Clin. exp. Immunol. (1989) 77, 144–150

Kinetics of pathogen-specific humoral response in *Treponema pallidum*-infected young and old inbred strain 2 guinea pigs

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(Accepted for publication 11 April)

SUMMARY

The kinetics of the humoral response to pathogen-specific polypeptides was examined in *Treponema pallidum*-infected young (3–5 months old) and old (10–20 months old) inbred strain-2 guinea pigs. Sera collected before and at various times after infection were pooled and examined by immunoblotting and two serologic tests (ELISA and FTA) before and after sequential adsorption with CNBr-activated sepharose coupled to normal rabbit proteins and antigens from five nonpathogenic treponemal species. Prior to adsorption the kinetics of the humoral response to *T. pallidum* antigens did not seem to differ significantly between the two groups. After adsorption, however, a delay in the appearance of detectable antibodies and a milder response to various pathogen-specific polypeptides was observed in the older group. After adsorption, a sharp drop in ELISA-TP, ELISA-TR and FTA titres occurred in both groups. Six months post-infection, between 9 and 10 pathogen-specific polypeptides (97, 57, 47, 45, 43, 39, 37, 33, 17, and 15 kD) were recognized by both groups. The effect of age and levels of natural treponemal antibodies on the clinical symptoms of primary lesions and humoral response to pathogen-specific polypeptides is discussed.

Keywords *Treponema pallidum* specific antibodies guinea pigs syphilis

INTRODUCTION

Although it is known that humoral and cellular mechanisms contribute to the pathogenesis and development of immunity in syphilitic infection, their nature and the time when they become operative during the course of the disease remain poorly understood. Several studies have demonstrated a wide range of reactivity of immune serum obtained in natural (Hanff *et al.*, 1982; Moskophidis & Muller, 1984) or experimental syphilis (Alderete & Baseman, 1980; Wicher, Jakubowski & Wicher, 1987a). To our knowledge, only two attempts have been made to remove cross-reacting antibodies in order to render immune serum pathogen-specific (Lukehart, Baker-Zander & Gubish, 1982; Wos & Wicher, 1986). This is relevant since many *Treponema pallidum* proteins have been implicated in the attachment of the pathogen to eukaryotic host cells (Alderete & Baseman, 1979; Fitzgerald, 1983; Thomas, Baseman & Alderete, 1985) and several reports have indicated a good correlation between appearance of treponemicidal and neutralizing antibodies and development of immunity (Turner & Nelson, 1950; Bishop & Miller, 1976).

Inasmuch as immunization with non-pathogenic treponemes has so far proven ineffective in preventing infection with *T. pallidum* (Guelperin, 1951; Miller, Whang & Fazzan, 1963; Izzat *et al.*, 1970; Graves, Drummond & Strugnell, 1984), relevant information could be extracted from the identification of pathogen-specific antibodies and their development during the course of infection.

We have demonstrated that the immunology of *T. pallidum* infection in susceptible guinea pigs mimics critical aspects of human syphilis. It evokes a wide spectrum of specific and non-specific treponemal antibodies (Wicher *et al.*, 1987a), production of circulating immune complexes, autoantibodies to host antigens (Baughn *et al.*, 1987) and a delayed resistance to reinfection. We have also provided evidence for an important role of cell-mediated immunity in protection against challenge with *T. pallidum* (Wicher *et al.*, 1987b).

Since the levels of natural anti-treponemal antibodies (Jakubowski *et al.*, 1987) and the development of clinical symptoms in guinea pigs are age related (Wicher *et al.* 1988), it was interesting to explore how these two factors influence and correlate with the development of pathogen-specific antibodies in young and old guinea pigs; this is the subject of the present report.

Presented in part at the Annual Meeting of the American Society for Microbiology, May 1988, Miami, FL.

