Secondary lesions in rabbits experimentally infected with *Treponema pallidum*

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SUMMARY   Thirty rabbits infected with 10^3 of either Nichols or Melbourne 1 strains of *Treponema pallidum* were observed for the development of secondary lesions, which appeared outside areas inoculated with viable treponemes. More rabbits infected with Melbourne 1 strain (eight of 15 rabbits) than were infected with the Nichols reference strain (three of 15 rabbits) developed secondary lesions. The mean (SD) incubation periods of secondary lesions were 52 (8) days for rabbits infected with Melbourne 1 and 56 (4) days for rabbits infected with Nichols strain. These mean incubation periods did not correlate with appreciably increased concentrations of immune complexes or glycosaminoglycans in the serum of infected rabbits.

Introduction

Rabbits have been used as the model of choice for experimental syphilis largely because the primary lesion, which develops after intradermal inoculation with low numbers of *Treponema pallidum,* histologically and morphologically resembles the chancre of syphilis in man.[1] The clinical similarity between natural syphilis in man and experimental syphilis in rabbits ususally ends, however, after the resolution of the primary lesion, as rabbits infected with the Nichols reference strain of *T pallidum* subspecies *pallidum*[2] do not commonly develop secondary syphilis lesions.

Though the pathogenesis of secondary syphilis has not yet been clarified, the generalised symptoms are thought to result from the growth of organisms that have disseminated from the primary site of infection. The reason for the widespread growth of *T pallidum* despite demonstrable activation of immune mechanisms at the primary stage of the disease[3 4] is not known; a number of investigations have suggested that the host immune response is suppressed at this time.[5-7]

Some studies have indicated that at least one of the symptoms of secondary syphilis, glomerulonephritis, is caused by the deposition of circulating immune complexes.[8-10] Circulating immune complexes may also mediate the suppressive effect of syphilitic serum on lymphocyte transformation seen in in vitro assays

of cell mediated immunity in natural syphlis in man and experimental syphilis in rabbits,[11 12] and the generalised nature of secondary syphilis may result from immunosuppression in vivo mediated by circulating immune complexes. Other reports have indicated that in vitro suppression of lymphocyte responsiveness is produced by an interaction of serum glycosaminoglycans with lymphocytes[6] or mitogens,[13] or both.

During a recent study of the ontology of immunity to chancre formation in experimental syphilis, rabbits were observed for the development of secondary lesions.[14] In this paper we show a difference in the potential of *T pallidum* strains to induce secondary lesions. We also investigated the relation between the formation of secondary lesions, serum concentrations of two postulated mediators of immunosuppression in syphilis (circulating immune complexes and glycosaminoglycans), and the development of immunity to chancre formation.

Materials and methods

T PALLIDUM ORGANISMS

The Nichols[15] and Melbourne 1[16] strains of *T pallidum* were maintained by passage in the testes of adult rabbits or stored frozen in minimum essential medium (Flow Laboratories, North Ryde, NSW, Australia) containing 10% (volume per volume) fetal calf serum and 15% (volume per volume) glycerol.

RABBITS

Adult male rabbits were bought from Monash University (Clayton, Victoria, Australia), housed

individually at 16-18°C, and fed antibiotic free food ad libitum.

INFECTION AND CHALLENGE INOCULATIONS
Thirty rabbits were injected intradermally on the dorsal midline 5 cm anterior to the tail with either 10^3 Nichols (15 rabbits) or Melbourne 1 (15 rabbits) strain of *T pallidum*. At $2\frac{1}{2}$, 5, $6\frac{1}{2}$, $7\frac{1}{2}$, and 10 weeks after infection the dorsal surfaces of three rabbits from each group were clipped free of hair and injected with eight inocula of 10^2 Nichols and eight inocula of 10^2 Melbourne 1 strain of *T pallidum* as described previously.[14] All infected rabbits were observed for lesions, which developed outside the challenge inoculation areas before and after challenge.

CIRCULATING IMMUNE COMPLEXES
We measured concentrations of circulating immune complexes at different times after infection by the method of Maret *et al.*[17] Serum samples were diluted to 5-8 mg protein/l with 0·1 mol/l borate buffer pH 8·4, mixed with an equal volume of 3·5%, 7%, or 10% (weight per volume) polyethylene glycol 6000 (BDH Chemical, East Kew, Victoria, Australia) made up in 0·1 mol/l borate buffer pH 8·4, and incubated at 4°C for 18 hours. The precipitate was then collected by centrifugation at 5000 × *g* for 30 minutes, and the supernatant was decanted. The precipitates were washed once with a solution of polyethylene glycol at the original concentration and dissolved in 0·1 mol/l sodium hydroxide. The absorbance at 280 nm was measured in a Perkin-Elmer 125 double beam spectrophotometer.

