Investigation of the neutralizing activity for Treponema Pallidum of neonatal rabbit basal serum taken at 2, 3, and 4 weeks of age

Helen Ceclie Mercier

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INVESTIGATION OF THE NEUTRALIZING ACTIVITY FOR
TREPONEMA PALLIDUM OF NEONATAL RABBIT BASAL SERUM
TAKEN AT 2, 3, AND 4 WEEKS OF AGE

A Thesis
Presented to the
Faculty of
California State University
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Helen Cecile Mercier
August 1987
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8/17/87
ABSTRACT

Evidence of neonatal rabbit resistance to symptomatic infection with *Treponema pallidum*, the etiological agent of human syphilis, at one week of age, and its decline as the animal approaches five weeks of age has been demonstrated. The present study was designed to examine the possible influence of a neutralizing factor(s) on neonatal resistance by determining the neutralizing activity of basal sera from 18 neonatal rabbits 2, 3, and 4 weeks of age. Three experimental runs, with two sera each from rabbits 2, 3, and 4 weeks of age were performed. A total of 15 adult New Zealand white rabbits were inoculated with suspensions containing a final inoculum of $1 \times 10^3$ *T. pallidum* (Nichols strain) per site. Positive and negative controls were run in parallel for a total of 18 inoculation sites per adult rabbit. Results of the study demonstrated an absence of detectable neutralizing activity for *T. pallidum* in the sera of 2, 3, and 4 week old rabbits. Serum neutralizing activity may not necessarily contribute to the resistance demonstrated by neonatal rabbits. The definitive mechanism(s) of natural resistance of neonates to syphilitic infection has yet to be defined.
ACKNOWLEDGEMENTS

I am pleased to have the opportunity to thank the very special people who have assisted me with this research project.

Dr. Darlene Gamboa, my major research professor, has been a motivating force throughout, providing the guidance I needed when learning new syphilis research techniques, while at the same time, leaving me "on my own" so that I could develop a sense of independence and self-confidence in the area of experimental animal research.

Dr. Ruth Wilson, my academic advisor and good friend, has been a constant source of encouragement, as well as a totally honest critic. Her delving questions into my research methods, results, and conclusions, have provided invaluable insights into the skills needed to write a paper that is not only informative, but understandable, as well. Dr. Wilson took the photographs used in this thesis manuscript, in addition to others used as data vouchers.

A special thank-you to Dr. Alexander Sokoloff for his patient and very careful reading of the manuscript, as well as for the academic excellence that he constantly demonstrated as a professor at CSUSB.

Dr. Charles McCammom has been a constant source of inspiration and praise during the seven plus years that I
have worked with him in the clinical laboratory. As a former syphilologist and member of my thesis committee, he has provided valued insights into the acquired form of the human disease.

Lastly, I would like to thank my mother and father, Rita and Lucien Mercier, for having instilled in me a sense of pride in a job well done, and a dedication to persevere in spite of difficulties. They have been the best and most important teachers of my life.
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INTRODUCTION

Scholars and medical historians have debated the mysterious origin of syphilis for nearly 500 years. Although this academic dispute over whether syphilis originated in the New World (Columbian Theory) or had been present in the Old World from time immemorial (Pre-Columbian Theory) continues, neither theory is entirely satisfactory. Whatever its origin, a great pandemic of syphilis occurred in all parts of Europe between 1493 and 1494, and by 1497 appeared even in the remote areas of Scotland (PHS 1968). At this time syphilis was a very acute disease, frequently fatal in the secondary stage. Physicians throughout Europe recognized it as a new and previously unknown condition and were reporting and diagnosing its symptoms as early as 1500. Fortunately, the extremely acute, severe form of syphilis quickly lessened to the more chronic form of today.

The causative organism of syphilis is a member of the order Spirochaetales of the family Treponemataceae. The many species of this order are widely distributed in nature with the overwhelming majority being free-living and saprophytic. Species have been described from a diversity of ecological habitats including soil, water, and the alimentary tracts of insects and amphibians. The spirochetes are generally defined as actively motile by means of a twisting corkscrew-like rotation.
The family Treponemataceae contains three genera, *Borrelia*, *Treponema*, and *Leptospira*. Within all three genera there are species parasitic or pathogenic for man, other mammals and/or birds. The genus *Treponema* contains four principal species of pathogenic organisms. These include *T. pallidum*, responsible for human syphilis, *T. pertenue*, the etiologic agent of yaws, *T. carateum*, responsible for pinta, and *T. paraluis* subsp. *cuniculi*, the causative organism of rabbit syphilis.

