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# Immune Serum Confers Protection Against Syphilitic Infection on Hamsters

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Pooled serum from hamsters immune to syphilitic infection conferred complete protection on recipient hamsters challenged with *Treponema pallidum* subsp. *endemicum*. Cutaneous lesions did not develop, and the recipients' lymph nodes weighed less than those of controls and contained no treponemes. Treponemicidal activity in the pooled immune serum was relatively high. When treponemes were incubated in immune serum and complement and the suspension was then inoculated into hamsters, recipients developed neither lesions nor enlarged lymph nodes teeming with treponemes. With hamsters already infected for several weeks, however, immune serum failed to impair or influence the progression of syphilis. Treponemes were eliminated only when immune serum was administered within a short time of syphilitic infection. These results demonstrate that hamsters develop an effective serum-mediated treponemicidal response, but this response is not sufficient to eliminate treponemes at the primary foci of infection.

The course of syphilitic infection and its eventual resolution is associated with the development of both humoral and cell-mediated responses. We have shown previously that T-cell-dependent immunity can mediate resistance to syphilitic infection (20). The involvement of humoral immunity in eliminating *Treponema pallidum* from the infected host's tissue is a subject of controversy (5, 11–13, 15, 24, 28, 29).

The humoral response appears to afford only modest protection. When the disease has progressed from a local lesion to secondary syphilis, 24% of patients develop relapsing secondary lesions, despite the development of antibodies that immobilize and inactivate *T. pallidum* (5, 11–13, 15, 16, 24, 29). Rabbits injected with killed *T. pallidum* cells (6, 12, 30) or with extracts of *T. pallidum* (10) develop Venereal Disease Research Laboratory, hemagglutination, and specific treponemal antibodies and yet are not protected against subsequent challenge with virulent treponemes. Serum and immunoglobulin from rabbits resistant to syphilitic infection confer limited protection on recipients challenged with *T. pallidum* (3, 7, 8, 17, 22, 25–27), and syphilitic rabbit serum has treponemicidal activity in vitro (4, 5). These findings suggest that humoral reponses are involved, but that such responses do not decide the outcome of syphilitic infection.

In the present study we have determined that serum from hamsters immune to infection with *T. pallidum* subsp. *endemicum*, the causative agent of endemic syphilis, can confer protection on recipients against challenge by the same strain. We present direct evidence that syphilitic hamsters mount an effective humoral immune response that can be treponemicidal.

## MATERIALS AND METHODS

**Animals.** Inbred LSH/Ss Lak hamsters were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Hamsters weighing 80 to 100 g were housed six per cage at an ambient temperature of 18°C, a condition which facilitates the development of cutaneous lesions (9). Before treponemal infection the hamsters were shaved, and they were maintained free of hair by clipping twice a week.

**Organism.** *T. pallidum* subsp. *endemicum* (formerly *T. pallidum* Bosnia A) was originally isolated from a dark field-positive chancre on the shaft of the penis of a 35-year-old patient who resided in Bosnia, Yugoslavia (28). The strain has been maintained by passage in hamsters. In our laboratory the hamsters' inguinal lymph nodes were removed aseptically 3 to 4 weeks after intradermal infection, teased apart in sterile saline, and filtered through 60-mesh stainless-steel screens. After centrifugation at 270 × *g* for 3 min to remove cellular debris, the number of treponemes in the supernatant was determined by dark-field microscopy.

**Infection of hamsters and evaluation of pathological changes.** Hamsters were infected intradermally at two sites in the inguinal region with treponemes suspended in RPMI 1640 medium. Cutaneous lesions generally developed 2 to 3 weeks after inoculation with $1 \times 10^5$ to $5 \times 10^5$ organisms. Two other pathological changes—increases in the weight of inguinal lymph

nodes and in the number of treponemes in the nodes—were also used to evaluate the host response to infection as previously described (21). The same criteria were used to evaluate the immune response of hamsters adoptively immunized with serum from syphilitic donors.

