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Effect of Pretreatment with *Mycobacterium bovis* (Strain BCG) and Immune Syphilitic Serum on Rabbit Resistance to *Treponema pallidum*

S. R. GRAVES AND R. C. JOHNSON*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

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Stimulation of the rabbit reticuloendothelial system with viable *Mycobacterium bovis* (strain BCG), and other agents, had no effect on the development of syphilitic lesions after intradermal or intravenous inoculation with graded doses of *Treponema pallidum* (virulent Nichol's strain; mean infective dose < 10). The simultaneous administration of immune syphilitic rabbit serum retarded the development of lesions, but this appeared to be due solely to the immune serum, suggesting no synergism between the activated reticuloendothelial system and the anti-*T. pallidum* antibodies. The administration of two doses of BCG enhanced syphilitic lesion development in the rabbit.

Syphilis, caused by infection with *Treponema pallidum*, is one of the remaining important and widespread bacterial diseases for which no vaccine is available. Apart from the serious and often fatal manifestations of tertiary syphilis, there is circumstantial evidence that prior infection with syphilis predisposes the host to earlier death from nonsyphilitic causes, possibly due to a lowering of general body defense mechanisms (4). There is an urgent need for a syphilis vaccine for use with "at-risk" populations. Before this can be achieved, the organism must be grown in vitro so as to obtain sufficient quantities of antigen. In addition, a clearer understanding is needed of the host's immune response to infection with *T. pallidum*, in particular an explanation for the unusually long delay in the development of natural immunity. Rabbits infected with *T. pallidum* take from 3 to 6 months before becoming resistant to reinfection (44), and in humans it is possible to reinfect syphilitic patients at various, but especially early, stages of the disease (7). Currently patients are usually treated before the development of immunity, leaving the patient susceptible to reinfection.

In the field of immunology, two components of host immunity are currently recognized: (i) humoral immunity, which is dependent on serum and secretory antibody, and (ii) cellular immunity, which is dependent on lymphocytes and macrophages. The role of antibodies (humoral immunity) in the evolution of a syphilitic infection in rabbits has recently been studied in several laboratories with almost identical results (32, 37, 43). Immune syphilitic rabbit serum, administered passively at the time of challenge and for a number of days thereafter, will not prevent the establishment of the infection. Lesions will not form while antiserum is being administered but develop once the antiserum is withdrawn. The lesions may be atypical in appearance and less severe after antiserum treatment. Of the various antibodies that develop during a syphilitic infection, reagenic (VDRL or Wasserman), Reiter protein complement fixing, immobilizing, or fluorescent, none correlates with immunity (25, 29, 42).

The role of cell-mediated immunity (CMI) in syphilis has not yet been established. Sixty-six percent of syphilitics in the tertiary stage of the disease show a delayed-type hypersensitivity skin reaction to an extract of *T. pallidum*, the Luotest (18). In vitro tests, presumably of CMI, on syphilitics have generally been positive (8, 13, 15, 26, 35), although some investigators have evidence of impaired CMI in some stages of the disease (13, 20, 28). In rabbits, a degree of immunity has been transferred with lymphocytes from immune rabbits (M. Metzger, personal communication), whereas a limited level of nonspecific resistance to *Listeria monocytogenes* was detected in rabbits 3 to 5 weeks after *T. pallidum* infection (36). In vitro tests for CMI were positive within 2 weeks (V. Wicher and K. Wicher, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M6, p. 67) or 3 weeks (Metzger, personal communication) of infection. These data suggest that CMI plays some role in the infection. In rabbits, however, the infection is never cleared, and viable *T. pallidum* remain in the lymph nodes for the life of the animal (44).

In this current investigation, we induced

CMI in rabbits against viable BCG before challenging them with low levels of *T. pallidum* both intradermally (i.d.) and intravenously (i.v.). In case opsonins were required for phagocytosis by the activated macrophages, some rabbits were passively inoculated with immune syphilitic rabbit serum before and after challenge. As well as BCG, other nonspecific stimulants of the reticuloendothelial system (RES) and of T-lymphocytes (thymus dependent) were tested for their ability to modify the course of *T. pallidum* infection in rabbits.