Although the individuals of all *Treponema* species are morphologically and serologically similar, the pathogenic forms can be distinguished antigenically from the non-pathogenic cultivatable strains of *Treponema* (PHS 1968). The suggested evolutionary history of the pathogenic species of the *Treponema* is a matter of speculation. Most authors believe that the pathologic species developed from free-living, non-pathogenic forms, and later adapted to their human or animal hosts. Although most suggest that all pathogenic *Treponema* were derived from a single source, speculators differ on how far back in the evolutionary scale the differentiation between the species occurred. Many contend that the *Treponema* pathogenic for humans are the same species modified only by various factors in the environment and the host (PHS 1968).

In 1905 two German scientists, Fritz Schaudinn and Eric
Hoffman, discovered *Treponema pallidum* (*Spirochaeta pallida*) in the primary sores of persons infected with syphilis. *Treponema pallidum* is a thin, delicate, spiral bacterium with 6 to 14 tight-body coils and is motile by means of endoflagella. It ranges in size from 6 to 15 microns in length, and has a uniform cylindrical thickness of about 0.25 microns. (Wistreich and Lechtman 1984).

The clinical manifestations of acquired syphilis are divided into three stages: primary, secondary, and tertiary. During the primary stage, a chancre develops within two to six weeks at the site of *Treponema* contact from which the treponemes quickly invade the blood stream and lymphatics, and are distributed throughout the body. Six to eight weeks after the appearance of the primary chancre, the secondary stage is characterized by the development of cutaneous and mucous membrane lesions. Additional symptoms include headache, fever, and generalized lymphadenopathy. A latent period follows which marks the end of the infectious period of syphilis. Although serological tests are positive during this latent phase, clinical symptoms of the disease are absent. The tertiary stage (one third of the cases go on to this) may take five to twenty years to appear. During tertiary syphilis, *T. pallidum* can invade and damage any organ of the body, e.g., gummas (lesions that may appear on any part of the body), and aneurysms of the aorta. Person-
ality disorders and/or paralysis may also occur due to invasion of the central nervous system.

Acquired syphilis has been well documented and described, but additional attention needs to be focused on the unborn victim, the conceptus. Congenital syphilis begins when *T. pallidum* crosses the placenta and infects the fetus. Although this infection was believed to occur only after the 18th week of gestation, when atrophy of the Langhan's cell layer (cYTOTROPHOBLAST) of the placenta takes place (Brown and Moore 1963, Peterson 1973, Sokol and Aroujo 1973), recent evidence indicates that treponemes can invade fetal tissue as early as the first trimester (Harter and Benirschke 1976, Grossman 1977). Stillbirth is likely if pregnancy occurs during the primary and secondary stages of syphilis. When pregnancy occurs during the tertiary stage, infected newborns may exhibit a variety of clinical manifestations ranging from asymptomatic infection, cold or flu-like symptoms, to fatal disease. On the other hand, the newborn may be totally unaffected (Stokes et al. 1944a, Crissey and Denenholz 1984).

Congenital syphilis is divided into two principal stages, early and late. This terminology refers to the time in the child’s life when symptoms appear. The effects of early syphilis, analogous to acquired secondary syphilis, appear before the age of two years and include skin and
mucous membrane lesions, hemolytic anemia, hepatosplenomegaly, and involvement of the skeletal and central nervous systems (Wistreich and Lechtman 1984). Over 50% of these infants have 'snuffles', a thick white or blood-tinged nasal discharge teeming with treponemes (Woody et al. 1963, Grossman 1977, Crissey and Denenholz 1984).

The clinical disease of late congenital syphilis is comparable to the tertiary manifestations of the acquired form. It becomes evident after the age of two years, and in many cases not until puberty. Pathognomic manifestations of congenital syphilis include interstitial keratitis, eighth nerve deafness, and dental deformities (Hutchinson's triad). Suggestive manifestations include bone destruction (sabre shins, saddle nose, frontal bossing, perforation of the palate, Clutton's joints), cutaneous lesions (rhagades, gummas), and, rarely, neurologic and cardiovascular involvement (Grossman 1977).