HEXURONIC ACID
Hexuronic acid was precipitated from serum from normal and infected rabbits with cetylpyridinium chloride (Ajax Chemicals, Sydney, NSW, Australia). A volume of 1 ml of serum was diluted to 5 ml with distilled water, and 10% (weight per volume) aqueous cetylpyridinium chloride was added dropwise until precipitation was observed. After the tubes had been incubated at 37°C for 20 minutes, the precipitate was collected by centrifugation at 1500 × *g* for 10 minutes, dissolved in 2 ml of 2 mol/l sodium chloride and precipitated again with 8 ml absolute ethanol overnight at 4°C. The precipitate was collected by centrifugation at 1000 × *g* for 10 minutes and resuspended in 2 ml distilled water. We measured the amount of hexuronic acid present in cetylpyridinium chloride precipitates by the method of Bitter and Muir.[18] To assess the efficacy of this method in recovering hexuronic acid from serum, we added 10 mg/l, 20 mg/l, 50 mg/l, or 100 mg/l hyaluronic acid (Helon; Pharmacia, North Ryde, NSW, Australia) to pooled normal rabbit serum that had previously been shown not to contain hexuronic acid.

STATISTICS
Student's *t* test was performed as described previously.[19]

Results
SECONDARY LESIONS IN INFECTED RABBITS
We defined as secondary lesions all nodular dermal indurations that developed outside the grid area where challenge inoculations were made. Table I shows the incidence and incubation periods of secondary lesions that developed in rabbits infected with *T pallidum*. Three of 15 rabbits infected with Nichols strain developed secondary lesions after challenge, in contrast with eight of 15 rabbits infected with the Melbourne 1 strain. The mean (SD) incubation periods of secondary lesions, which were constant and independent of the time before challenge, were 56 (4) days for the Nichols strain and 52 (8) days for the Melbourne 1 strain of *T pallidum*. Two of three rabbits infected with Melbourne 1 strain six and a half weeks before challenge developed "satellite" secondary

TABLE I *Incidence and incubation periods of secondary lesions in rabbits infected with 10^3 Nichols or Melbourne 1 strains of Treponema pallidum and challenged with both strains.*

Time between infection and challenge (weeks)	Strain used for infection	No of rabbits developing lesions /No challenged	Incubation period† (days)	
			Absolute	Mean (SD)
$2\frac{1}{2}$	Nichols	0/3		
	Melbourne 1	2/3	42, 50	46
5	Nichols	1/3	54	
	Melbourne 1	2/3	42, 53	47
$6\frac{1}{2}$	Nichols	2/3	54, 61	57
	Melbourne 1	2/3	55, 64	58
$7\frac{1}{2}$	Nichols	0/3		
	Melbourne 1	2/3	50, 64	57
10	Nichols	0/3		
	Melbourne 1	0/3		
Total	Nichols	3/15	54-61	56(4)
	Melbourne 1	8/15	42-64	52(8)

* All dermal lesions that appeared outside the marked grid where challenge inoculations were made were defined as secondary lesions.
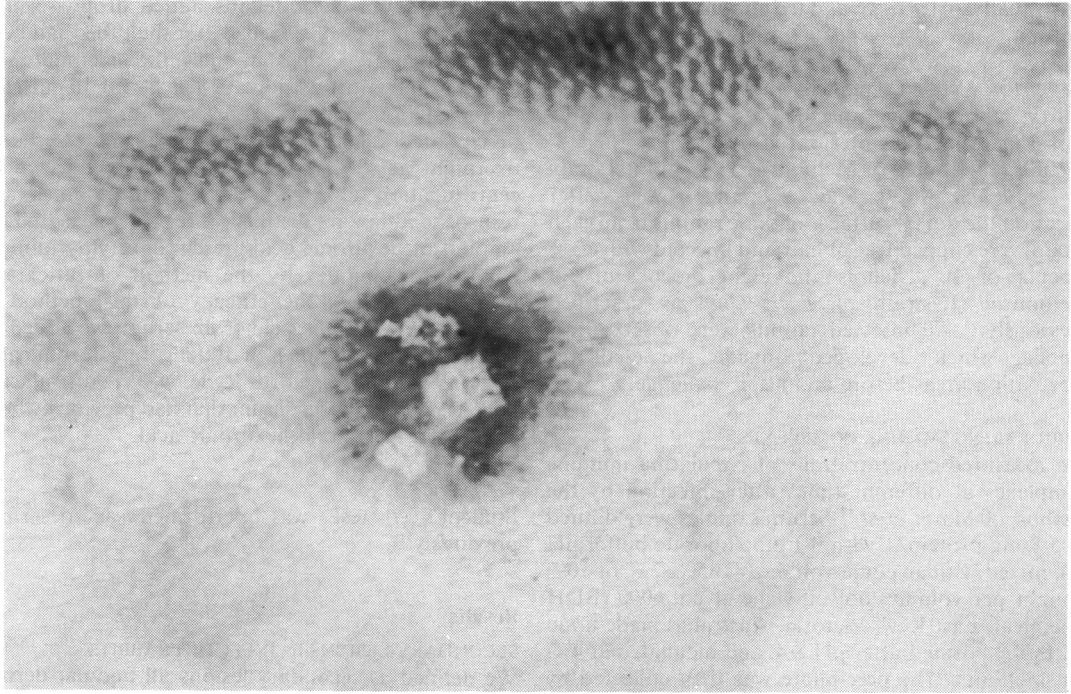† Period between initial infection and first detected induration.

