Median Infective Dose of *Treponema pallidum* Determined in a Highly Susceptible Guinea Pig Strain

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The median infective dose of *Treponema pallidum* subsp. *pallidum* and the production of immunity to reinfection in C4D guinea pigs have been determined with 10^1 to 10^6 organisms per infective dose. The mean infective dose is 10^2 organisms, and immunity—in those animals that demonstrated lesions—developed after 4.5 months postinfection.

The suitability of the guinea pig as an alternative model for venereal syphilis has been gradually established (11, 19). We have examined inbred strains 2 and 13, outbred strains Hartley and Albany, and a strain deficient in the fourth complement component (C4D). We have found that the C4D animals are the most susceptible to *Treponema pallidum* infection and develop the most severe clinical symptoms among the various strains of guinea pigs (21). Possibly because of their high susceptibility, the clinical course of *T. pallidum* infection in C4D animals is practically unaffected by the factors of age, sex, and site of inoculation (22). Moreover, like humans, rabbits, or inbred strain 2 guinea pigs, *T. pallidum*-infected C4D animals respond with production of circulating immune complexes and autoantibodies to fibronectin and creatine kinase (2). Although C4D is not strictly an inbred strain, we have demonstrated that differences in histocompatibility within the strain seem to be negligible, as indicated by mixed lymphocyte cultures and prolonged (at least up to 6 months posttransfusion) survival of cells adoptively transferred (24). This is consistent with the concept that graft-versus-host reaction is very mild or unnoticeable in guinea pigs (14). Webster et al. (15) reported the establishment of a successful allogeneic chimeric state in C4D guinea pigs (Hartley→C4D) for more than 1 year. The C4D guinea pig has also a great potential for experimental congenital syphilis (23) and for establishing a guinea pig-adapted strain of *T. pallidum* (20). The potential usefulness of the C4D guinea pig for experimental syphilis calls for determination of the median infective dose (ID_{50}) of *T. pallidum* and examination of how the various infective doses relate to the development of resistance to reinfection.

The C4D strain was identified in 1970 in the National Institutes of Health (5) within the multipurpose outbred guinea pig colony during a course of immunization for preparation of immunoglobulin allotype-specific antisera. These animals have genetically controlled total deficiency of the fourth component of complement. The animals' reproduction, lifespan, health status, and susceptibility to infectious agents are similar to those of the complement-sufficient strains of guinea pigs. The C4D animals were widely distributed to research institutions by a generous policy of the National Institutes of Health and M. M. Frank (4) and contributed greatly to various studies, especially in the complement field (3).

(The work described below was presented in part at the Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 1988.)

The C4D strain, obtained from the National Institutes of Health by our institution in 1975, has a restricted genetic polymorphism, as it was started with one male and six females. The animals used for our experiments were 3- to 4-month-old males. They were housed individually in air-conditioned quarters (18 to 20°C) and fed antibiotic-free food and water ad libitum.

T. pallidum subsp. *pallidum* Nichols was obtained from testes of infected Nys Flemish Giant rabbits at the peak of orchitis (ca. 10 days). The organisms were extracted into phosphate-buffered saline (PBS) (10 ml per testis), and tissue and cell debris was removed by centrifugation at 200 × *g*. Various concentrations (10^6 to 10^1 organisms [10]) of *T. pallidum* were prepared in a diluent consisting of *T. pallidum*-free inflammatory testis fluid. The diluent was prepared by extraction of orchitic tissue in PBS (10 ml per testis) and centrifugation at 300,000 × *g* for 1 h. The effective removal of treponemes from the testis extract was verified by its injection (1 ml per testis) into testes of two normal rabbits. All 60 animals (six groups of 10 animals each), under anesthesia (Ketaset; Bristol Laboratories, Syracuse, N.Y.), were infected intradermally in a depilated hind leg (right site) with one of the various concentrations (10^1 to 10^6 organisms) of *T. pallidum*. During the infection (lasting ca. 2 h), the treponemal suspensions were kept at room temperature and frequently stirred. Single animals from the six groups were selected alternately for infection. Infected areas were depilated once a week and examined daily or every other day for the appearance of lesions.

On the basis of previous experience (19), we elected to examine the development of immunity 4.5 months after primary infection in three groups of animals injected with 10^1, 10^2, or 10^3 organisms. The animals were challenged with $4 × 10^7$ *T. pallidum* in a 0.1-ml volume, injected intradermally in the depilated (left) hind leg; this inoculum consistently produced 100% symptomatic lesions in normal C4D animals. Sera collected under sedation (Ketaset) monthly for a period of 4 months postinfection and 4 months postreinfection were examined for presence of antibodies by fluorescent treponemal antibody adsorption test (FTA-ABS), enzyme-linked immunosorbent assay (ELISA) with solubilized *T. pallidum* as an antigen, and immunoblot. All of these procedures were done in a manner similar to that described previously (18).

