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Humoral response in *Treponema pallidum*-infected guinea pigs: I. Antibody specificity

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SUMMARY

Young male inbred strain 2 guinea pigs were infected intradermally with 8×10^7 *Treponema pallidum* extracted from a rabbit orchitis, and 5 months later reinfected with 10^7 *T. pallidum*. Ninety percent of the animals developed symptomatic lesions after initial infection but none on challenge. Immunoblotting of sera obtained at intervals after infection or reinfection showed antibodies against *T. pallidum* antigen (TP), nonpathogenic treponemes — *T. phagedenis* biotype Reiter (TR), *T. refringens* strain Noguchi (TN), and *T. vincentii* (TV) — as well as normal rabbit serum (NRS) and normal rabbit testes extract (NRT). Antibodies reacting with TP were detected as early as 17 days (five polypeptides) and steadily rose (at 3 months 17 polypeptides were seen). Cross-reacting antibodies to TR, TN, TV, or rabbit proteins decreased within 3 to 5 months. After reinfection, the antibodies to NRS increased more sharply than the anti-treponemal antibodies. Adsorption with TR and NRS of sera obtained after infection or reinfection produced a reduction of antibodies to TP by 75–87%.

Keywords experimental syphilis guinea pig model

INTRODUCTION

The host range for experimental syphilis has been extended to the guinea pig (Wicher & Jakubowski, 1964a, b; Wicher, Wicher & Wang, 1976; Pierce, Wicher & Nakeeb, 1983; Wicher, Wicher & Gruhn, 1985; Wicher & Wicher, 1985) which offers new approaches for exploration of experimental syphilis and is a useful model for evaluation of potential vaccines.

We have earlier reported (Wicher *et al.*, 1976; Pierce *et al.*, 1985) that the antitreponemal antibodies produced by guinea pigs infected with *T. pallidum* are of the IgG class; no antitreponemal IgM or cardiolipin antibodies have been detected. This observation has recently been confirmed (Pavia & Niederbuhl, 1985). Peritoneal lymphocytes from infected guinea pigs respond *in vitro* to *T. pallidum* antigen and transiently to normal rabbit testes extract (Wicher & Wicher, 1985). The latter observation was not unexpected, since the *T. pallidum* suspensions are extracted from rabbit testes.

In this report the specificity of the humoral response to infection with *T. pallidum* in a kinetic fashion, is explored in detail.

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MATERIALS AND METHODS

Animals and infection. Young, male, inbred strain 2 guinea pigs (300–450 g) were obtained from the Wadsworth Center's animal facilities. The animals were individually housed at a controlled temperature of 18–20°C and given antibiotic-free food and water *ad libitum.*

Treponema pallidum (Nichols strain) was obtained from testes of infected New York State Flemish Giant, (NYS [FG]) rabbits. The rabbits had been killed with T-61 given i.v. (American Hoechst, Somerville, NY) at the peak of orchitis (9–12 days). The organisms were extracted from minced testes by using 10 ml of phosphate-buffered saline (PBS)/testis (Wicher *et al.*, 1983).

An inoculum of *T. pallidum*, 8×10^7 treponemes in 0·2 ml of PBS, was injected i.d. into the scrotal region of 20 sedated (0·2 ml Ketaset given i.m.) guinea pigs. The animals, kept in air-conditioned quarters, were monitored for clinical symptoms and blood for antibodies were collected under sedation with Ketaset at various intervals. On the fifth month the animals were reinfected i.d. with 10^7 *T. pallidum.*

Treponemal antigens. Four types of treponemal antigens were prepared from: *T. pallidum* (TP); *T. phagedenis* biotype Reiter (TR); *T. refringens* strain Noguchi (TN); and *T. vincentii* strain N-9 (TV). (The abbreviations here designate the antigens, not the organisms.)