FIGURE 1 *Satellite secondary lesions around the healing site of primary infection in one of two rabbits infected with 10³ Melbourne 1 strain Treponema pallidum for six and a half weeks (bar represents 1 cm).*

lesions at a radius of 2 cm around the initial site of infection (fig 1).

CIRCULATING IMMUNE COMPLEXES IN INFECTED RABBITS

We used 3%, 7·5%, and 10% polyethylene glycol to precipitate immune complexes from serum from normal (pre-infection) rabbits and those infected with 10³ Melbourne 1 or Nichols strains of *T pallidum* for up to 10 weeks (fig 2). The 7·5% and 10% polyethylene glycol caused more precipitation from normal serum samples than 3% polyethylene glycol, thus increasing the "normal" baseline for material that was absorbed at 280 nm. We saw no increase above the "normal" baseline in material precipitable by polyethylene glycol at concentrations of 3% and 7·5% (weight per volume) over the 10 week period. We did, however, see an apparent rise in the mean amount of material precipitated by 10% polyethylene glycol in serum from rabbits infected with Melbourne 1 strain six weeks after infection, but the large variability of individual rabbit responses meant that the result was not significant (p>0·05).

HEXURONIC ACID

We detected no hexuronic acid in cetylpyridinium chloride precipitates of serum from normal or infected rabbits two to 10 weeks after infection with Nichols or
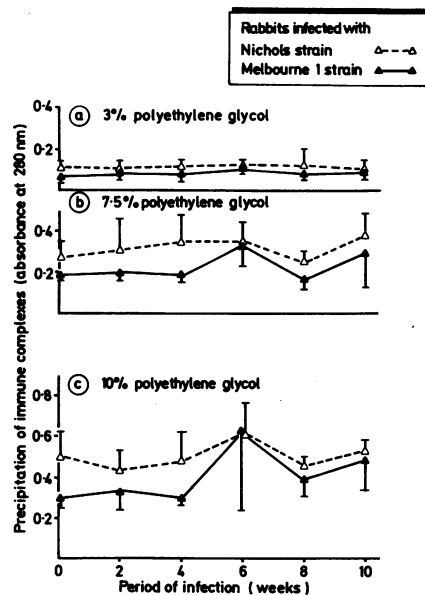

FIGURE 2 *Mean precipitation of immune complexes from serum of rabbits infected with 10³ Nichols (four rabbits) or Melbourne 1 (four rabbits) strains of Treponema pallidum immediately before infection (0 weeks) and at two weekly intervals afterwards (bars represent standard deviations from mean).*

Melbourne 1 strains of *T pallidum*. We established the efficacy of cetylpyridinium chloride precipitation by adding to normal rabbit serum 5 mg/l to 50 mg/l hexuronic acid (10 mg/l to 100 mg/l purified hyaluronic acid), 62% to 90% of which was recovered by cetylpyridinium chloride precipitation (table II).