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TABLE 1. Determination of ID_{50} of *T. pallidum* in groups of 10 C4D young adult, male guinea pigs

Infective dose	No. of animals with lesions	Time (days) to onset of lesions[a]		Duration (days) of lesions	
		Range	Mean	Range	Mean
10^1	0				
10^2	5	51-86	66.4	61-140	75
10^3	8	26-64	39.3	35-105	71.4
10^4	8	17-30	22.9	34-118	61.0
10^5	10	11-30	18	26-110	50.4
10^6	10	12		54-140	70.8

[a] Animals infected with 10^1 organisms exhibited no lesions during 4 months of observation.

After primary infection, the number of demonstrable lesions and their times of development varied with the infective dose (Table 1). None of the animals infected with 10^1 organisms developed clinical symptoms throughout the 4-month observation period. Fifty percent of animals infected with 10^2 *T. pallidum* developed typical ulcerative dermal lesions within 51 to 86 days [mean, 66.4 days]; hence, the ID_{50} for the C4D guinea pig is considered to be 10^2 organisms. Groups of animals infected with 10^3 or 10^4 *T. pallidum* demonstrated dermal lesions in 80% of the animals, and those receiving 10^5 or 10^6 organisms demonstrated dermal lesions in 100% of the animals. The higher the infective dose was, the shorter the incubation time was, but the difference in the duration of the lesion was not dose dependent. Regardless of the size of the inoculum, the development and characteristics of the lesion were similar: an indurated papule which evolved into a more severe chancrelike lesion with centrally located superficial or deep necrosis. Once the lesions developed, their sizes (9- to 15-mm diameter) were not significantly different among the various groups. After a variable period, the necrotic lesions began to recede, until total regression and healing occurred.

Various numbers of *T. pallidum* were found in the lesions by dark-field microscopy; the number varied with the time of infection but not with the size of the initial inoculum. Thus, more treponemes were seen (an average of one organism per field, magnification of ×100) in the early stage of lesion development than during the regression period (an average of 0.2 organisms per field, or one organism in every five fields).

Reinfection of the three groups previously infected with 10^1, 10^2, and 10^3 organisms evoked a strong delayed type hypersensitivity reaction characterized by redness, induration, and increase in local body temperature. The reaction appeared 24 to 48 h after injection and lasted up to 10 days. Typical ulcerative dark-field-positive lesions developed in all animals previously injected with 10^1 organisms and in those five animals which were asymptomatic after primary infection with 10^2 organisms. None of the animals previously infected with 10^3 treponemes developed typical lesions after reinfection, suggesting that all were immune. In most animals that developed lesions after reinfection, their onset was in some degree hindered by the prolonged delayed type hypersensitivity reaction.

After primary infection, the FTA-ABS titers correlated with the size of the inoculum but did not differ significantly between symptomatic and asymptomatic animals within a group (Fig. 1). Animals infected with 10^6 organisms showed FTA-ABS titers of 128 ± 44 (mean ± standard deviation)
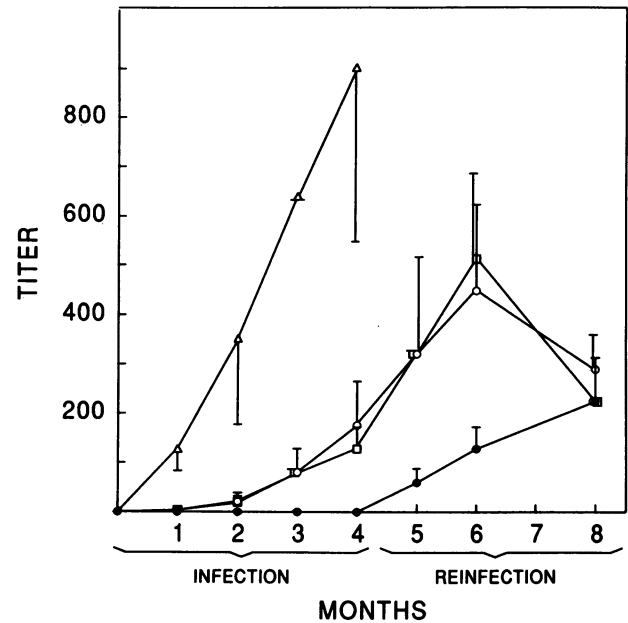

FIG. 1. FTA-ABS test. Titers of treponemal antibodies in C4D guinea pigs infected with 10^6 (△), 10^3 (□), 10^2 (○), and 10^1 (●) *T. pallidum*. Sera of individual animals were examined monthly for a period of 4 months postinfection, and those initially with 10^3 to 10^1 organisms were also examined after reinfection. Results are expressed as group means ± standard deviations.

after 1 month and 896 ± 350 after 4 months of infection, whereas animals infected with 10^2 and 10^3 organisms were serologically negative after 1 month and reached titers between 128 ± 44 and 176 ± 88, respectively, after 4 months of infection. Animals infected with 10^1 *T. pallidum* were negative by FTA-ABS throughout the 4-month period, but the ELISA with solubilized *T. pallidum* as the antigen (Fig. 2) demonstrated a gradual increase (titers up to 1,000) in treponemal antibody titers, indicating a relatively mild but active infectious process.