We found that BCG-induced CMI (as shown by enhanced carbon clearance and delayed-type hypersensitivity to tuberculin) gave no measurable resistance to *T. pallidum* infection. In conjunction with immune syphilitic serum, lesion retardation occurred but was probably due solely to the immune serum. *T. pallidum* was not susceptible to BCG-activated rabbit macrophages.

MATERIALS AND METHODS

Pretreatment of rabbits with immune serum. The immune serum used was a pool obtained from several rabbits approximately 6 months postinfection. They were shown to be immune by the failure of a lesion to develop after i.d. challenge with 10^6 *T. pallidum*. The serum was not heat inactivated. Each experimental rabbit received 60 ml of the immune syphilitic rabbit serum, i.v., in six 10-ml amounts. In protocol A, the first serum dose was given 4 h before challenge and every 2nd day thereafter for a total of 10 days. In protocol B, the first serum dose was given 2 days before challenge and daily thereafter until 3 days after challenge.

Pretreatment of rabbits with BCG. Each rabbit received either one or two doses of viable BCG (Glaxco). In the first case, 2 mg of BCG per rabbit i.v. (22) was followed by challenge 4.5 weeks later. In rabbits treated with two doses of BCG, the second dose (10 mg of BCG per kg i.v.) was given 5 weeks after the first and followed by challenge 1 week later. Rabbits were skin tested for delayed hypersensitivity to tuberculin (purified protein derivative), 250 test units per 0.1 ml, given 4 weeks after the first BCG dose.

Test for RES activity. Rabbits pretreated with BCG were tested for enhanced RES activity by the clearance of colloidal carbon from the blood (14). Eight milligrams of carbon per 100-g rabbit was injected i.v., and blood samples (0.02 ml) were taken 10, 20, and 30 min thereafter into 2.0 ml of water. The phagocytic index was determined as the slope of the straight line $\log_{10}$(optical density at 710 nm) against time.

Preparation of challenge T. pallidum. *T. pallidum* was propagated in the testes of large male New Zealand rabbits that had previously been shown to be free of reagenic antibody (RPR card test; Hynson, Wescott and Dunning, Inc., Baltimore, Md.). Approximately 5×10^7 viable *T. pallidum* was inoculated per testis, resulting in a well-developed orchitis within 8 to 11 days. Rabbits were housed at 16 to 19 C. Elution of *T. pallidum* from the minced orchitic testis involved three sequential 5-min washings with a total of 10 ml of sterile 5% bovine serum albumin (Reheis Chemical Co., Chicago, Ill.) in saline. The pooled washings, collected under sterile nitrogen, had a concentration of approximately 10^7 to 10^8 *T. pallidum* per ml as determined by counts in a Petroff-Hauser chamber. Dilutions of the suspension were prepared in 5% bovine serum albumin-saline, under nitrogen, and inoculated into the experimental rabbit within 1 h of the original harvest.

Challenge with T. pallidum. Male Dutch Belt rabbits were used in all experiments. They were previously shown to be free of reagenic antibody. The were housed individually at 16 to 19 C and fed an antibiotic-free diet. Each rabbit had its back shaved and reshaved when necessary. For i.d. challenge, the back was marked into areas for duplicate injections of variously sized inocula (1 to 10^4 *T. pallidum*). Each site received a volume of 0.1 ml i.d. For i.v. challenge, either 10^3 or 10^5 *T. pallidum* was inoculated. Three rabbits were used in each experimental group. After infection, the rabbits were examined daily for the development of dermal lesions on the shaved back and the time of first appearance of a lesion was noted.

Statistical analysis. The student *t* test was used to compare the time of appearance of lesions in different groups of rabbits and the number of lesions per rabbit after i.v. challenge. It was also used to compare rabbit phagocytic index, after various treatments, with normal rabbits.

Other attempts at RES stimulation. Although BCG was the main RES stimulant used in this study, others were also used, but not in conjunction with immune syphilitic serum. Endotoxin (lipopolysaccharide B) from *Escherichia coli* O127.B8 was given i.v., 250 μg/kg, 48 h before *T. pallidum* challenge. *Corynebacterium parvum* (heat killed) was given i.v., 15 mg/rabbit, 6 days before challenge. *Bordetella pertussis* was also given i.v., 5×10^{10} cells/rabbit, 6 days before challenge. In these groups of rabbits, the extent of RES stimulation was not tested for by carbon clearance.