TABLE II *Recovery of added hexuronic acid from pooled normal rabbit serum by cetylpyridinium chloride precipitation (results represent mean of two samples)*

Amount added (mg/l)	Amount (%) recovered (mg/l)
5	4 (80)
10	9 (90)
25	18 (72)
50	31 (62)

Discussion

Rabbits infected with 10^3 Nichols or Melbourne 1 strains of *T pallidum* develop primary syphilitic lesions after similar incubation periods (Strugnell, unpublished observation), and infection with either strain induces sustained immunity to chancre formation five to seven and a half weeks after inoculation (Strugnell *et al* unpublished observation).[14] This immunity to chancre formation is effective against both the strain used to infect the rabbits initially (the homologous strain) and the heterologous strain (Strugnell, unpublished results).[14]

Turner and Hollander reviewed other criteria that may be used to differentiate between strains of *T pallidum*.[20] These criteria included the degree of histological damage associated with dermal and testicular primary lesions, the degree to which lymph nodes are affected, the development of extended lesions, and the presence of local lesions in experimentally infected rabbits. More recently, *T pallidum* strains have been shown to vary in their susceptibility to erythromycin.[21] In the study reported here we found that rabbits infected with a relatively recent isolate of *T pallidum*, Melbourne 1 strain, developed secondary lesions more often than rabbits infected with the Nichols reference strain. The incubation periods of secondary lesions that developed in rabbits infected with either Nichols or Melbourne 1 strain were constant. All lesions developed between six and nine weeks after infection, when sustained immunity to chancre formation is first established by superinfection with low numbers of *T pallidum*.

The morphology of the secondary lesions varied between minor maculopapular indurations to lesions that progressed to ulceration similar to the primary chancre. Two rabbits that were challenged when secondary lesions most commonly developed (about six and a half weeks after initial infection) developed satellite secondary lesions around the healing primary chancre. This phenomenon may be evidence of so called "local immunity" in syphilis.

We were unsuccessful in attempts to show an association between the development of secondary lesions and serum concentrations of two postulated mediators of syphilitic immunosuppression, circulating immune complexes and glycosaminoglycans. Furthermore, hexuronic acid, a component of glycosaminoglycans,[22] was not detected in the serum of infected rabbits at any time after infection. This finding was inconsistent with previous reports of "mucopolysaccharides" in the serum of rabbits infected with syphilis.[6][13] The maximum concentrations of glycosaminoglycans found in the previous studies, 64 mg/l, and 1 g/l,[6][13] were higher than the minimum sensitivity of the method used to recover hexuronic acid used in our study (5 mg/l). An explanation for the discrepancy may be that both previous studies used rabbits that were infected intratesticularly[6][13] or intravenously.[13] Intravenous inoculation of rabbits with viable *T pallidum* invariably leads to the development of syphilitic orchitis (Adams and Baughn, unpublished observation). We have shown previously that, after they have been infected with *T pallidum*, rabbit testes increase the synthesis of macromolecules resembling glycosaminoglycans proportionately to the extent of disease.[23] Thus the glycosaminoglycans detected in previous reports were possibly synthesised by and released from infected foci within the testes. The rabbits used in the present study were infected intradermally, and the degree of pathological change generated by a single dermal lesion may have been insufficient to release detectable concentrations of glycosaminoglycans into the serum.

Maret *et al* used precipitation with polyethylene glycol to assay immune complexes in syphilitic rabbits.[17] In contrast to serum from rabbits infected with *T pallidum* intravenously and intratesticularly, serum from intradermally infected animals did not precipitate with polyethylene glycol above levels obtained with normal rabbit serum. The authors stated, however, that in limited tests of serum from people with secondary syphilis, polyethylene glycol precipitation detected increased concentrations of circulating immune complexes. Baughn *et al* used the Raji cell and Clq binding assays and found a correlation between concentrations of circulating immune complexes and disseminated lesions. We observed an apparent rise in the mean level of material precipitable by polyethylene glycol during the period when secondary lesions first developed, six weeks after infection, but the large variability in responses by individual animals meant that the result was not significant.

Though this study did not elucidate the pathogenic

mechanisms responsible for the development of symptoms associated with secondary syphilis, the observation that secondary lesions were often induced in rabbits infected with the Melbourne 1 but not the Nichols strain of *T pallidum* emphasises two important points concerning the rabbit model of syphilis. Firstly, the Nichols strain of *T pallidum*, which is commonly used in syphilis research, may not be typical of current environmental *T pallidum* strains in its virulence for rabbits; passage through rabbits in the 70 years since its isolation[24] may have lead to its attenuation. Secondly, under appropriate conditions of selection of strain and method of infection, the rabbit may be used in studies of a dermal syndrome that resembles secondary syphilis in man. Studies are continuing in this laboratory to assess the histopathology of secondary lesions in rabbits.

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