC i.v. at various time intervals after infection with *T. pallidum*. Spleen cells from these animals and from uninfected control rabbits, sensitized with SRBC at the same time, were obtained 7 days later. These cells were (i) used directly in the Jerne plaque assay to measure the effect of sensitization in vivo, or (ii) cultured with and without SRBC for 4 days to quantitate the secondary immune response in vitro. In vivo sensitization of syphilitic rabbits caused moderate enhancement of IgM-secreting cells and a striking reduction in IgG PFC; results of a representative study are shown in Table 1. Cells from control rabbits cultured for 4 days in vitro in the presence of SRBC showed marked increases in both IgM and IgG PFC. By contrast, cells obtained from rabbits early in the course of syphilis (≤21 days after treponemal challenge and before the appearance of generalized lesions) appeared to mount a normal IgM response but had greatly reduced numbers of IgG PFC (Table 1). Similar results were obtained with cells from the other five experimental rabbits sensitized with SRBC 3, 7, or 14 days after i.v. infection with *T. pallidum*. When PFC were calculated per spleen, the results paralleled PFC per 10^6 cells (data not shown). Different results were obtained with six experimental rabbits sensitized with SRBC at the height of generalized

TABLE 1. *Alterations in the primary in vivo and secondary in vitro anti-SRBC response*[a]

Response	Ig	Uninfected	Infected
Primary, in vivo	IgM	282 ± 23	612 ± 108[b]
	IgG	649 ± 43	26 ± 43[b]
	IgM	394 ± 37	538 ± 94[c]
	IgG	426 ± 51	18 ± 33[c]
Secondary, in vitro	IgM	280; 42,867	675; 36,067[b]
	IgG	180; 25,000	35; 1,866[b]
	IgM	356; 49,485	1,590; 7,520[c]
	IgG	210; 31,000	20; 200[c]

[a] Values for primary responses indicate PFC per 10^6 cells ± standard deviation. Those for secondary responses indicate PFC per culture, the first value referring to a culture system to which no SRBC were added, and the second value referring to a culture system to which SRBC were added. See text for further details.

[b] SRBC (2×10^9) were given i.v. 11 days after infection with *T. pallidum*, and the in vivo assay was performed on day 18. The control animal was sensitized i.v. with 2×10^9 SRBC, and the assay was carried out 7 days later.

[c] SRBC (2×10^9) were given i.v. 28 days after infection with *T. pallidum*, and the in vivo assay was performed on day 35. The uninfected control animal was sensitized i.v. with 2×10^9 SRBC, and the assay was carried out 7 days later.

syphilitic eruption (28, 35, or 42 days after i.v. challenge), in that decreases both in secondary IgM and IgG responses in vitro were observed. The results of a representative experiment are shown in Table 1.

Further evidence that cells from infected rabbits were incapable of normal responses was obtained in experiments in which rabbits received two doses of SRBC at 14-day intervals. Under these conditions, control rabbits have a predominantly IgG response in vivo (Table 2); this response was greatly decreased with infection. IgM PFC were increased during syphilis. Differences between control and infected rabbits were shown not to be due to shifts in the kinetics of the response. Results of sensitization in vitro paralleled the in vivo observations; the IgG response predominated in cells from control rabbits, and this response was greatly decreased in cell cultures from syphilitic rabbits (Table 2).

We also addressed the question of whether circulating suppressor cells might contribute to the failure of the syphilitic rabbits to mount normal IgG responses. The last three lines of Table 2 show that the addition of autologous PBL to splenic lymphocytes did not suppress the in vitro responses either in control or syphilitic rabbits; in fact, PBL alone from control rabbits exhibited good secondary responses.

In a further attempt to detect circulating suppressor cells, 6 to 9 months were allowed to pass between the two sensitizing doses of SRBC (13). Four syphilitic rabbits were infected with *T. pallidum* 21 days before the initial sensitization. Seven days after the second dose, control and infected rabbits were sacrificed and their in vivo and in vitro plaque responses were studied as in the previous experiments. One representative experiment is shown in Table 3. Control rabbits had depressed IgM and IgG PFC in vivo when compared to animals that had received two closely spaced injections of antigen. The animals which had received their first dose of SRBC during infection, however, exhibited typical secondary in vivo responses, with IgG PFC predominating.