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MATERIALS AND METHODS

Animals and infection

Ten young adult (3–5 months old) and 10 old (10–20 months old) male inbred strain 2 guinea pigs were infected intradermally in the pubic area and one hind leg with 5×10^7 *T. pallidum* per site as reported elsewhere (Wicher *et al.*, 1988). The guinea pigs were bled before infection and 7 days, and 1, 2, 3, 4, 5, and 6 months after infection for serology. They were examined throughout the experimental period for clinical symptoms.

Infection and bleeding were performed with the animals under sedation (0·3 ml ketaset; Bristol laboratories, Syracuse, NY). At the end of the experimental period they were killed with Euthanasia agent T-61 (American Hoechst, Sommerville, NJ).

Treponemal antigens

Antigens of six treponemal strains were used: *T. pallidum* subsp. *pallidum*, Nichols (TP); *T. phagedenis* biotype Reiter (TR); *T. vincentii* strain N-9 (TV); *T. refringens* strains Noguchi (TN); *T. minutum (TM)* and *T. denticola* (TD). (The abbreviations here designate the antigens, not the organisms.) The nonpathogenic treponemes were kindly provided by Dr Robert M. Smibert from the Virginia Polytechnic Institute, Blacksburg, VA. The treponemes were cultured in our laboratory and processed for antigens as previously reported (Wicher *et al.*, 1987a).

Rabbit protein antigens

Normal rabbit testes extract (NRT) and normal rabbit serum (NRS) were the same preparations as previously described (Wicher *et al.*, 1987a).

Western blot

Twenty microlitres (20 μg) of solubilized treponemal antigen (TP, TR, TV), and rabbit proteins (NRS, NRT) were electrophoresed on 12% polyacrylamide gels in a discontinuous Tris-glycine system in a Mini Protean II Slab cell with 25 mM Tris, 192 mM glucine and 20% methanol buffer (pH, 8·3) at 100 V for 1 h.

Since levels of natural treponemal antibodies are low in young guinea pigs (Jakubowski *et al.*, 1987), pools prepared with serum samples taken before infection and 7 days post-infection were used as probes at 1 : 4 dilution, and all other specimens were used at 1 : 10 dilution. For comparative purposes, samples from old guinea pigs were diluted similarly. Bound antibodies were identified with 1 : 1000 dilution of horseradish peroxidase-conjugated protein A with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) and H_2O_2 as substrates. Low-molecular-weight standards (Bio-Rad) were run in parallel. Molecular weights were determined by the method of Weber and Osborn (1969).

ELISA

Serum samples from each group of animals were pooled according to the time of collection and examined in duplicate or triplicate by ELISA using microplate wells precoated with 0·1 ml of either solulilized (5 μg/ml) TP, sonicated (10 μg/ml) TR or TV, (5 μg/ml) NRT or 1 : 1000 diluted NRS as antigen (Wicher *et al.*, 1987a). In some experiments a pool of sonicated (10 μg/ ml) TR, TN, TV, TM and TD was also used.

Fluorescent treponemal antibody test (FTA)

This test was performed with heat-inactivated sera as reported elsewhere (Wicher, Wicher & Gruhn, 1985).

Immunoadsorption

CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) coupled to NRS, NRT, and individual (TR) or pooled (TV, TN, TM, TD) treponemal antigens (8–10 mg/ml) was prepared according to manufacturer's instructions and used for sequential adsorption of pooled serum samples. Six to nine adsorptions with each antigen were necessary to totally remove cross-reacting antibodies (Wos & Wicher, 1986).

RESULTS

Clinical course of infection

Age definitely affected the character of primary lesions. Progressive ulcerative chancre-like lesions developed predominantly in young adults, whereas old animals developed predominantly non-progressive papular lesions. The clinical course and plausible reasons for the age-related difference have been discussed elsewhere (Wicher *et al.*, 1988).

Kinetics of the humoral response.