**Counting of treponemes in lymph nodes.** The approximate number of treponemes per lymph node was determined by the procedure of Miller (14). Briefly, duplicate slides of each homogenized lymph node were prepared, and 120 fields per slide were examined for treponemes by dark-field microscopy. When the number of treponemes in the suspensions was less than $10^3$, lymph nodes were centrifuged at 10,000 × *g* for 20 min, suspended in 0.02 ml of RPMI 1640 medium, and examined by dark-field microscopy.

**Preparation of immune and normal sera.** Sixty hamsters were infected intradermally at two sites in the inguinal region with $10^5$ *T. pallidum* subsp. *endemicum*. At 10 to 16 weeks after infection the hamsters were treated with penicillin (4,000 U) to terminate infection. After another 2 weeks five hamsters were reinfected intradermally with *T. pallidum* subsp. *endemicum* and were resistant to the development of further syphilitic lesions. The remaining animals were bled by intracardiac puncture to obtain serum. Concomitantly normal serum was obtained from 55 hamsters 14 days after treatment with penicillin.

The pooled immune and pooled normal sera were sterilized by filtration (0.45-μm filter; Millipore Corp., Bedford, Mass.) and stored at −20°C until use. The pooled immune serum had a microhemagglutination *T. pallidum* antibody titer of 10,240, whereas normal serum had no microhemagglutination *T. pallidum* antibody titer.

**Passive transfer of resistance.** Recipients were injected intravenously with immune or normal serum (0.5 ml) at 3-day intervals for 3 or 4 weeks. Three days after the first injection the hamsters were challenged intradermally with $10^5$ *T. pallidum* subsp. *endemicum* organisms.

**Assay of treponemicidal activity.** Duplicate 0.1-ml portions of heat-inactivated, prereduced immune and normal sera and their serial twofold dilutions in RPMI-1640 medium were supplemented 1:4 with guinea pig complement (GIBCO Laboratories, Grand Island, N.Y.) and inoculated with $10^5$ *T. pallidum* subsp. *endemicum* organisms in flat-bottomed 96-well microtiter dishes (Costar, Cambridge, Mass.) at a final volume of 0.2 ml per well. Only the 1:16 suspension was injected into normal hamsters. These suspensions were incubated in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, Mich.) at 34°C for 5 h. After incubation the percentage of motile treponemes, displaying a corkscrew or flexing motion, was determined in some of these suspensions. Approximately 35% of *T. pallidum* subsp. *endemicum* organisms are motile when extracted from freshly harvested lymph nodes of syphilitic hamsters and examined immediately by dark-field microscopy. The remaining suspensions (0.1 ml) were injected intradermally at two sites in the inguinal region.

**Serological test.** The Sera-Tek treponemal antibody test, manufactured by Fujizoki Pharmaceutical Co., Ltd., Tokyo, Japan, was obtained from Ames Co. (Elkhart, Ind.). The test was performed as specified by the manufacturer, except that pooled serum obtained from the syphilitic hamsters was serially diluted with absorbing diluent to yield quantitative titers.

**Statistical analysis.** All results were tested by analysis of variance. The Fisher least-significant-difference test (23) was used to examine pairs of means when a significant F ratio indicated reliable mean differences. The alpha level was set at 0.05 before the experiments started.

## RESULTS

**Development and transfer of humoral immunity.** Seven groups of four hamsters each were injected intravenously with serum obtained from hamsters at various intervals after infection. An eighth group received normal serum. Subsequently all recipients were challenged.

Infection with *T. pallidum* subsp. *endemicum* produced lesions in most animals that had been adoptively immunized with serum from hamsters infected for less than 8 weeks (Table 1). These sera, however, contained sufficient antitreponemal activity to delay the onset of lesions and significantly decrease ($P \leq 0.01$) the weight and number of treponemes in the lymph nodes, compared with recipients of normal serum. In contrast, hamsters passively immunized with serum from hamsters infected for more than 8 weeks had no lesions. As a corollary, their lymph nodes weighed less and contained few or no treponemes.