The discovery of penicillin by Fleming in 1929 and its development by Florey and his associates in 1941 (Stokes et al. 1944b) as a powerful chemotherapeutic agent during World War II brought about a dramatic reduction in the incidence of congenital syphilis between the years 1947 and 1957 (Saxoni et al. 1967, Peterson 1973). However, we are beginning to see a re-emergence of this form of the disease (Brown and Moore 1963, Robinson 1969, Tan 1973, Teberg and

In approximately 60% of human cases, congenital syphilis is latent (PHS 1968), identified only by reactive serological tests. This asymptomatic period contributes to the difficulty in early diagnosis of congenital syphilis. Perhaps this is a pattern of human resistance similar to that seen experimentally in fetal and neonatal rabbits. The works of earlier researchers, Uhlenhuth and Mulzer (1913), Grigoriew (1929), Bessemans and Van Canneyt (1932), Seiffert (1934), Kemp and Rosahn (1937), Kemp and Fitzgerald (1938), and Pautrizel et al. (1957) represent efforts to understand the course of the experimental congenital and neonatal disease. The results of the past reports are confusing at best because differences in experimental design hamper comparison. However, one point shines through the confusion, either from careful data interpretation or by each author's declaration -- fetal and neonatal rabbits demonstrate resistance to \textit{T. pallidum} infection. This was definitively demonstrated by Gamboa and Miller (1984) in neonatal rabbits. In 1985, following high doses of repeated intravenous injection of \textit{T. pallidum}, Fitzgerald unequivocally
demonstrated the passage of *T. pallidum* from infected does to fetal rabbits. Again, Fitzgerald's results lend themselves to interpretation as resistance. In contrast, Festenstein and Bokkenheuser (1961), and Festenstein et al. (1967), upon inoculation of the newborn animals, demonstrated a runting syndrome indicative of susceptibility.

The studies of Gamboa and Miller (1984) and Gamboa et al. (1984) provided evidence of neonatal rabbit resistance to symptomatic infection at one week of age, and monitored its decline as the animal approached five weeks of age. The possible influence of a serum neutralizing factor(s) of one week old neonatal basal serum upon resistance was presented (Gamboa and Miller 1984). This potential correlation was based on the presence of neutralizing activity in sera of one week old neonates and its absence in sera of five week old animals.

The present study was designed to examine the potential influence of neutralizing activity on neonatal resistance by determining the neutralizing activity of basal sera from neonatal rabbits 2, 3, and 4 weeks of age.
MATERIALS AND METHODS

Rabbits

Adult (> 6-month-old) male New Zealand white (NZ-W) rabbits with nonreactive Venereal Disease Research Laboratory (VDRL) serologic tests were used throughout the study. The rabbits were maintained at 18 to 20°C and were given antibiotic-free food and water ad libitum.

Treponema pallidum

Treponema pallidum (Nichols strain) were obtained from infected animals provided by Dr. James N. Miller, UCLA Treponemal Research and WHO Laboratory, Los Angeles, California, where they are maintained by intratesticular passage. Normal animals were infected by inoculation of 1.0 ml/testis of a suspension containing a minimum of $2 \times 10^7$ treponemes/ml. At the height of orchitis development, approximately 9 days, the animals were sacrificed by intracardiac injection of a lethal amount of T-61 Euthanasia Solution (National Laboratories Corporation, Somerville, NJ) and the testes were aseptically removed (Fig. 1).

The testes were sliced longitudinally and the treponemes were harvested in heat-inactivated (56°C for 30 minutes) normal rabbit serum (HI-NRS). The suspension was centrifuged at 250xg for 7 minutes to remove gross cellular debris (Fig. 2). Treponemal concentration was calculated
using darkfield microscopy and the suspension was adjusted to 1x10⁶ T. pallidum/ml in HI-NRS.

**Control and Test Sera**

The test sera consisted of eighteen serum samples, each from a different neonatal rabbit (6 each from 2, 3, and 4 week old neonates). These sera were taken from test rabbits used by Gamboa and Miller (1984) and stored at -76 C. Three separate experimental runs, with two sera each from rabbits 2, 3, and 4 weeks old, were performed.