Unadsorbed sera. The molecular analysis of the humoral response in both groups of guinea pigs demonstrated (Fig. 1) that while the pattern of gradual increase in the humoral response to *T. pallidum* epitopes is aparently similar in both young and old guinea pigs, the kinetics of the humoral response to nonpathogenic treponemal antigens is quite different. The number of TP peptides recognized by preinfection sera increased from two (57 and 66·2 kD, Fig. 1a) in young or five (45 to 66·2 kD, Fig. 1b) in old guinea pigs, to approximately 17 to 18 polypeptides (15 to > 100 kD) in both groups at 4 and 6 months after infection.

The number of nonpathogenic treponemal polypeptides recognized by old guinea pigs' natural antibodies differed substantially from those recognized by natural antibodies from young animals (8–10 peptides from 24 to 97·4 kD *versus* 2–3 peptides, from 45 to 57 kD). The immune reaction was also more intense in the former (Fig. 1b). More interestingly, the pattern of polypeptides recognized by the old group of guinea pigs, in general terms, did not change throughout the experimental period.

However, infection of young guinea pigs elicited antibodies to a lower number of cross-reacting polypeptides, some of them with no counterparts in the old group (Fig. 1a). Indeed, at 6 months post-infection, when the animals were 9 to 11 months old, the sera reacted with a pattern substantially different from that of old-group pre-infection sera at a similar age.

A temporary drop in the levels of antibodies to nonpathogenic treponemes was aparent in the immunoblot at 1 and 2 months post-infection although more pronounced in the old group (Fig. 1). This was quantitatively confirmed by ELISA-TP at 1 and 2 months and by ELISA-TR at 1 month post-infection (Fig. 2). ELISA run simultaneously with sonicated and solubilized pathogenic and nonpathogenic treponemes consistently showed that the antibody titres against nonpathogenic treponemes were 1–2-fold higher when sonicated rather than solubilized antigens were used. On the contrary, a greater enhance-

