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Effect of syphilitic rabbit sera taken at different periods after infection on treponemal motility, treponemal attachment to mammalian cells in vitro, and treponemal infection in rabbits

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SUMMARY The time course of antibody synthesis during syphilis was studied in experimentally infected rabbits. A rapid antibody response was seen; the rabbits became positive in both the rapid plasma reagin (RPR) test and *Treponema pallidum* haemagglutination assay (TPHA) by nine days after infection. Treponemal immobilising antibodies were also seen as early as nine days after infection. Antibody inhibition of treponemal attachment to baby rabbit genital organ (BRGO) cells in culture occurred with immune sera taken 30 days after infection but not earlier. When *T pallidum* was mixed with immune syphilitic rabbit sera taken at different stages of the infection and used to infect normal rabbits the rabbits became partially resistant to *T pallidum* only when the treponemes were mixed with sera taken at least 30 days after syphilitic infection. This appearance correlated well with the development of antibodies which blocked attachment of *T pallidum* to host cells. These antibodies may be involved in the resistance to reinfection which develops in syphilis as the disease progresses.

Introduction

Syphilis occurs both as an acute and a chronic infection. Large quantitites of antibodies are formed and some may be involved in opsonisation[1] or be directly bacteriocidal for *Treponema pallidum*.[2] Several attempts have been made at passive immunisation using immune serum and resulting in partial protection in the recipient rabbits.[3,4] Antibodies do interfere with the attachment of *T pallidum* to mammalian cells[5,6] and these factor(s) may be important in eventually producing immunity to the bacterium. Fitzgerald and coworkers have suggested that *T pallidum* may attach to tissue cells through a mucopolysaccharidase and that antibodies against this enzyme may prevent attachment to tissue cells.[7] They found that antibodies which reacted with commercial hyaluronidase were present in syphilitic sera.[8,9] Baseman and Hayes[10] showed that immune sera contained antibodies against the major surface proteins of *T pallidum*, presumably including those proteins concerned in treponemal attachment to mammalian cells.

Although the reports quoted above have shown the importance of humoral immunity in syphilis, the treponemes are not eliminated from primary or secondary lesions until a cellular infiltration occurs, mainly of T lymphocytes.[11] Numerous non-specific antibodies are formed during the course of syphilis which may have nothing to do with immunity.[12] Some of these may be autoantibodies. For example, antiheart antibodies have been reported in experimental syphilis.[13] In this study we report the effect of sera collected during syphilitic infection on the attachment of treponemes to cultured mammalian cells in vitro and on infection in vivo. Our aim was to determine when humoral immunity begins and whether antibodies produced early in infection may be involved in enhancement of the disease process by stimulating treponemal attachment to host cells.

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Materials and methods

SOURCE OF *T PALLIDUM*

T pallidum (Nichols) was propagated in adult male rabbits as described[14] and the treponemes extracted anaerobically.[15] Eagle's minimal essential medium (EMEM) with 10% fetal calf serum was used in all experiments, as it had previously been shown to be superior to three other tissue culture media.[16]

RABBIT SERA

Three rabbits were injected with approximately 5×10^7 viable *T pallidum* per testis. The rabbits were bled before infection to provide normal rabbit serum (NRS) and periodically thereafter (from 5 to 150 days) to provide syphilitic rabbit sera (SRS). The control rabbit was injected with 5×10^7 heat-killed *T pallidum* per testis and bled similiarly. Sera were stored separately at $-70°C$ until all were ready for experimental use. They were then sterilised by filtration (Millipore 0·45 μm).

TISSUE CULTURE CELLS

We had previously examined various cell lines for attachment of *T pallidum* and the baby rabbit genital organ (BRGO) cell line was superior to others tested.[16] Thus BRGO cells were used for all experiments. This primary cell line was isolated in our laboratory and maintained in EMEM with 10% FCS and 10 mmol/l HEPES without antibiotics at 37°C. The medium was changed every three days and the confluent monolayers subcultured using phosphate buffered saline containing 0·025% trypsin (Sigma) and 0·001% ethylenediamine tetra-acetic acid (EDTA) (Sigma) to remove cells attached to the tissue culture flask.

SEROLOGICAL TESTS

The rapid plasma reagin (RPR) test (Hynson, Wescott and Dunning, Baltimore) and the *T pallidum* haemagglutination (TPHA) test (Fujizoki Pharmaceutical Co Ltd, Shinjuku-ku, Tokyo) were performed according to the manufacturer's direction.

The presence of treponemal immobilising antibiotics in the sera was determined by mixing freshly harvested motile treponemes (7×10^6/ml) with individual SRS at a final serum concentration of 20% and incubating them microaerophilically at 35°C for 24 hours. Examination by dark field microscopy of 100 treponemes at random enabled a percentage motility to be determined for the culture. Since reducing agents interfere with the activity of antibodies in sera[17] they were excluded from the medium.