These results suggest that the degree of protection conferred on recipients by immune serum correlates well with the progress of syphilitic disease in the donor hamsters at the time the immune serum is obtained.

**Quantitation of treponemicidal activity.** Pooled immune serum (microhemagglutination *T. pallidum* titer, 10,240) was obtained from hamsters infected for 10 to 16 weeks. Six groups of three or four hamsters each were injected intravenously with this serum, undiluted or in serial twofold dilutions, at 3-day intervals for 24 days. Three days after the first injection the hamsters were challenged with *T. pallidum* subsp. *endemicum*. Concomitantly a seventh group of three hamsters received normal serum and was infected with *T. pallidum* subsp. *endemicum* (Table 2). In all hamsters receiving immune serum or its dilutions (up to eightfold), lesions failed to develop, and the lymph node weights were significantly less ($P \leq 0.1$) than in controls. More importantly, no treponemes were detected in their lymph nodes, even after dark-field examination of extracts concentrated by centrifugation. Further dilutions of immune serum, however, decreased anti-*T. pallidum* subsp. *endemicum* activity. Lesions developed, and the weights and number of treponemes in the lymph nodes increased. All recipients of normal serum developed lesions, and their lymph nodes contained $10^5$ treponemes.

TABLE 1. Increase in protection conferred by transfer of immune serum from donors infected for increasingly longer times with *T. pallidum* subsp. *endemicum*[a]

| Length of infection (weeks) | Lesions | | Lymph nodes | |
|---|---|---|---|---|
| | No./total sites | Development time (days) | Weight (mg) | Treponemes ($\times 10^3$) |
| 0 (normal) | 8/8 | 13 ± 1 | 19 ± 2 | 1,938 ± 1.2 |
| 1 | 8/8 | 17 ± 1 | 11 ± 1 | 39 ± 0.1 |
| 2 | 8/8 | 18 ± 1 | 11 ± 2 | 39 ± 0.1 |
| 4 | 6/8 | 23 ± 1 | 9 ± 1 | 21 ± 0.1 |
| 6 | 4/8 | 25 ± 1 | 8 ± 2 | 17 ± 0.1 |
| 8 | 0/8 | | 5 ± 1 | 0.4 ± 0.1 |
| 10 | 0/8 | | 6 ± 1 | 0 |
| 16 | 0/8 | | 5 ± 2 | 0 |

[a] *T. pallidum* subsp. *endemicum* organisms were inoculated at two sites in each hamster. Animals were sacrificed 40 days after infection. All values in columns 3 through 5 are given as means ± standard errors ($n$ = 4 hamsters per group).

Immune serum and its dilutions (up to 32-fold) also killed *T. pallidum* subsp. *endemicum* in vitro. Treponemes were incubated anaerobically with immune serum and complement for 5 h and inoculated into hamsters. Recipients of suspensions of treponemes and immune serum diluted 16-fold developed no lesions, and their lymph nodes weighed less than those of controls and contained no treponemes (Table 3). When these experiments were replicated, similar results were obtained.

**Effect of immune serum on progression of syphilis.** These same lots of immune (microhemagglutination *T. pallidum* titer, 10,240) and normal sera were administered to hamsters 2, 3, 6, and 12 weeks after infection. Recipients of immune or normal serum showed no significant differences in development of cutaneous lesions or in lymph node weights (Table 4). The weights and numbers of treponemes in the lymph nodes of recipients of immune serum were generally lower than for recipients of normal serum, although these differences were statistically significant only at week 2 ($P \leq 0.05$). Administration of immune serum to hamsters infected for 1 week was partially successful in reducing the number and size of lesions and the weight and number of treponemes in the lymph nodes. Administration of undiluted immune serum within a short time of syphilitic infection, however, prevented the development of lesions (Table 4). The animals' lymph nodes weighed less than those of controls and contained no treponemes. When these experiments were partially replicated with new lots of immune serum, similar results were obtained. Occasionally small numbers of treponemes (<100) were detected in the lymph nodes of hamsters passively immunized with undiluted immune serum and then infected within 3 days.