Spleen cells alone from both control and syphilitic animals had similar responses in vitro (Table 3). Incubating spleen cells and PBL at a ratio of 1:1 indicated that PBL from uninfected rabbits suppressed the immunoglobulin responses of autologous spleen cells. Nylon wool filtration of PBL eliminated this suppressive effect; in fact, nylon wool filtration of PBL substantially increased their IgG PFC. This suppressive effect of PBL was not observed during in vitro incubation of cells from infected rabbits. In other studies (data not shown), varying the number of PBL caused a proportional degree of suppression with the most pronounced effect of IgG PFC, but only in the case of uninfected rabbits. Similar results were obtained with four other rabbits (data not shown), irrespective of whether they had been sensitized with one or two doses of SRBC at various times between 7 and 35 days after *T. pallidum* infection. Thus, rabbits that had been sensitized with SRBC during the early course of their infection did not appear to develop a population of circulating suppressor cells as did similarly sensitized uninfected animals.

Differential counts and immunofluorescent staining revealed no significant differences between cell preparations from uninfected and infected rabbits. Spleen cell preparations con-

TABLE 2. *Altered secondary responses when two sensitizing doses of SRBC were administered at the height of syphilitic lesions*[a]

Response	Ig	Spleen	PBL	Uninfected	Infected
Secondary, in vivo	IgM			204 ± 27	604 ± 68
	IgG			884 ± 76	68 ± 68
Secondary, in vitro	IgM	4		765; 17,600	385; 6,460
	IgG			700; 59,200	45; 680
	IgM	2		660; 19,400	400; 5,320
	IgG			425; 53,400	0; 280
	IgM	2	2	670; 17,400	365; 5,140
	IgG			450; 53,000	40; 420
	IgM	3	1	680; 23,400	405; 5,100
	IgG			690; 50,800	0; 300
	IgM		2	425; 3,300	155; 1,080
	IgG			15; 14,300	5; 120

[a] SRBC (2×10^9) were injected i.v. on days 21 and 35 of *T. pallidum* infection. The uninfected control received two injections of SRBC 14 days apart. The assay was performed 7 days after the second injection of SRBC. Values for in vivo responses indicate PFC per 10^6 cells ± standard deviation. Those for in vitro responses indicate PFC per culture, the first value referring to a culture system to which no SRBC were added, and the second value referring to a culture system to which SRBC were added. See text for further details.

TABLE 3. *Effect of autologous PBL and nylon wool-treated PBL on the secondary anti-SRBC response of spleen cells*[a]

Response	Ig	Cells[b]			Uninfected	Infected
		Spleen	PBL	NW-PBL		
Secondary, in vivo	IgM				105 ± 8	185 ± 11
	IgG				78 ± 15	715 ± 64
Secondary, in vitro	IgM	4			915; 32,000	1,070; 61,000
	IgG				370; 7,600	315; 30,800
	IgM	2			875; 28,400	1,290; 54,130
	IgG				450; 6,800	185; 31,734
	IgM	2	2		410; 10,267	925; 53,733
	IgG				45; 666	435; 29,267
	IgM		2		10; 280	240; 400
	IgG				5; 20	125; 200
	IgM	2		2	470; 29,733	1,035; 51,867
	IgG				370; 7,200	390; 33,200
	IgM			2	100; 1,680	335; 440
	IgG				0; 460	35; 160

[a] Each infected rabbit was injected i.v. with 4×10^7 *T. pallidum* and 21 days later with 2×10^9 SRBC. Six months later, a second i.v. injection of SRBC was administered. The uninfected control received SRBC at the same times, and the assay was performed 7 days after the second injection of SRBC. Values for in vivo responses indicate PFC per 10^6 cells ± standard deviation. Those for in vitro responses indicate PFC per culture, the first value referring to a culture system to which no SRBC were added, and the second value referring to a culture system to which SRBC were added. See text for further details.