Treponema pallidum suspensions in PBS were filtered through 0·8 μm pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, CA). The organisms were washed exhaustively in PBS, and the suspension adjusted to a concentration of 10^{10} organisms/ml and sonicated (MSE sonicator; MSE Inc., Westlake, OH) to complete disruption (Wos & Wicher, 1985). For Western blots the sonicated antigens were dissolved with 2% sodium-N lauryl sarcosine for 30 min at 37°C. The antigen preparations were divided into aliquots and stored at −60°C.

Cultivable treponemes were cultured in basic spirochaetal medium supplemented with 10% inactivated whole rabbit serum (Hanson & Canefax, 1964). The treponemes were washed three times with PBS, adjusted to 2×10^{10} organisms/ml, and processed as described for TP antigen. All treponemal antigens used in these studies were from the same batch of harvested treponemes and were used at the same concentration.

Rabbit protein antigens. Normal rabbit testes extract (NRT) was prepared from minced testes extracted in 10 ml of PBS/testis, as has been reported for infected rabbits (Wos & Wicher, 1985). The NRT and pooled sera from healthy adult rabbits (NRS) were used as control antigens and were stored at −60°C until used. For Western blots these reagents were processed like the treponemes.

Western blots. The dissolved treponemal and rabbit preparations (20 μ g) were separated on 10% polyacrylamide slab gels with a discontinuous Tris-glycine buffer system (Laemmli, 1970). The proteins were electrophoretically transferred (Towbin, Staehelin & Gordon, 1979) to 0·20 μm pore-size nitro-cellulose paper (Schleicher and Schuell, Keene, NH) in a Bio-Rad transblot cell with 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8·3) at 8 V/cm for 3 h at 4°C. Transferred antigens were probed with a 1:20 dilution of pooled or individual guinea pig sera. Bound antibodies were identified with horseradish peroxidase-conjugated protein A (Zymed Laboratories, Burlingham, CA) with O-dianisidine as a substrate (Eastman, Rochester, NY; Wos & Wicher, 1985). Molecular-weight standards (Pharmacia Fine Chemicals, Piscataway, NJ), were run in parallel (Weber & Osborn, 1969).

Immunoadsorption. Cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) coupled with TR, NRS, or TP (8–10 mg/ml) was prepared according to the manufacturer's instructions and used for sequential adsorption of pooled serum samples (Wos & Wicher, 1986). Adsorption with each antigen was repeated, and aliquots of serum were taken after each adsorption for retesting.

Serology. To measure specific and cross-reactive antibodies, sera from 10 infected guinea pigs were examined as individual or pooled samples by three tests: fluorescent treponemal antibody, microhaemagglutination and ELISA.

Fluorescent treponemal antibody test (FTA). The FTA test was performed with heat-inactivated sera appropriately diluted in PBS or in commercial sorbent (Beckman, RIA/Diagnostic Dept., Fullerton, CA) as described by Wicher *et al.* (1985).

Microhaemagglutination test (MHA-TP). The Sera-Tek MHA-TP was performed as specified by the manufacturer (Ames Division, Miles Laboratories, Elkhart, IN)

Enzyme-linked immunosorbent assay (ELISA). ELISA was carried out in Immulon-I flat-bottomed microplates (Dynatech, Alexandria, VA) essentially as described (Voller, Bidwell & Bartlett, 1980). One hundred microlitre volumes of sonicated TP, TR or NRS antigens (5, 3, or 60 μg/ml respectively) in carbonate buffer were placed in the microplate wells and kept overnight at 4°C. Unadsorbed or adsorbed sera and positive and negative controls were diluted in PBS-Tween and added in 100 μl volumes to duplicate wells. After 2 h incubation the wells were washed with PBS-Tween and incubated with 100 μl of alkaline phosphatase-conjugated rabbit antiserum to guinea pig immunoglobulins (heavy-and light-chain-specific, Miles Laboratories, Naperville, ILL). The antiserum was used at a 1:1000 dilution, which had been determined as optimal in pilot experiments. After 2 h incubation the plates were washed again, and 100 μl of *p*-nitrophenyl-phosphate (Sigma Chemical Company, Saint Louis, MO) at a concentration of 1 mg/ml in 10% diethanolamine buffer was added. After an additional 30 min incubation the reaction was stopped with 50 μl of 3 M NaOH. Absorbance was determined at 410 nm in a Microelisa Minireader (Dynatech). A positive result was an optical density of $>0{\cdot}2$ which is above 2 s.d. of the mean, determined from 50 non-reactive sera. The end-point titre was the reciprocal of the last serum dilution with an absorbance $\geqq 0{\cdot}2$.