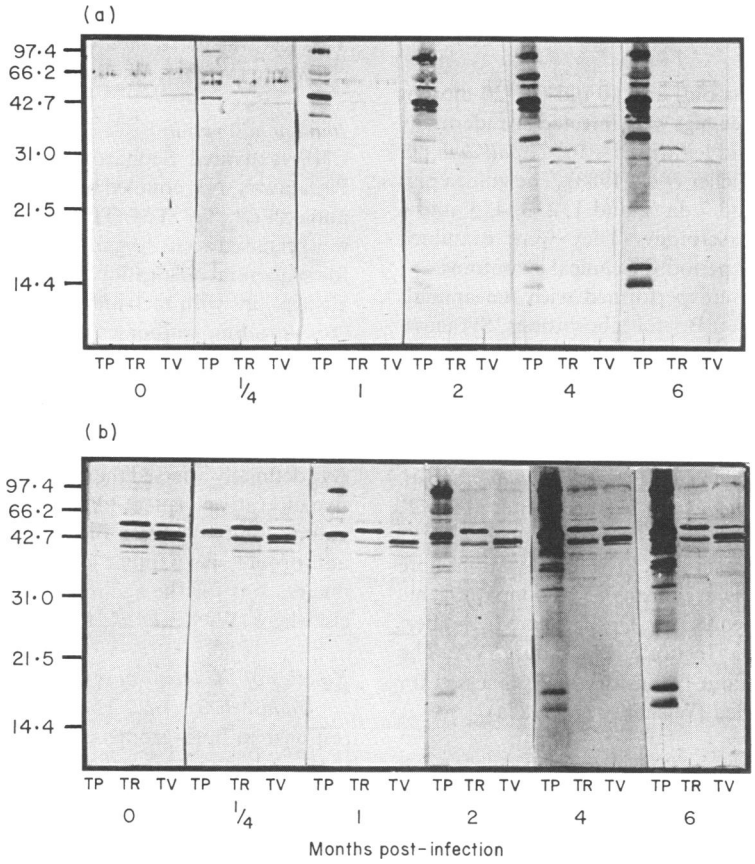

Fig. 1. Kinetics of the humoral response to *T. pallidum* infection in young (a) and old (b) guinea pigs. Protein antigens of *T. pallidum* subsp. *pallidum* (TP), *T. phagedenis* biotype Reiter (TR) and *T. vincentii* (TV) reacted with guinea pig antisera as demonstrated by Western blot. Pooled sera collected before (0) and one week (1/4) after infection were used at dilution 1:4. Pooled sera collected 1, 2, 4, and 6 months post-infection were used at dilution 1:10.

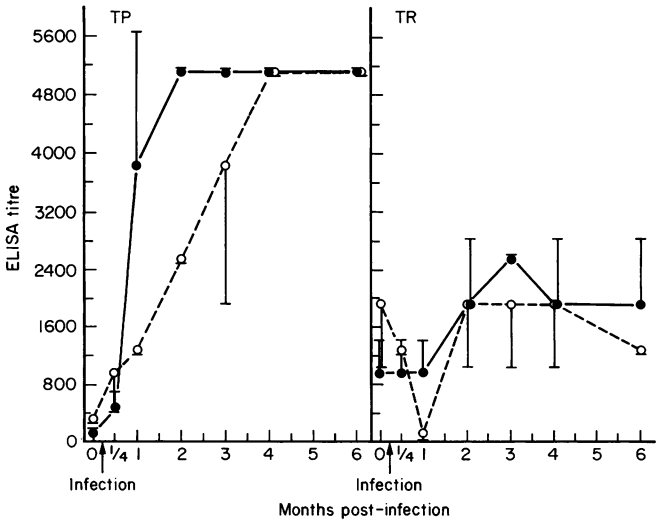

Fig. 2. ELISA titres of pooled sera from 10 young (●) and 10 old (○) guinea pigs collected before (0) and after infection, examined with solubilized TP and sonicated TR antigens. Results expressed as mean ± s.d. of two determinations done with each pool of sera.

ment in the antigen-antibody reaction (antibody titre 2–4-fold higher) was observed when solubilized rather than sonicated TP was used as antigen (unpublished observations). Therefore, all determinations by ELISA were done using solubilized TP and sonicated nonpathogenic treponemes as antigens.

As previously reported (Baughn *et al.*, 1987; Wicher *et al*, 1987a), the reaction against rabbit proteins reached its maximum between 1 and 2 months post-infection, and decreased thereafter (Table 1).

Adsorbed sera. Aliquots of pooled sera (as shown in Fig. 1), collected 7 days, and 2, 4, and 6 months post-infection, were exhaustively adsorbed with NRS, NRT, TR and a pool of 4 nonpathogenic treponemes (TV, TN, TM and TD) and again examined by immunoblot (Fig. 3 a, b) ELISA and FTA tests (Table 1).

After adsorption, no reaction was detected by Western blot with NRS and NRT (data not shown) or with TR or TV. Nevertheless, a progressive increase in the number of pathogen-specific antibodies developed during the course of infection, though with a noticeable delay in the old compared with the young group of guinea pigs (Fig. 3, Table 2).

Adsorbed sera from young adult guinea pigs reacted with a 47-kD polypeptide after one week and with additional polypep-

Table 1. Humoral response to *T. pallidum* before and after sequential adsorption with rabbit proteins and cross-reacting treponemal antigens