After reinfection, both groups initially infected with 10^2 or 10^3 organisms demonstrated similar patterns of FTA-ABS titers (Fig. 1): a gradual increase reaching a maximum titer of 512 ± 175 or 448 ± 175, respectively, at 2 months following reinfection and decreasing thereafter. The same sera reacted in ELISA to soluble *T. pallidum* antigen with an immediate increase of titers which remained on the same level (group infected with 10^3 organisms) or started to decrease (group infected with 10^2 organisms) within a few weeks. The relatively restricted response observed with FTA-ABS was anticipated, since the animals of either group (injected with 10^2 or 10^3 organisms) may have developed a substantial degree of immunity after primary infection. The extremely low humoral response to reinfection in the group initially injected with 10^1 organisms (Fig. 1), however, was unexpected, particularly since all of these animals, as well as five normal controls, developed typical ulcerative lesions after challenge with 4×10^7 organisms.

Molecular analysis of the humoral response, with pooled sera collected 4 months after primary infection with 10^1, 10^2, and 10^3 organisms and 1 to 4 months after reinfection, was done by immunoblot (Fig. 3). The number of *T. pallidum* polypeptides recognized by the antisera depended on the number of organisms used for primary infection. Four
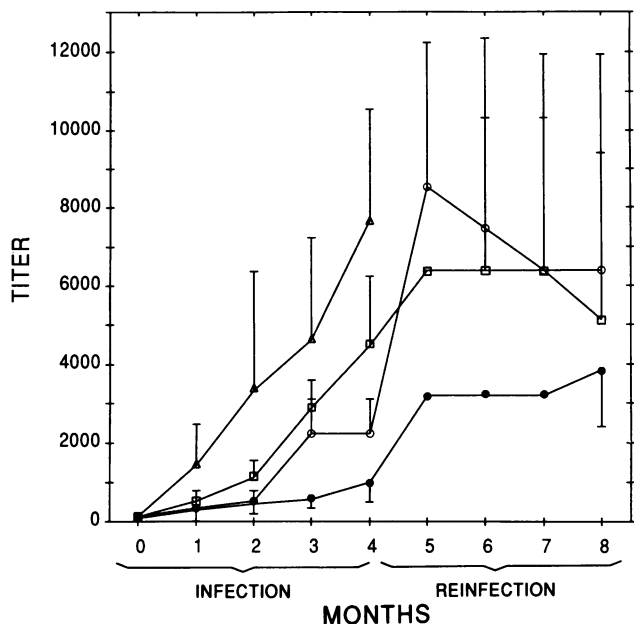
FIG. 2. ELISA. Solubilized *T. pallidum* (0.5 µg per well) was used as an antigen. The antibody titer has been defined as the reciprocal value of the highest dilution of serum giving an absorbance value of ≥0.2. The symbols and times of examination are the same as in Fig. 1, and the results are expressed as group means ± standard deviations.

months after infection, the sera from animals infected with 10^1 microorganisms did not react with *T. pallidum* polypeptides (Fig. 3A, lane 1). However, sera from animals infected with 10^2 organisms recognized the 47-kDa polypeptide (Fig. 3B, lane 1), and sera from those infected with 10^3 microorganisms recognized 47-, 45-, 39-, and 37-kDa polypeptides (some of the lines are practically invisible but were present on the nitrocellulose paper). During the first 2 months after reinfection, the group injected with 10^1 organisms produced antibodies reacting with a single polypeptide of 47 kDa and at the third month produced antibodies to five *T. pallidum* proteins (47 to 38 kDa). The animals infected with 10^2 or 10^3 *T. pallidum*, after reinfection, showed also antibodies to increasing numbers of *T. pallidum* polypeptides from 47 to
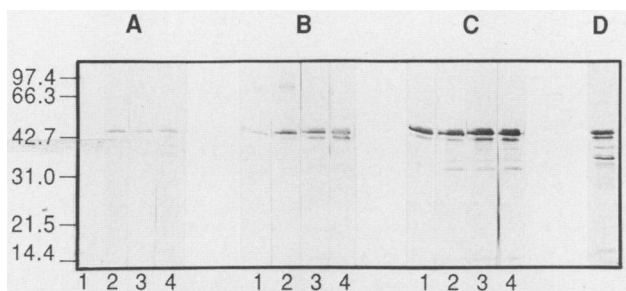

FIG. 3. Immunoblots of pooled sera from C4D animals infected with 10^1 (A), 10^2 (B), or 10^3 (C) *T. pallidum*, examined 4 months after infections (lanes 1) and 1 month (lanes 2), 2 months (lanes 3), and 3 months (lanes 4) after reinfection. *T. pallidum* Nichols has been used as an antigen [5 µg/20 µl], and sera were diluted 1:20. For comparison, results with pooled sera from animals infected with 10^6 *T. pallidum*, taken 4 months after infection, are presented in panel D.