Attempts to stimulate T-lymphocytes. Phytohemagglutinin M, 5 mg/kg, and concanavalin A, 1 mg/kg, were both given i.v. to separate groups of rabbits 48 h before *T. pallidum* challenge. The degree of lymphocyte stimulation was not mesured.

RESULTS

Immune syphilitic rabbit serum. Immune serum, protocol A, significantly retarded the development of syphilitic lesions after both i.d. (Tables 1–3) and i.v. (Table 4) challenges. The retardation was effective after i.d. challenge of 10^4, 10^3, and 10^2 *T. pallidum* and i.v. challenge with 10^5. The number of i.d. inoculation sites developing into lesions was also reduced at 10^3, 10^2, and 10 *T. pallidum* challenges. In protocol A, the immune serum was administered until

TABLE 1. *Development of dermal syphilitic lesions in rabbits treated with immune syphilitic serum and subsequently challenged with Treponema pallidum, intradermal route*

Challenge dose	Untreated rabbits		Immune syphilitic serum-treated rabbits			
			Protocol A[a]		Protocol B[b]	
	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)
10^4	6/6	13.8 ± 0.7	6/6	22.8 ± 4.7 $P < 0.005$	ND[e]	ND
10^3	6/6	18.0 ± 2.2	4/6	27.5 ± 2.2 $P < 0.001$	ND	ND
10^2	11/12	21.4 ± 6.6	2/6	32.0 ± 7.0 $P > 0.050$	6/6	26.7 ± 5.6 $P > 0.050$
10	13/18	27.5 ± 6.0	0/6		4/6	27.2 ± 5.4 $P > 0.050$
1	6/12	26.8 ± 2.6	ND	ND	3/6	24.7 ± 5.5 $P > 0.050$

[a] 60 ml of immune syphilitic rabbit serum given per rabbit, i.v., in six 10-ml amounts. The first dose was given 4 h before challenge and every 2nd day thereafter for a total of 10 days.

[b] 60 ml of immune syphilitic rabbit serum given per rabbit, i.v., in six 10-ml amounts. The first dose was given 2 days before challenge and daily thereafter until 3 days after challenge.

[c] Numerator, Number of positive syphilitic lesions. Denominator, Number of sites inoculated with *T. pallidum*.

[d] Days after challenge at which lesions first appeared; ±1 standard deviation.

[e] ND, Not done.

TABLE 2. *Development of dermal syphilitic lesions in rabbits treated with viable BCG (one dose) and subsequently challenged with Treponema pallidum, intradermal route*

Challenge dose	Untreated rabbits		1× BCG-treated rabbits[a]			
			Without immune syphilitic serum		With immune syphilitic serum (protocol A)[b]	
	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)
10^4	6/6	13.8 ± 0.7	6/6	18.0 ± 4.9 $P > 0.50$	5/6	20.8 ± 4.8 $P < 0.010$
10^3	6/6	18.0 ± 2.2	6/6	19.7 ± 4.0 $P > 0.050$	5/6	24.4 ± 3.1 $P < 0.010$
10^2	11/12	21.4 ± 6.6	6/6	24.2 ± 2.7 $P > 0.050$	2/6	27.5 ± 4.9 $P > 0.050$
10	13/18	27.5 ± 6.0	5/6	30.0 ± 4.6 $P > 0.050$	4/6	27.7 ± 2.9 $P > 0.050$
1	6/12	26.8 ± 2.6	ND[e]	ND	ND	ND

[a] 2 mg of BCG per rabbit, i.v., followed by *T. pallidum* challenge 4.5 weeks later.

[b] See footnotes *d*, Table 1.