Guinea pig group	Time post-infection	Antigen	ELISA Serum Unadsorbed	ELISA Serum Adsorbed	FTA Serum Unadsorbed	FTA Serum Adsorbed
Young adult (3–5 mo)	1 wk	TP	480±226	20±0	<10	<10
		TR	960±452	10±0		
		NRS	640±0	<5		
	2 mo	TP	5120±0	30±14	320±0	10±0
		TR	1920±905	5±0		
		NRS	1920±905	<5		
	4 mo	TP	5120±0	80±0	480±226	20±0
		TR	1920±905	10±0		
		NRS	960±452	<5		
	6 mo	TP	5120±0	320±0	480±226	20±0
		TR	1920±905	10±0		
		NRS	320±0	<5		
Old (10–20 mo)	1 wk	TP	960±452	20±0	20±0	<10
		TR	1280±0	<5		
		NRS	480±226	<5		
	2 mo	TP	2560±0	40±0	320±0	20±0
		TR	1920±905	10±0		
		NRS	1280±0	<5		
	4 mo	TP	5120±0	120±56	480±226	20±0
		TR	1920±905	<5		
		NRS	640±0	<5		
	6 mo	TP	5120±0	240±113	640±0	30±14
		TR	1280±0	7·5±3·3		
		NRS	640±0	<5		

Results are presented as mean ± s.d. of 2 determinations.
TP, *T. pallidum* subsp. *pallidum*; TR, *T. phagedenis* biotype Reiter; NRS, normal rabbit serum.

tides of molecular masses of 45, 39, 37, 17, and 15 kD 2 months post-infection. After 4 and 6 months of infection young animals responded with antibodies against nine to ten polypeptides; two minor antigens of 97 and 57 kD, the major antigenic component 47 kD which sometimes migrates as a broad band in the 47- to 45-kD position (doublet?) and six polypeptides of 43, 39, 37, 33, 17, and 15 kD. A similar pattern of specific humoral response was demonstrable with the adsorbed sera old guinea pig on the month 6 post infection, except that the reaction with antigenic polypeptides of molecular masses lower than 47 kD was much less intense; this explains the delayed detection of most specific antibodies in this group.

The effectiveness of the adsorption was verified by ELISA using TP, TR and NRS as antigens (Table 1). The reaction against TR and NRS was negligible whereas the antibody response to TP dropped from a titre of 1:5120 to titres ranging from 1:80 to 1:320 in the young and old guinea pigs serum pools on months 4 and 6 post infection. Similarly, the FTA titres dropped from 3- to 5-fold in both groups.

DISCUSSION

The relevance of pathogen-specific antibodies to the process of resistance developed during the course of *T. pallidum* infection has so far not been elucidated. Indirect evidence, however, strongly suggests that antibodies developed against surface antigens participating in the attachment of the pathogen to the host cells (Alderete & Baseman, 1979; Fitzgerald, 1983; Thomas, Baseman & Alderete, 1985) or directed against flagellar antigens (Blanco *et al.*, 1986 a, b; 1988) may be important in limiting the progress of the disease.

In the present study, except for antibodies reacting with 37-kD peptide that were detectable in the young group already 2 months post-infection, specific antibodies reacting with assumingly critical immunogens of molecular masses 45, 37 and 33 kD were definitely present in both young and old animals between 4 to 6 months post-infection at the time when the animals were fully protected against challenge with *T. pallidum* (Wicher *et al.*, 1987b).

The biological role of the remaining TP-specific antibodies developed in the guinea pig, however, is less clear. Clinical findings suggest that antibodies appearing early in the course of infection may not play a major role in controlling the pathogen (Musher, Baughn, Knox, 1979). Antibodies within this category may include those directed against the 47-kD protein. Antibodies against this strongly immunogenic component appear