Immune rabbit serum (IRS) obtained from male NZ-W rabbits infected with T. pallidum a minimum of 3 months prior, and immune to intradermal challenge, was used as the positive neutralizing control. Normal rabbit serum (NRS) from VDRL nonreactive male NZ-W rabbits susceptible to T. pallidum infection was used as the negative neutralizing control. Paralleling test sera, IRS and NRS control sera were stored at -76 C until needed, at which time they were brought to room temperature for further manipulations. (Both IRS and NRS were kindly supplied by Dr. James Miller.)

Viability controls were suspensions of 1x10⁶ T. pallidum/ml in HI-NRS inoculated at 0 hours and following the 16 hour incubation period. These were used to gauge the viability of virulent organisms prior to and following the incubation period.
Micro-neutralization Assay (micro-NZ)

Micro-neutralization assays were performed by the method of Gamboa and Miller (1984). Briefly, test and control sera were aliquoted into appropriately labeled sterile and stoppered test tubes (Fig. 3) and kept on ice (Fig. 4). Test and control suspensions were prepared with 90 μl sera together with 10μl of a 1x10⁶ T. pallidum/ml suspension (Figs. 5, 6). These were incubated for 16 hours at 34°C in an anaerobic atmosphere of H₂ and CO₂ (Fig. 7) (BBL Gas Pak Anaerobic Systems, Becton Dickinson Co., Cockeysville, MD). Test and control suspensions were diluted by the addition of 0.9 ml HI-NRS just prior to injection for a final suspension of 1x10⁴ T. pallidum/ml. Each site received 0.1 ml suspension for a total inoculum of 1x10³ T. pallidum/site. Positive (+) neutralization was indicated by the absence of lesion development, negative (-) neutralization by the appearance of typical lesions within the appropriate incubation period established by the (-) controls. Delayed lesion development was indicative of partial (+/-) neutralization. Representative lesions of the micro-NZ assay are pictured in Figures 14-24.

Inoculations

Five VDRL nonreactive male NZ-W rabbits with "good" backs (Fig. 8) (as opposed to "bad" backs, Fig. 9) were obtained in advance of each experimental run (Bio Robotics,
Van Nuys, CA) and their backs were shaved just prior to the time of inoculation. Each rabbit was inoculated in the designated pattern illustrated in Figure 10. Each of the test and control suspensions were drawn into sterile 1 ml leur lok tuberculin syringes (Fig. 11), and administered for a total of five replicate inoculation sites, one site on each rabbit back (Fig. 12). All animal backs were clipped and monitored daily for lesion development. Incubation periods and lesion diameters (Fig. 13) and durations were recorded daily. Aspirates of representative lesions were examined for motile treponemes by darkfield microscopy. VDRL serology tests were performed on all test animals upon termination of each experimental run.

**Statistical analysis**

The incubation periods of neutralization lesions were analyzed by the Student's t test. The differences in the results were considered to be significant if \( p < 0.05 \).
Figs. 1-2. Extraction of *Treponema pallidum* from rabbit testes. --1. Testes infected with a minimum suspension of $7 \times 10^7$ *T. pallidum*/ml/testis removed for maceration. --2. *T. pallidum* suspension following extraction from testes, centrifugation, and removal of gross cellular debris.
Figs. 3-6. Dilution procedures for the micro-NZ assay.

3. Pipetting of 10μl aliquots of a 1x10^6 Treponema pallidum/ml suspension.

4. Serum samples were immediately placed on ice.

5. The addition of 90μl of test or control serum.

6. Preparation of test and control suspensions for 16 hour incubation period.
Fig. 7. Anaerobic atmosphere jar, 34°C incubator, and syringe labels.
Figs. 8-9. Rabbit back characteristics.--8. Test rabbit with a good back, i.e., smooth skin with no hair patterns after shaving. Black dots mark inoculation sites.--9. A rabbit with hair patterns that make lesion interpretation difficult.
NOS. 2, 3, AND 4 = SERUM FROM 2, 3, & 4 WEEK OLD RABBITS