15 kDa. Though immunoblotting is not a quantitative technique, the gradual increase in the antibody activity was well represented.

The various concentrations of *T. pallidum* were prepared in *T. pallidum*-free inflammatory testis fluid to ensure identical conditions for all concentrations of *T. pallidum*. The diluent did not contain residual *T. pallidum*, as established by intratesticular injection in rabbits. The presented data allow us to conclude that the C4D animals have a susceptibility close to that of humans (50 organisms [9]) and rabbits (25 organisms [8]). Approximately 100 *T. pallidum* caused symptomatic infection and immunity in 50% of the animals, and even 10 microorganisms could produce asymptomatic infection. Animals infected with 10^2 organisms, with or without dermal lesions, produced treponemal antibodies within 2 months, and those which developed lesions were immune to reinfection after 4.5 months. While the humoral response to reinfection in the groups of animals initially infected with 10^2 and 10^3 organisms followed a general pattern previously seen in immune animals (16, 24), we cannot explain the low humoral response to reinfection in animals injected with 10^1 *T. pallidum*. After reinfection, 100% of these animals developed typical ulcerative lesions no different from those developed in control animals or in those infected with 10^6 organisms, and yet, antibody levels examined by two quantitative tests clearly showed a significantly ($P < 0.001$, Student's t test) depressed humoral response. Since a mild but steady increase in titers in the ELISA with solubilized *T. pallidum* as an antigen was observed after infection, we are tempted to speculate that some degree of low-dose tolerance induction may have occurred during this stage; this, however, remains to be further explored.

The delayed type hypersensitivity reaction observed after reinfection in animals infected with 10^1 to 10^3 organisms was likely against rabbit proteins present in the inoculum used for reinfection, since it was of the same degree of severity regardless of the number of organisms used for primary infection. The ID_{50} for C4D animals is the lowest among the five strains examined in our laboratory (12, 21). The ID_{50} of *T. pallidum* in the Hartley strain has been earlier determined to be 10^5 microorganisms (12) and is similar to that in both inbred strains 2 and 13 (11).

In addition to its high susceptibility to *T. pallidum*, the C4D animal presents other advantages. Primary lesions in these animals are more severe and more prolonged than in any of the other strains of guinea pigs, thereby affording an experimental model for the exploration of protective mechanisms of immunity, particularly those of a cell-mediated nature (24). Several studies have indicated that T lymphocytes from C4D guinea pigs respond as well as T lymphocytes from inbred strain 13 (both guinea pig leukocyte antigens identical, including the I-region) in recognition of antigens and mitogens. C4D guinea pig lymphocytes also function normally as both the responder and stimulator populations in a mixed lymphocyte reaction (4). Antigen- or mitogen-pulsed macrophages from C4D animals are able to effectively stimulate T-cell proliferation, suggesting a normal capability for mitogen and antigen presentation (4). Although genetically deficient in the C4 component, the animals possess a normal alternate pathway which allows them to fix complement components beyond C4 and to generate biologically active components in the absence of C4 (7). Therefore, C4D guinea pigs are capable of mediating several in vivo biologic functions, including normal complement-dependent inflammatory responses and Arthus reactions (6). Studies of

the bactericidal and opsonic properties of C4D animals indicated that despite diminished opsonic and bactericidal activities, their overall host defenses are not substantially impaired (13).

The high susceptibility of C4D animals and their relatively long gestation period (ca. 70 days) make these animals an appropriate model for congenital syphilis. We are presently exploring the production of immune complexes and rheumatoid factor in C4D neonates born to syphilitic mothers (23). This phenomenon has been observed in human congenital syphilis (discussed in reference 1), but its mechanism is unknown. C4D guinea pigs are also a good model for examination of the antigenicity and mechanisms of protection (humoral versus cellular) by using recombinant treponemal antigens (17). Extension of the studies with recombinant antigens may lead to isolation of a T-cell clone expressing immunity to an identified *T. pallidum* peptide. Finally, the availability of the highly susceptible C4D strain and the resistant Albany line offers new potentials for studies of the genetic mechanism controlling susceptibility or resistance to *T. pallidum* infection (25).

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