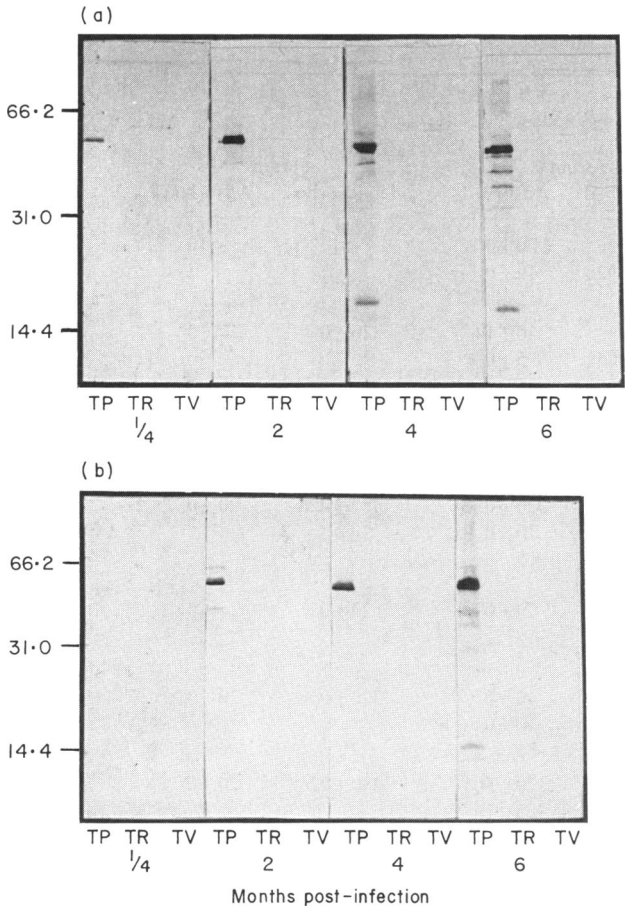

Fig. 3. Kinetics of pathogen-specific humoral response in young (a) and old (b) guinea pigs. Pooled sera collected 1 week, and 2, 4, 6 months post-infection were sequentially adsorbed with CNBr-activated Sepharose coupled to NRS, NRT and five non-pathogenic treponemes, concentrated to original volume and examined by immunoblot with TP, TR and TV as antigens. Molecular-weight standards (kD) are shown.

very early after infection in rabbit (Hanff *et al.*, 1983) and in young guinea pigs as shown in this report. More interestingly, no particular role has been attached to the early appearance of specific antibodies directed against *T. pallidum* peptides of molecular masses 15 and 17 kD. Hensel, Wellensiek & Bhakdi (1985) proposed to use these two low-molecular-mass polypeptides as an immunologic marker for human syphilis regardless of its stage. This consideration was based on observations of their own and those of other investigators, that antibodies to these two polypeptides are produced very early in the course of infection (Hanff *et al.*, 1982; 1983), do not seem to react with peptides of similar molecular mass in nonpathogenic treponemes, and most of all, are not present in normal human sera (Hensel, Wellensiek & Bhakdi, 1985). The implication is that these two antibodies are specific for *T. pallidum*.

In the guinea-pig model, antibodies against 15- and 17-kD polypeptides were clearly evident after one week of infection, and did not react with similar peptides of TR, TV, TN or TD (the last two not shown). However, as evident from the immunoblots (Fig. 3 a, b), substantial amount of antibodies reacting with 17-kD and most of those reacting with 15-kD polypeptides were removed by sequential adsorption with various nonpathogenic treponemes. Evidently, the two peptides consist of specific and cross-reacting epitopes. Moreover, cross-reacting epitopes are obviously expressed on polypeptides with different molecular masses.

We are not certain whether antibodies reacting late on the months 4 and 6 post infection with the two minor bands of 97 and 57 kD are directed exclusively to *T. pallidum* antigens and/or to host proteins avidly bound to TP (Peterson, Baseman & Alderete, 1983; Fitzgerald *et al.*, 1984; Fitzgerald & Repesh, 1985).