RESULTS

Typical dark-field-positive lesions appeared in 18 of 20 infected guinea pigs after 7–15 days of infection. The signs of infection followed the course previously described (Wicher *et al.*, 1985).

Qualitative assessment of specific and cross-reactive responses. The humoral response before and after 17, 30, and 90 days of infection was examined by immunoblotting with a pool of 10 sera at a 1:20 dilution and with TP, NRT, and NRS as antigens (Fig. 1, Table 1). Preinfection sera diluted 1:20 did not react against these antigens. At 17 days after infection the guinea pigs responded weakly to TP but strongly to NRT and NRS, with multiple polypeptides at 80–90 kD (NRT and NRS) and 47 kD (NRS). At 30 days, the antiserum recognized 10 TP polypeptides, ranging from 18 to 90 kD, and the reaction to NRT and NRS was still very strong. At 60 days (Fig. 2, Table 1) 11 TP polypeptides were recognized; two were new at 39 and 45 kD, and one had disappeared at 90 kD. At 90 days (Fig 1, Table 1) the antiserum recognized 17 TP polypeptides from 14 to 80 kD; and the intensity of the reactions with NRT and NRS had definitely decreased.

The specificity of the antibodies was also examined against other treponemal antigens (TR, TN, and TV) as well as TP (Fig. 2). Sera obtained during early infection (2 months) reacted most strongly with TP (11 polypeptides) but also with TR, TN, and TV (15, 14, and 13 polypeptides respectively). After 5 months of infection the antisera reacted with only a few polypeptides of the nonpathogenic treponemes but still very strongly with TP. Reactions with some TP polypeptides (e.g. at 18, 45–49, and 70 kD) seemed even more intense than after 2 months, suggesting steady antigenic stimulation.

Quantitative assessment of specific and cross-reactive immune responses. Contrary to the results of the immunoblotting tests, approximtely 60% of the preinfection sera, when examined without sorbent, were positive by FTA. In ELISA approximately 10% of these sera reacted with TP and approximately 50% with TR (data not shown). The sera for both tests were diluted in PBS rather than sorbent and the reactions are due to natural antibodies (Jakubowski *et al.*, 1987). Such antibodies were not found earlier (Wicher *et al.*, 1976; 1985; Pierce *et al.*, 1983) because the sera were diluted with sorbent or because higher starting dilutions ($\geqslant 10$) were applied.

One month after infection the pooled unabsorbed serum was positive by ELISA against TP, TR, and NRS and by FTA, with FTA giving the highest titre (Fig. 3). MHA-TP results were still negative. Adsorption with NRS decreased the ELISA titres from 80 to 20 (TP) or 10 (TR) and the FTA titre from 160 to 40. Further adsorption with TR lowered the ELISA titre to 10 (TP) or removed it completely (<5, TR), while the FTA titre decreased to 20. The remaining reactivity by ELISA (TP) and FTA was removed by adsorption with TP.

For sera collected after 3 months of infection ELISA showed more antibodies against TP and TR but fewer against NRS. The FTA titre increased to 640, and the sera were positive by MHA-TP. Adsorption with NRS removed all reactivity against this antigen but decreased only 2-fold the