## DISCUSSION

The results of this investigation showed that serum from hamsters immune to syphilitic infection can confer complete protection on recipient hamsters challenged with *T. pallidum* subsp. *endemicum*. Relatively high treponemicidal activity was found in serum of hamsters infected 10 weeks and more. Administration of immune serum to hamsters already infected for more

TABLE 2. Effect of dilution of immune serum on pathology of recipient hamsters challenged with *T. pallidum* subsp. *endemicum*[a]

| Serum dilution | Lesions | | Lymph nodes | |
|---|---|---|---|---|
| | No./total sites | Development time (days) | Weight (mg) | Treponemes ($\times 10^3$) |
| Undiluted | 0/8 | | 5 ± 2 | 0 |
| 1:2 | 0/6 | | 5 ± 2 | 0 |
| 1:4 | 0/6 | | 5 ± 1 | 0 |
| 1:8 | 0/6 | | 6 ± 4 | 0 |
| 1:16 | 4/6 | 24 ± 5 | 10 ± 4 | 4 ± 1 |
| 1:32 | 3/6 | 18 ± 4 | 18 ± 17 | 50 ± 4 |
| Normal serum | 6/6 | 15 ± 2 | 11 ± 1 | 96 ± 10 |

[a] *T. pallidum subsp. endemicum* organisms were inoculated at two sites in each hamster. Animals were sacrificed 10 days after the controls developed lesions. Lymph node values are given as means ± standard errors ($n$ = 3 or 4 hamsters per group).

TABLE 3. Pathology of hamsters inoculated with a suspension of *T. pallidum* subsp. *endemicum* incubated in normal or immune serum[a]

| Treponemes incubated in: | Lesions | | Lymph modes | |
|---|---|---|---|---|
| | No./total sites | Development time (days) | Weight (mg) | Treponemes ($\times 10^3$) |
| Normal serum | 4/6 | 32 ± 6 | 17 ± 5 | 90 ± 8 |
| Immune serum | 0/6 | | 7 ± 1 | 0 |

[a] *T. pallidum* subsp. *endemicum* organisms were incubated in serum with complement for 5 h. A 16-fold dilution of the suspension was then inoculated at two sites in each hamster, and the animals were sacrificed 10 days after controls developed lesions. Values in columns 3 through 5 are means ± standard errors ($n$ = 3 hamsters per group).

than 2 weeks, however, failed to impair the progress of the disease. Treponemes were eliminated only when immune serum was administered within a short time (3 days) of syphilitic infection. These results suggest that antibody-mediated immunity is involved in hamsters' resistance to syphilitic infection.

Similar observations have shown that immune serum from syphilitic immune rabbits can confer protection against challenge with *T. pallidum* Nichols (3, 7, 8, 17, 22, 25–27, 31). In that case, when treatment with immune serum was discontinued, lesions (chancres) developed. We have also shown (19) that partial protection can be conferred on hamsters with immune serum. It inhibited the development of lesions, but did not prevent infection; the inguinal lymph nodes contained a measurable number of treponemes. In the present investigation no syphilitic lesions or other pathological changes were detected after administration of immune serum, even when the serum was diluted eightfold.

These apparent differences in treponemicidal activity of syphilitic immune serum may be related to its quality. It is possible that infected hamsters and rabbits have a period of maximum serum treponemicidal activity. Pooling immune sera from animals at different stages of disease may dilute its activity.