Pre-infection sera from old guinea pigs reacted more intensively and with a larger number of peptides than pre-infection serum samples from young animals. More interestingly, this different pattern of humoral response remained throughout the experimental period. Perhaps for the same reason a substantial drop in the levels of serum antibodies (ELISA-TR, ELISA-TP) was observed on the months 1 and 2 post infection with the old group only. Thus, the old animals containing a high level of natural antibodies apparently manifest a more intense immune clearance of cross-reacting antigens. It is also likely that age and/or prior colonization with nonpathogenic treponemes evoking high levels of natural antitreponemal antibodies are responsible for the smouldering nature of primary lesions (Wicher *et al.*, 1988) and conse-

Table 2. Kinetics of pathogen-specific antibody response in young and old guinea pigs infected with *T. pallidum*

Guinea pig group	Time post-infection	*T. pallidum* polypeptides (kD)								
		97	57	47–45	43	39	37	33	17	15
Young adult	1 wk			++						
(3–5 mo)	2 mo		<±*	++++		±	<±		<±	<±
	4 mo	+	±	++++	+**	+	±	±	++	±
	6 mo	±	+	++++	++	++	++	+	++	+
Old	1 wk									
(10–20 mo)	2 mo		+	+++		+				
	4 mo		<±	+++		<±	<±		<±	
	6 mo	<±	±	++++	+**	+	+	±	+	±

* < ± Definite reaction after probing the blot, but barely visible in the photograph.
** As a broad band extending from 47 to 43 kD.

quently, milder and delayed production in *T. pallidum*-specific antibodies in the old group.

However, *T. pallidum* infection of young animals, in which colonization with nonpathogenic spirochetes is nil or very limited, as evident by lack or low levels of antitreponemal antibodies (Jakubowski *et al.*, 1987), apparently affected differently the subsequent antibody response to specific and nonspecific treponemal antigens. As shown by the adsorbed sera, the humoral response against TP-specific epitopes seems definitely stronger in sera from young as compared with sera from old animals. Consequently, most of the pathogen-specific antibodies were detected earlier in the former.

The number of pathogen-specific polypeptides reacting with the immune guinea pig serum is higher than that reported by Lukehart, Baker-Zander & Gubish (1982) and by Wos & Wicher (1986) for immune rabbit serum. Lukehart, Baker-Zander & Gubish (1982), working with *T. pallidum*-infected rabbits, reported that after adsorption of 3 ml of 9–10 month immune serum with a high concentration (1·4 g) of intact TR, the antiserum reacted by immunoblot with three TP polypeptides of molecular masses 48, 14, and 12 kD. It is feasible that the molecular masses of these three TP peptides correspond to the most recently accepted 47 kD, 17 kD and 15 kD (Norris *et al*, 1987), which agrees with our present report.

By sequential adsorption with several nonpathogenic treponemes of a rabbit antiserum produced by adjuvant immunization with solubilized *T. pallidum* antigen, Wos & Wicher (1986) were able to remove antibodies reacting with 31 out of 34 polypeptides. The adsorbed antiserum did not react with any non-pathogenic treponemes but reacted with three TP polypeptides of molecular masses 36, 34 and 27 kD, and the FTA titre dropped drastically from 1:5120 to 1:5. More interestingly, despite the large number of antibodies produced in the latter study, only three pathogen-specific peptides could be recognized by the adsorbed antiserum and they totally differed from the three peptides reacting with the adsorbed antiserum obtained from the same animal species but infected with *T. pallidum* (Lukehart, Baker-Zander & Gubish, 1982). Thus, we may expect differences in biologic activity from antisera produced by different protocols of immunization.

Although the various stages of syphilis are not clearly delineated in the experimental model, the kinetics of the pathogen-specific humoral response in guinea pig does not differ significantly from that observed with extensively adsorbed *T. pallidum*-infected human and rabbit sera collected at various stages of the disease (Wicher, Zabek & Wicher, manuscript in preparation). We may therefore reason that if the humoral arm of the immune response plays a role in the resistance developed during the course of infection, it may be of similar nature in natural and experimental syphilis.

ACKNOWLEDGMENTS

The technical assistance of David Contois and the secretarial help of Valarie Harris are gratefully acknowledged. We thank Dr Murray King for reading the manuscript and editorial comments. This work was supported by NIH grant AI-21833 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

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