[b] Values indicate number of cells $\times 10^6$ per culture. NW-PBL, Nylon wool-treated PBL.

tained 85 to 94% lymphocytes, with 40 to 52% of the cells bearing immunoglobulin on their surfaces. PBL preparations usually contained 60 to 75% lymphocytes before nylon wool filtration and 90 to 98% lymphocytes after treatment. Nylon wool removed primarily monocytes and neutrophils and did not appear to affect the proportion of immunoglobulin-bearing lymphocytes which ranged from 32 to 43% before or after filtration.

DISCUSSION

In an earlier study (2), we demonstrated that the primary and secondary in vivo IgG responses to a heterologous antigen (SRBC) were severely depressed throughout the first 2 months of syphilitic infection. The present study extends those findings and suggests that secondary in vitro responses to SRBC are also abnormal in *T. pallidum*-infected rabbits. At the height of infection, during the period when the treponemal burden in the tissues is maximal, spleen cells failed to respond in culture with IgG or IgM PFC.

The nature of the unresponsiveness may be multifactoral. Increased numbers of treponemes and greater amounts of mucoid material or acidic mucopolysaccharides are apparent at the time when dermal lesions are progressing (7, 28). Treponemes and/or their products could be competing with SRBC for uncommitted macrophages. Overloading of macrophages might inhibit their ability to process and present SRBC antigens to lymphocytes. Although this could account for the in vivo unresponsiveness of infected spleen cells, it is difficult to envision antigen carryover on the surface of macrophages being sufficient after extensive washing to produce the observed in vitro effects.

Treponemal antigen-antibody (Ag-Ab) complexes could also contribute to the observed immunosuppression (1, 4, 5, 16). The rabbit is known to mount early responses to nontreponemal and treponemal antigens so that, by the third week of infection, complexes formed in vivo in antigen excess might paralyze macrophages, rendering them incapable of ingesting SRBC. Several findings are consistent with this hypothesis: (i) carry-over of complexes on the surfaces of spleen cells is much greater than that of antigen (16, 21); (ii) complexes formed in antigen excess have been shown to suppress in vitro responses to a greater degree than complexes formed at equivalence (5, 16); and (iii) circulating immune complexes can be detected in the serum of rabbits with disseminated syphilitic lesions using C1q binding (9) and the Raji cell technique (26) (Baughn, Musher, and Tung, unpublished data).

Antigen and particularly Ag-Ab complexes could also impair the functional activity of B- or T-lymphocytes (1, 12, 13, 19, 20, 24). Complexes formed in critical ratios can "freeze" membrane receptors of Ag-reactive cells (12, 13), and may

reduce secondary responses in vitro (16). Retention of treponemal Ag-Ab complexes on the surface of T cells of infected rabbits might "freeze" the cell membrane and result in the unresponsiveness to SRBC seen when these cells are placed in tissue culture. Consistent with this hypothesis is evidence suggesting that other aspects of T-cell function are impaired during the early stages of infection with *T. pallidum* (18, 27, 31, 32).

Previous studies have shown that one long-term effect of sensitization in rabbits is the presence of circulating lymphocytes which specifically suppress immune responses (14). Our study, which demonstrates suppression of the immunoglobulin PFC response to SRBC in normal uninfected rabbits and ablation of this suppression by nylon wool filtration, supports this finding. The finding that rabbits which had active *T. pallidum* infection at the time of initial sensitization respond 6 to 9 months later to a booster dose of antigen with a seemingly normal, unsuppressed secondary response further suggests that the induction of an immune response to SRBC is abnormal during active syphilis; this abnormality may be at the level of antigen processing. No evidence was obtained during the course of these studies to support the possibility that *T. pallidum* infection suppresses immunoglobulin responses by inducing a population of functional suppressor T cells.

The relation between the observed abnormalities in the immune response to SRBC and the early appearance of treponemal and nontreponemal antibodies in syphilis remains to be elucidated, as does the relation between any of these findings and the progression of infection despite a humoral response. This study further emphasizes the complexity of immunological responses to *T. pallidum* infection.

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