[c, d, e] See Table 1.

the 10th day after challenge. In contrast, protocol B was an attempt to build up a high level of antibody at the time of introduction of the *T. pallidum*. The same volume of immune serum was administered, within a 5-day period, but only until the 3rd day after challenge. In protocol B, the immune syphilitic serum had no significant retarding effect on the development of lesions or on the number of inoculation sites becoming positive. The distribution of the immune serum over the longer time period, 10 days, as compared with 3 days after challenge was much more effective in modifying the course of the infection. This suggests that it was the continued presence of antibodies that was effective, probably by slowing the growth

TABLE 3. *Development of dermal syphilitic lesions in rabbits treated with viable BCG (two doses) and subsequently challenged with Treponema pallidum, intradermal route*

Challenge dose	Untreated rabbits		2× BCG-treated rabbits[a]			
			Without immune syphilitic serum		With immune syphilitic serum (protocol B)[b]	
	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)	Positive lesions[c]	Lesion appearance[d] (days)
10^4	6/6	13.8 ± 0.7	ND[e]	ND	ND	ND
10^3	6/6	18.0 ± 2.2	ND	ND	ND	ND
10^2	11/12	21.4 ± 6.6	9/10	16.4 ± 6.3 $P > 0.050$	6/6	21.0 ± 3.7 $P > 0.050$
10	13/18	27.5 ± 6.0	6/10	16.8 ± 4.4 $P < 0.005$	6/6	25.5 ± 2.7 $P > 0.050$
1	6/12	26.8 ± 2.6	6/10	22.8 ± 2.6 $P < 0.025$	3/6	28.3 ± 4.6 $P > 0.050$

[a] 2 mg of BCG per rabbit i.v. followed by 10 mg of BCG per kg i.v. 5 weeks later and challenge with *T pallidum* 1 week thereafter.
[b, c, d, e] See Table 1.

rate of the treponeme and delaying the onset of the lesion. Some destruction of the inoculum probably also occurred as shown by the reduced number of inoculation sites that developed into lesions at lower challenge doses.

BCG treatment. Rabbits treated with one dose of BCG showed no significant modification of their syphilitic infection after either i.d. or i.v. challenge. However, these rabbits were shown to have considerable enhancement of their phagocytic indexes (Table 5) and presumably enhanced CMI as well. Delayed hypersensitivity to tuberculin (purified protein derivative) was also present at the time of challenge. Nevertheless, any enhanced blood clearance of the treponemes after i.v. challenge, arising from the BCG activation of the liver and spleen phagocytes, was not detected. The number of dermal lesions and their time of appearance were similar to the control rabbits.

When immune syphilitic serum, protocol A, was given to the BCG-treated rabbits, the effect on lesion development was essentially the same as that observed with injection of immune syphilitic serum alone.

Rabbits treated with two doses of BCG showed significantly faster development of lesions after i.d. challenge (10^2, 10, and 1 *T. pallidum*) and a greatly increased number of dermal lesions (average 12.7) compared with control rabbits (average 2.7) after i.v. challenge with 10^3 *T. pallidum*. The enhanced infection in these rabbits was neutralized by immune syphilitic serum (protocol B) so that the infection proceeded normally (Table 3) even though immune syphilitic serum (protocol B) itself had no significant effect on lesion development in rabbits not treated with BCG (Table 1).

Other treatments. Presumed nonspecific stimulation of the rabbit RES with endotoxin (11, 45), *C. parvum* (1, 2, 46), and *B. pertussis* (45) of the rabbits' T-lymphocytes with phytohemagglutin M and concanavalin A (17) did not affect the course of the syphilitic infection after i.v. challenge with 10^6 *T. pallidum*. Dermal lesions appeared at day 22 or 23, as in the controls, and evolved in the normal manner.

DISCUSSION

The role of the phagocyte in the course of syphilitic infection is unknown. The virulence of strains of *T. pallidum* has been associated with the amount of mucoid (hyaluronic acid-like) material produced in the syphilitic lesion. Turner (42) suggests that this material impedes normal cellular defenses. It may well be part of an antiphagocytic treponeme outer coating. A protective outer covering has been postulated (9) and an outer membrane has recently been observed (16). The fact that *T. pallidum* is not completely cleared from the host (44) suggests that either phagocytosis is defective in some way or that *T. pallidum* survives inside the macrophages and other phagocytic cells in a way analogous to the true intracellular bacteria, e.g., *Mycobacterium tuberculosis* and *L. monocytogenes* If the latter is the case, intraphagocytic *T. pallidum* could account for the development of the disease in the host by aiding dissemination throughout the body (giving rise to secondary lesions) and possibly protecting the treponeme against the effects of serum