ATTACHMENT OF *T PALLIDUM* TO BRGO CELLS

T pallidum (2×10^7/ml) in serum free medium were mixed with individual SRS for between 30 and 60 minutes at a final serum concentration of 20%. The mixtures were then coincubated with BRGO cells in tissue culture for one hour. Coverslips were then removed from the Leighton tubes and unattached treponemes washed off with medium. The number of treponemes attached per BRGO cell was determined by observing between 30 and 60 BRGO cells at random from duplicate or triplicate tissue culture tubes by dark field microscopy.

SYPHILITIC RABBIT SERUM (SRS) MODIFICATION OF *T PALLIDUM* VIRULENCE

Different concentrations of *T pallidum* (10^3 to 10^7) were mixed with individual SRS for between 30 and 60 minutes at a final serum concentration of 50%. The remaining virulence of the *T pallidum* was determined by intradermal inoculation of 0·1 ml of culture into six sites on the shaved backs of each of two rabbits as described.[14] The course of the infection was followed and the latent period determined as the time between inoculation and the appearance of induration at the inoculation site. There is an inverse relationship between the latent period of infection and the number of virulent *T pallidum* remaining in the sample inoculated into the rabbit.[12]

Results

ANTIBODIES IN SYPHILITIC RABBIT SERA (SRS) AND THE EFFECT OF SRS ON TREPONEMAL MOTILITY IN CELL FREE MEDIUM

Sera taken from nine days after intratesticular infection (5×10^7 treponemes/testis) showed positive results in both the RPR and TPHA tests (fig 1). Sera taken later than 40 days after infection showed the highest titres in both tests.

Immobilising antibodies were present in sera only nine days after infection and reached maximum concentrations by 20 days after infection (fig 2). Thus the rabbits mounted a very rapid humoral immune response against *T pallidum* in the form of immobilising antibodies. The titre remained high until the last sample was taken 150 days after infection, indicating a long lasting antibody response.

EFFECT OF SRS ON THE ATTACHMENT OF TREPONEMES TO INTACT BRGO CELLS UNDER MICROAEROPHILIC CONDITIONS

Since inhibition of attachment to mammalian cells has been suggested as a possible important role of antibody in syphilis[7] the effect of individual and

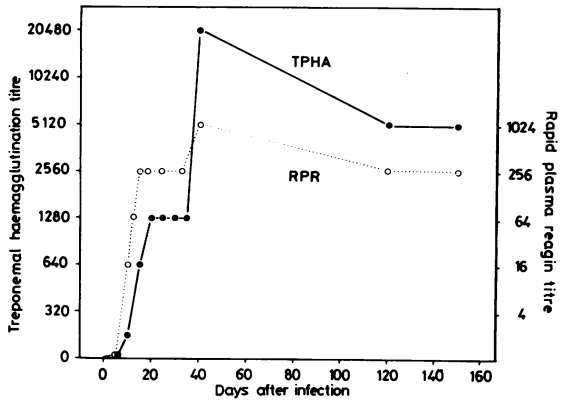

FIG 1 *Antibody titres by the rapid plasma reagin (RPR) and Treponema pallidum haemagglutination assay (TPHA) tests in syphilitic rabbit sera (SRS) during syphilitic infection.*

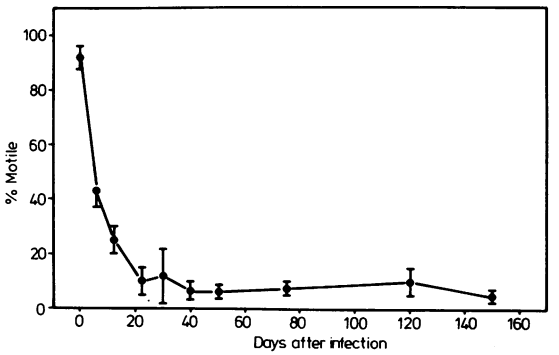

FIG 2 *Effect of syphilitic rabbit sera (SRS) on the motility of T pallidum in cell-free medium. Each point is the mean of six samples (SD).*

sequential syphilitic rabbit sera on attachment of treponemes to BRGO cells was determined.

The data showed that there was no significant enhancement of treponemal attachment to BRGO cells associated with early SRS when compared with normal serum (fig 3). Sera taken 30 days after infection, however, greatly inhibited treponemal attachment to BRGO cells by up to 70% compared with normal serum. Since the motility of the treponemes at the time of measurement was approximately 80–90% the inability of treponemes to attach to BRGO cells was probably not due to a lack of treponemal motility. SRS taken between 30 and 150 days after infection gave similar inhibition, indicating that the factor(s) (presumably antibody) which blocked treponemal attachment to the host cells was continually synthesised.