Humoral immunity developed gradually in syphilitic hamsters (Table 1). Recipients passively immunized with serum from hamsters infected for only 1 week had antitreponemal activity. Their lymph nodes weighed less and contained significantly ($P \leq 0.01$) fewer treponemes than did those of controls. Serum treponemal activity increased between 2 and 8 weeks after infection. Immune serum obtained after 10 weeks of infection conferred complete protection on recipients to syphilitic challenge. When treponemes were incubated in immune serum and complement and then inoculated into hamsters, recipients developed neither lesions nor enlarged lymph nodes teeming with treponemes. These results clearly demonstrate that hamsters develop an effective serum-mediated antitreponemal response.

Infection of the LSH hamsters with *T. palli-*

TABLE 4. Effect of immune serum on progression of syphilis in hamsters infected for various times with *T. pallidum* subsp. *endemicum*[a]

| Serum administered | | Lesions | | Lymph nodes | |
|---|---|---|---|---|---|
| Weeks after infection | Type | No./total sites | Diameter (mm) | Weight (mg) | Treponemes ($\times 10^3$) |
| 0 | Normal | 8/8 | 12 ± 1 | 22 ± 2 | 1,424 ± 79 |
| | Immune | 0/8 | 0 | 9 ± 3 | 0 ± 0 |
| 1 | Normal | 8/8 | 15 ± 2 | 30 ± 2 | 441 ± 24 |
| | Immune | 3/8 | 6 ± 1 | 15 ± 10 | 37 ± 9 |
| 2 | Normal | 8/8 | 17 ± 2 | 45 ± 7 | 1,271 ± 56 |
| | Immune | 8/8 | 17 ± 1 | 35 ± 10 | 403 ± 15 |
| 3 | Normal | 8/8 | 35 ± 4 | 78 ± 10 | 112 ± 13 |
| | Immune | 8/8 | 32 ± 3 | 61 ± 2 | 64 ± 1 |
| 6 | Normal | 8/8 | 50 | 77 ± 33 | 118 ± 1 |
| | Immune | 6/6 | 34 ± 4 | 68 ± 38 | 83 ± 1 |
| 12 | Normal | 4/4 | 50 | 34 ± 12 | 136 ± 13 |
| | Immune | 4/4 | 50 | 25 ± 10 | 91 ± 12 |

[a] *T. pallidum* subsp. *endemicum* were inoculated at two sites in each hamster. Immediately or at 1, 2, 3, 6, or 12 weeks after infection, groups of 3 or 4 hamsters were inoculated with normal or immune serum. Values in columns 4 through 6 are means ± standard errors.

*dum* subsp. *endemicum* is characterized by chronic cutaneous lesions and lymph nodes teeming with treponemes that persist for 6 to 9 months (21). Animals infected for 10 weeks or longer obtain quick resolution of their lesions by treatment with penicillin and are thereafter resistant to reinfection (18). The inability of hamsters to eliminate *T. pallidum* subsp. *endemicum* in the absence of antimicrobial treatment is a paradox, as their serum can kill treponemes in vitro and confer complete protection on recipients. In this study the progression of syphilitic disease was not impaired when these animals were infused with additional treponemicidal serum (Table 4). These results suggest that hamsters can develop the humoral immune components necessary to resist superinfection, but these components are unable to destroy and eliminate *T. pallidum* at the primary foci of infection. Treponemes are killed only when immune serum is administered within a short time of syphilitic infection (Tables 1, 2, and 4).

The protracted nature of syphilitic infection in hamsters despite the development of substantial humoral and cell-mediated immunity (20) implies that treponemes are protected from destruction. Alderete and Baseman (1) and Baseman and Hayes (2) have presented evidence that host proteins are both loosely and avidly associated with the outer envelope of *T. pallidum* Nichols. Treponemes could evade direct cell killing, antibody-mediated phagocytosis, or complement-mediated lysis by coating themselves with protective "self" antigens or antibodies. Additional investigations are needed to examine these and other possible mechanism(s) by which treponemes evade an effective humoral response.

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