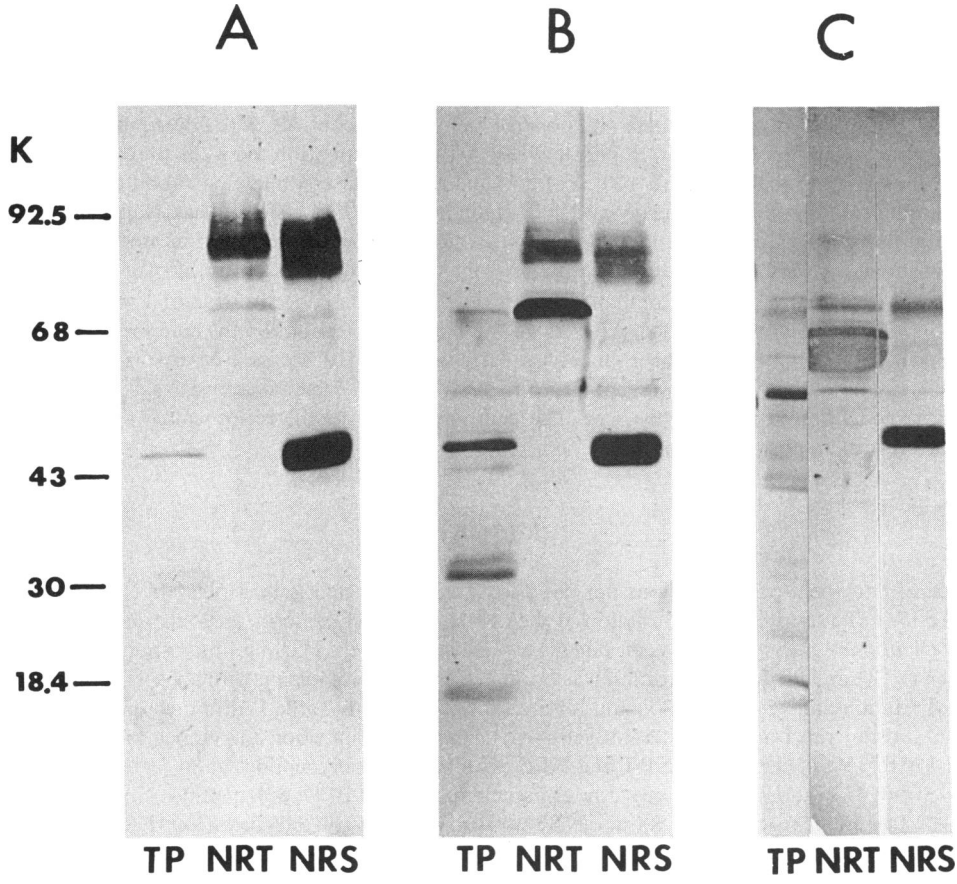

Fig. 1. Western blot analysis of TP, NRT, and NRS reacted with 1:20 dilution of guinea pig serum obtained after (A) 17, (B) 30, and (C) 90 days of infection with *T. pallidum*.

Table 1. Appearance of antibodies in sera of guinea pigs infected with *T. pallidum*

Day of Infection	FTA titre Mean	FTA titre Range	TP polypeptides (kD)
17*	27	10–80	18 32 35 47 90
30*	120	40–160	18‡ 32 35 42 47 49 55 70 90
60†	184	80–320	18‡ 32 35 39 42 45 47 49 55 70
90*	216	80–320	14 18 22 32 35 40 42 45 47 49 52 55‡ 60 70 75 80
150†	180	40–320	18‡ 32 35 37 39 45 47 49 55 70

* Fig. 1.
† Fig. 2.
‡ Possible doublet.