TABLE 4. *Development of dermal syphilitic lesions in rabbits treated with viable BCG and/or immune syphilitic serum and subsequently challenged with Treponema pallidum, intravenous route*

Challenge dose	Untreated rabbits		Immune syphilitic serum-treated rabbits (protocol A)		Viable BCG-treated rabbits							
					1× BCG treatment				2× BCG treatment			
					Without immune syphilitic serum		With immune syphilitic serum (protocol A)		Without immune syphilitic serum		With immune syphilitic serum (protocol B)	
	Lesions/rabbit[a]	Lesion appearance[b] (days)	Lesions/rabbit[a]	Lesion appearance[b] (days)	Lesions/rabbit[a]	Lesion appearance[b] (days)	Lesions/rabbit[a]	Lesion appearance[b] (days)	Lesions/rabbit[a]	Lesion appearance[b] (days)	Lesions/rabbit[a]	Lesion appearance[b] (days)
10^5	2, 0, 0	21, —, —	1, 3, 0	31, 32, —	1, 2, 0	21, 25, —	1, 3, 0	31, 32, —	ND	ND	ND	ND
10^3	3, 5, 0 avg: 2.7	24, 26, —	ND	ND	ND	ND	ND	ND	12, 17, 9 avg: 12.7 $P < 0.001$	20, 20, 25	2, 2	26, 28

[a] Number of dermal lesions per infected rabbit.
[b] Days after challenge at which lesions first appeared.

TABLE 5. *Phagocytic indexes of Dutch Belt rabbits after various treatments*

Treatment	Phagocytic index	
	Mean	Range
None	0.011	0.007–0.015
One BCG dose;[a] no *T. pallidum* infection	0.033	0.019–0.042 $P < 0.025$
Two BCG doses and *T. pallidum* infection[b]	0.027	0.020–0.032 $P < 0.010$
T. pallidum infection[c]	0.020	0.012–0.029 $P > 0.050$

[a] Tested 4.5 weeks after 2 mg of viable BCG was given i.v.
[b] First BCG dose was 2 mg i.v. followed 5 weeks later by 10 mg of BCG/kg i.v. *T. pallidum* infection (10^3 i.v. or 10^2 i.d.) was 1 week thereafter. Tested 6 weeks after *T. pallidum* inoculation.
[c] Tested 6 weeks after *T. pallidum* inoculation (10^3 i.v. or 10^2 i.d.).

antibody (38) and adverse extracellular redox conditions.

The use of viable BCG to nonspecifically enhance the phagocytic and bactericidal activity of macrophages against intracellular microorganisms, unrelated to mycobacteria (5, 23), may have given a clue as to the role of these host cells in the course of a syphilitic infection. That no significant retardation of the disease process was noted suggests that enhanced destruction of *T. pallidum* did not occur. Either phagocytosis is not part of the normal host response to the virulent bacterium or else BCG was an inappropriate antigen with which to attempt to stimulate the rabbit macrophages to exhibit enhanced *T. pallidum* uptake and digestion. The failure of the immune syphilitic serum to act synergistically with the activated macrophages of the rabbit suggests that any opsonins present in the serum were ineffective. In this regard, Metzger and Michalska (personal communication) found no correlation between the immune status of immunized rabbits and their serum level of opsonic antibody, as measured in their *T. pallidum* opsonophagocytic test.

Electron microscopic studies have shown *T. pallidum* within a variety of cells, including macrophages (3, 19, 29, 38, 39). However, they are often morphologically degraded (19) and may have been phagocytosed after they were no longer viable. By far the greatest majority of *T. pallidum* were extracellular (3, 19). The bacterium does not appear to be an obligate intracellular parasite, at least not in the early stages of the infection. This may explain the failure of activated macrophages to limit the infection. *T.*

pallidum virulence may be a result of its ability to resist phagocytosis per se or else due to its ability to survive within phagocytic cells.