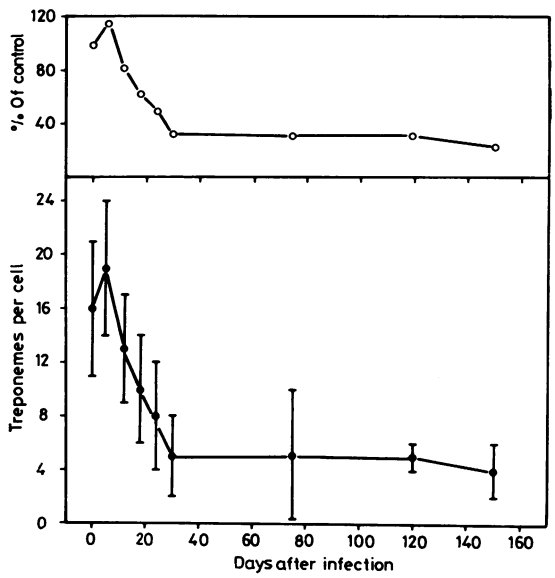

FIG 3 *Effect of SRS on the attachment of treponemes to intact baby rabbit genital organ (BRGO) cells under microaerophilic conditions.* ○——○ *—treponemes per cell in the presence of SRS/treponemes per cell in the presence of normal rabbit serum × 100;* ●——● *—mean number of treponemes per BRGO cell. Each point is the mean of 30-40 determinations (SD).*

EFFECT OF SRS ON TREPONEMAL INFECTION IN VIVO

An attempt was made to correlate the two different antibody responses (antibodies inhibiting treponemal motility and antibodies inhibiting treponemal attachment to the host cells) to actual resistance to infection in the rabbits.

When 10^7or 10^6 treponemes (prereacted with SRS) were inoculated into the rabbits there were no significant differences in the latent periods of infection (fig 4). A significant difference was observed, however, when 10^5, 10^4, or 10^3 treponemes (prereacted with SRS) were inoculated into the rabbits, provided that the sera were taken no earlier than 30 days after infection. The sera reduced the virulence of the *T pallidum* inoculum as shown by a substantial lengthening of the latent period of infection (fig 4).

Discussion

These results indicate that antibody present in serum from rabbits infected with *T pallidum* was probably contributing appreciably to the onset of immunity

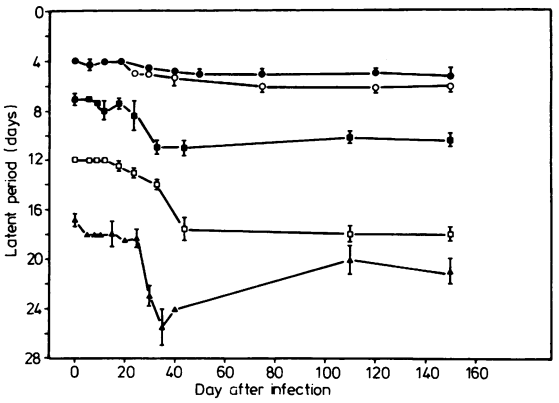

FIG 4 *Effect of SRS on infection with T pallidum in vivo. Number of T pallidum injected per site (after reaction with SRS): ●—10^7; ○—10^6; ■—10^5; □—10^4 ▲—10^3. Each point is the mean of 4-12 lesions on 1-3 rabbits.*

by 30 days after infection. Previous published reports have supported a role for antibody in immunity.[3 4 12 18-24] Rabbits generally start to become resistant to superinfection about two months after infection, although they may not become completely immune until after three months or later.[12] When the time course of appearance of *T pallidum* immobilising antibodies and tissue culture cell attachment blocking antibodies were investigated the immobilising antibodies appeared much earlier in infection. They reached appreciable concentrations only nine days after infection and a plateau level by 20 days after infection. The cell-attachment-blocking antibodies appeared much later and did not reach an appreciable concentration until 30 days after infection. Both antibody responses were long lasting and they showed no tendency to decay over the 150 days' observation period. Thus the cell-attachment-blocking antibodies correlated more closely with the onset of immunity in the rabbits than did the immobilising antibodies.

Immobilising antibodies were presumably treponemicidal in vitro but probably not so in vivo. They appeared well before immunity to superinfection developed. At the time immune serum brought about an appreciable reduction in attachment of *T pallidum* to host cells in the in vitro assay, the same serum was also showing a distinct delaying effect on lesion formation by *T pallidum*. Although many other antibodies are formed during syphilis this correlation suggests that the same antibodies which block attachment to host cells may contribute appreciably to the onset of immunity. How *T pallidum* circumvents this blocking effect of antibody to establish secondary lesions is unknown.

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