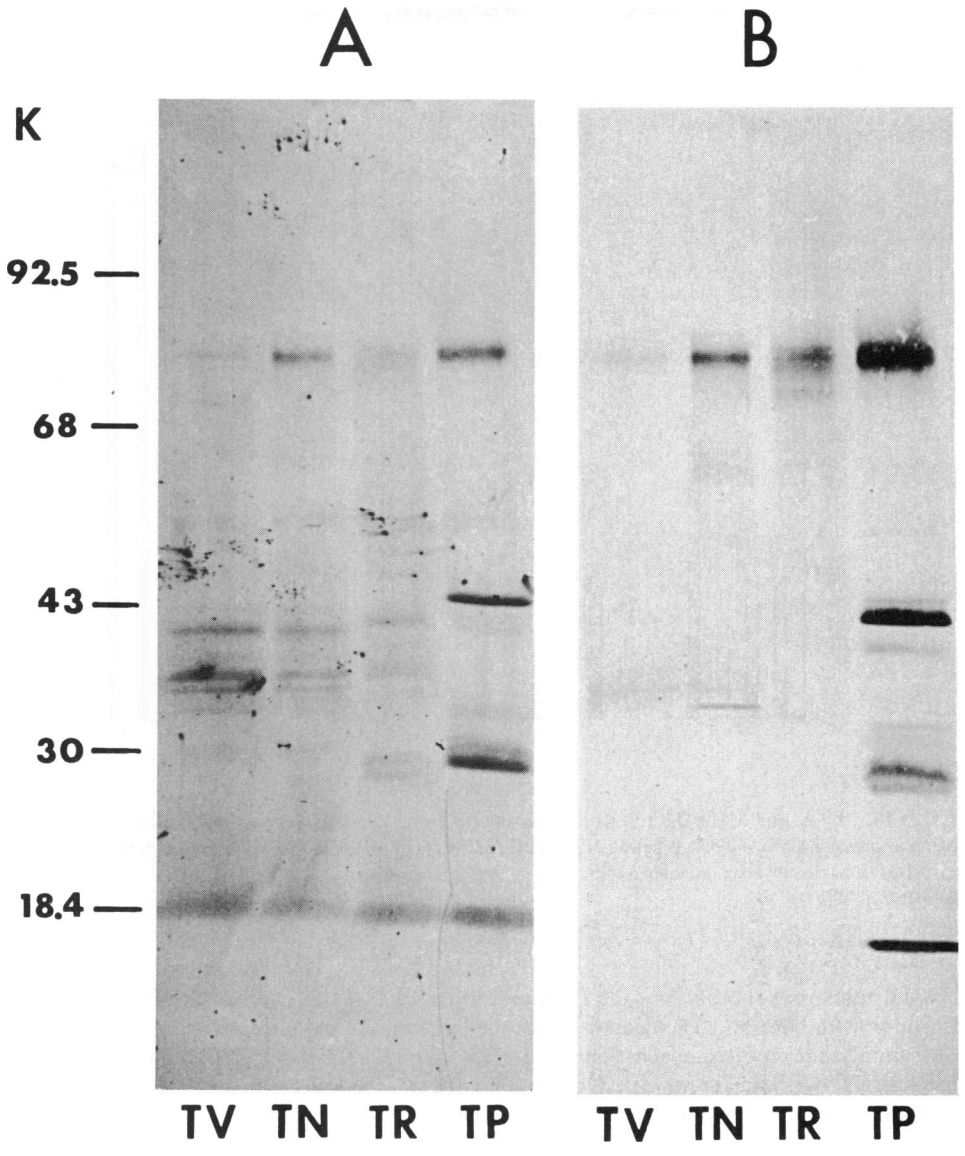

Fig. 2. Western blot analysis of TP, TR, TN, and TV reacted with 1:20 dilution of guinea pig serum obtained after (A) 2, and (B) 5 months of infection with *T. pallidum*.

ELISA titres against TP and TR, and the FTA titre 4-fold; it did not affect the MHA-TP titre. Adsorption with TR further decreased the antitreponemal reactivity in all tests but did not fully remove the reaction against TR.

For sera collected after 5 months of infection ELISA showed a further increase of antitreponemal antibodies and less reaction against NRS. Adsorption with NRS affected slightly the reaction with TR but not with TP by ELISA. The MHA-TP titre was unchanged, but the FTA titre dropped 4-fold. Further adsorption with TR substantially reduced the antitreponemal antibodies but left some reactivity against TR.