The demonstration of increased carbon clearance in the BCG-treated rabbits is not a measure of skin macrophage activation, and this is not an ideal parameter for following an i.d. *T. pallidum* infection. However, after i.v. inoculation of *T. pallidum*, the phagocytic cells of the liver and spleen are the ones initially involved in clearance. In the latter case, no modification of syphilitic lesion development occurred after i.v. challenge of the rabbits treated once with BCG. The phagocytic cells rapidly removed the carbon particles (Table 5) but not the *T. pallidum* (Table 4). Thus, one of the determinants of *T. pallidum* virulence seems to be its ability to resist phagocytosis, even in the presence of immune serum.

Other agents that are thought to stimulate enhanced phagocytosis in the rabbit, e.g., endotoxin (11, 45) and *C. parvum* (1, 2, 46), or increase the number of circulating leukocytes, e.g., *B. pertussis* (45), were without effect on the course of syphilis in the rabbit when given before an i.v. challenge. Because CMI and macrophage activation are considered to proceed via initial stimulation of thymus-dependent lymphocytes (T-cells), two agents that nonspecifically stimulate T-cell mitogenesis in vivo in mice were also tested (17). Phytohemagglutinin M and concanavalin A, when given i.v. before an i.v. *T. pallidum* challenge, failed to modify the syphilitic infection.

The fact that BCG-induced CMI fails to offer any protection against virulent *T. pallidum* does not rule out the involvement of CMI in the evolution of this disease in the host. CMI against *T. pallidum* probably requires specific *T. pallidum* antigen induction. Such specificity has been observed in murine tularemia (10) and human toxoplasmosis (6). There is considerable evidence implicating CMI in syphilis. Some of the earlier work on syphilis (34, 40) suggests the importance of a cellular mechanism, possibly in conjunction with antibodies, in limiting the course of the infection. More recent work using various in vitro tests point to the development of delayed-type hypersensitivity and possibly also CMI during the course of the infection (8, 13, 15, 20, 21, 26, 28, 35).

Schell and Musher (36) have shown a degree of nonspecific CMI induced in rabbits infected with *T. pallidum* as measured by enhanced resistance to the growth of *L. monocytogenes* in the rabbit liver but not the spleen. There is, however, evidence that points to a failure of CMI to develop adequately during syphilitic infection (13, 20, 28, 48). Although this may be a nonspecific deficiency, as indicated by lymphocyte depletion of the T-dependent areas of spleen and lymph nodes (12, 21, 41,) after *T. pallidum* infection, it is more likely to be specific against *T. pallidum*, since syphilitics do not show reduced skin reactivity to other antigens (48) nor do their lymphocytes inadequately transform in the presence of other antigens (28). It has been suggested that the development of CMI by the host induces the onset of the latent stage of the disease, corresponding to the massive decline in numbers of detectable *T. pallidum* (36, 42). But the CMI response generated by the rabbit in conjunction with the humoral response does not appear to be able to destroy all the *T. pallidum*. Medici (24) has postulated that the antibodies of the humoral response are antagonistic to the antitreponemal CMI in a situation similar to the immunological enhancement of certain tumors. The situation in syphilis, especially paresis (general paralysis of the insane), also resembles lepromatous leprosy. In both cases, the chronicity of the infection is associated with high level of circulating antibody and specific CMI unresponsiveness (47, 48). In lepromatous leprosy, there is evidence that the defect in CMI is specific and not a general failure of T-cell function (47). On the basis of the chronicity, delayed-type hypersensitivity and granuloma formation characteristic of syphilis, a World Health Organization scientific group (47) has suggested that CMI may be involved in the host-*T. pallidum* relationship.

It is possible that the postulated failure to develop an adequate CMI in rabbits is due to the initial localization of *T. pallidum* in the cooler tissues, e.g., skin or testes, where the temperature is closer to the *T. pallidum* growth optimum, resulting in an inadequate transformation of the host's lymphocytes in these cooler tissues. In the armadillo, it has been demonstrated (33) that susceptibility to *Mycobacterium leprae* is due to the lower body temperature resulting in reduced lymphocyte transformation and a depressed CMI.

The reason for the enhanced *T. pallidum* infection after the second BCG dose is probably due to high-dose immunological paralysis of the rabbit RES, resulting in a diminished CMI response to *T. pallidum* and hence a more severe syphilitic infection. A similar enhancement of rabbit syphilis has been reported after a prior vaccinia infection (30, 31).

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