At the end of the 5th month of infection the animals were reinfected i.d. with 10^7 *T. pallidum*, and 3 weeks later their sera were collected, pooled, and adsorbed. In this sample the ELISA-NRS and

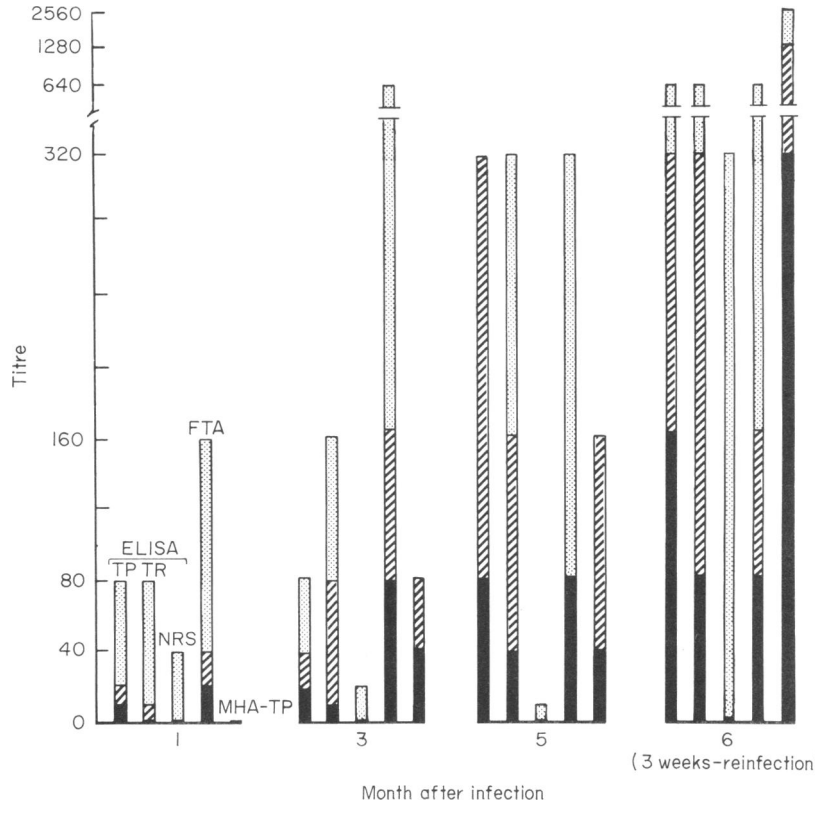

Fig. 3. ELISA, FTA, and MHA-TP titres of a pool of sera from 10 guinea pigs, pooled according to time of bleeding and sequentially adsorbed with CNBr-activated Sepharose coupled to NRS, TR, or TP. Whole bars, unadsorbed sera; dotted bars, adsorbed with NRS; shaded bars, adsorbed with TR; and black bars adsorbed with NRS and TR.

MHA-TP titres rose sharply, but the FTA and other ELISA titres increased only moderately. Adsorption with NRS and TR again demonstrated substantial cross-reactivity.

Assuming that repeated adsorptions with NRS and TR removes all antibodies except those specific for TP, we estimate that specific anti-TP antibodies constitute between 13 to 25% of the total antibodies elicited by infection with *T. pallidum*.

DISCUSSION

Infection of guinea pigs with *T. pallidum* obtained from rabbit testes evoked an immune response against treponemal antigens and rabbit proteins.

Qualitative analysis by immunoblotting revealed the contrasting immunogenicity of treponemal antigens and contaminating rabbit proteins. The initial response was, for the most part, a strong reaction to NRT and NRS polypeptides and a rather weak reaction to five TP polypeptides. As the infection progressed, the antibodies to rabbit proteins decreased, while the reactions to specific and cross-reacting treponemal antigens were augmented.

In the present study some antibodies elicited by the *T. pallidum* infection reacted exclusively with nonpathogenic treponemal antigens, e.g. four polypeptides at 50–55 kD in TR or two polypeptides below 70 kD in TV (Fig. 2). A similar pattern was observed earlier in rabbits (Wicher, Wos & Wicher, 1986). We do not have an experimentally proven explanation for this phenomenon.

After 5 months of infection the response to TP continued to rise (as judged by the intensity of the line of precipitation), but the reaction to non-pathogenic treponemal antigens was substantially diminished. It is likely that selected organisms from the heterologous population of *T. pallidum* used in the inoculum had actively propagated in the infected guinea pig. Alternatively, a selection of high affinity antibodies may have occurred during the course of infection.

The specific polypeptides recognized are the 22 kD, 37 kD and possibly the 47 kD polypeptide (Figs 1 & 2). This assumes that the reaction with NRS at the 47 kD region (Fig. 1) is coincidental and that the 47 kD TP polypeptide has no rabbit antigenic determinants.

The wide spectrum of immune responses was confirmed by quantitative serological tests. Tests without inhibiting sorbents were included to reflect the actual responses in the infected animals.

The amount of antibody specific for *T. pallidum* was negligible during the first months of infection, when the bulk of antibodies elicited could be readily adsorbed by CNBr-Sepharose coupled with NRS or TR (Fig. 3). The transient character of the sensitization to rabbit proteins, observed earlier in the lymphoproliferative response (Wicher & Wicher, 1985), was confirmed here by ELISA with NRS. These antibodies decreased with time and were negligible by the end of the 5th month of infection, when the response to TP was well established. This pattern is consistent with continuous sensitization to an exponentially multiplying microorganism compared with a single inoculum containing rabbit proteins.

The importance of this transient sensitization to rabbit proteins, however, should not be underestimated. Such immunogenic proteins have a priming effect. Indeed, reinfection after 5 months produced a 2-fold increase in the ELISA antitreponemal titres versus a 10-fold increase against NRS. The ELISA-NRS titre remained high for 2 months after reinfection (data not shown). The moderate increase in antibodies against TP after reinfection is perhaps consistent with the poor immunogenicity of treponemal antigens or restricted multiplication of the pathogen in an immune host.

This study clearly indicates that collection of serum around the 5th month of infection is the most appropriate for passive transfer experiments. It also cautions against the use, for the same purpose, of immune serum obtained from recently (1–2 months) reinfected animals since in such sera the antibody levels to rabbit proteins are disproportionately higher than to TP. Moreover such sera contain circulating immune complexes (Baughn *et al.*, 1987) which may interfere in adoptive immunity.

The guinea pig is not unique in displaying responses to antigens other than those specific to *T. pallidum*. It took a long time to learn that the rabbit produces, during the course of infection with *T. pallidum*, more antibodies to nonpathogenic treponemes than to *T. pallidum* itself (Wicher *et al.*, 1986). Indeed, when sera from infected rabbits were adsorbed consecutively with TR, TN, and TV, only three TP polypeptides reacted with the adsorbed antiserum (Wos & Wicher, 1986). Lukehart, Baker-Zander & Gubish (1982) have earlier reported three TP polypeptides reacting specifically with TR antigen-adsorbed antiserum. A similar cross-reactivity must occur in *T. pallidum*-infected man and other animals.

The heterogenous humoral response in *T. pallidum*-infected hosts is further complicated in man and rabbit by the formation of immune complexes and by autoimmune reactions to cardiolipin (Milgrom & Witebsky, 1962), fibronectin, laminin, collagen (Fitzgerald *et al.*, 1984) and muscle creatine kinase (Casavant, Wicher & Wicher, 1978; Strugnell *et al.*, 1986). Immune complexes were found in sera from experimentally infected rabbits (Baughn, Tung & Musher, 1980; Baughn & Musher, 1983) and naturally infected humans (Baughn *et al.*, 1986). Circulating immune complexes and antibodies to fibronectin and creatine-kinase were also found in *T. pallidum*-infected guinea pigs (Baughn *et al.*, 1987). Whether all these antibodies and immune complexes play a role in the development of clinical syphilis is not known. The overwhelming production of cross-reacting antibodies may interfere with, rather than enhance, the activity of the specific antibodies. A thorough analysis and a critical evaluation of specific compared with nonspecific treponemal antibodies is an awaiting challenge.

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