A THRU F = NEONATES NOS. 1 - 6

HI = HEAT-INACTIVATED SERUM
WITHOUT HI = UNHEATED SERUM

NRS = NORMAL RABBIT SERUM

IRS = IMMUNE RABBIT SERUM

VC = TREPONEMA PALLIDUM VIABILITY
  0 CONTROL AT ZERO INCUBATION

VC = T. PALLIDUM VIABILITY
16 CONTROL FOLLOWING 16 HOURS INCUBATION

Fig. 10. Inoculation pattern for test rabbits.
Figs. 11-12. Inoculation procedure.--11. Sterile, labeled luer lok syringes each containing either incubated test or control suspensions of $1 \times 10^4$ Treponema pallidum/ml. Only 17 syringes appear because zero hour viability control injections had been administered.--12. Inoculation of one test animal with 0.1 ml T. pallidum suspension at the VC site.
Fig. 13. Example of measurement of lesion diameter and inspection of lesion development.
RESULTS

Micro-neutralization assays performed with neonatal basal sera from 2, 3, and 4 week old rabbits failed to demonstrate neutralizing activity for Treponema pallidum. Table 1 summarizes the results of this study. Among the neonate groups 96-100% of inoculated sites developed lesions from unheated serum preparations. There were no significant differences among incubation periods (number of days from the day of inoculation to the first day of lesion development) of lesions using sera from the three neonatal age groups, nor were there any significant differences among sera from adult NRS control groups and neonatal groups. The mean incubation period for lesion development among neonate groups ranged from 16.4 ±1.8 to 17.4 ±2.1 days and the mean incubation period of the NRS was 18.6 ±4.4 days. However, in two instances (3 wk-HI and 4 wk-HI) the examination of data on individual sera showed either notably delayed or totally absent lesion development which is not evident from the summarized data on Table 1.

Aliquots of both control and test sera were examined for a heat-labile component(s) by heat-inactivating (56°C for 30 minutes) sera prior to use in the micro-neutralization assays. As expected, the immune rabbit serum controls neutralized T. pallidum (no lesions) when serum was not heated and resulted in either no lesions (54% of sites) or
delayed lesions when heat-inactivated. Interestingly, in one experimental run, one serum sample from each of the 3 and 4 week old heat-inactivated neonatal age groups demonstrated partial neutralization by the absence of lesions at 3 of 5 sites and 2 of 5 sites respectively. Lesions that did develop from these serum samples were notably delayed.

The neonates whose basal sera were used for these assays were infected with *T. pallidum* following extraction of their basal serum by Gamboa and Miller (1984). The resistance among the neonates they inoculated was not uniform. Some neonates developed atypical dermal lesions at one or both inoculation sites while others remained free of lesions. The atypical designation was defined as any lesion that was small, indurated, nonulcerative and of short duration as compared to adult controls inoculated similarly. As shown in Table 2, no apparent correlation was demonstrable between the development or absence of atypical lesions among neonates and the neutralizing activity of their basal sera in any of the age groups. Both heat-inactivated and unheated serum samples from the seven which developed atypical lesions (+ neonates), failed to neutralize the treponemes at 95% of the inoculated sites. Likewise, sera from 11 that had not developed lesions (− neonates), failed to demonstrate neutralizing activity at 97% of the inoculated sites.

Figures 14-24 follow the progressive lesion development
of three representative animals from a total of fifteen used to test for the neutralizing activity in neonatal rabbit serum. These figures illustrate the first appearance of erythema (day 15 post-inoculation, Figs. 14, 15) and continue through the healing stages (day 48 post-inoculation, Fig. 24).

Aspirates of representative lesions routinely drawn just prior to ulceration (Fig. 17), and selected from both test and control sites, demonstrated actively motile treponemes by darkfield microscopy. Upon termination of the experiments, all test animals had converted to reactive VDRL serologies.
TABLE 1. Neutralizing activity of neonatal basal sera from 2, 3, and 4 week old rabbits.

<table>
<thead>
<tr>
<th>Rabbit Age</th>
<th>Serum</th>
<th>No. of Serum Samples</th>
<th>No. of Lesions/No. of Sites c Inoculated (%)</th>
<th>Incubation Period d Mean ± SD</th>
<th>Incubation Period Mean ± SD</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Unheated Sera</td>
<td></td>
<td>Heat-Inactivated Sera</td>
</tr>
<tr>
<td>2 wk basal</td>
<td>6</td>
<td>29/30 (96)</td>
<td>17.0 ± 2.4</td>
<td></td>
<td>30/30 (100) 16.6 ± 2.5</td>
</tr>
<tr>
<td>3 wk basal</td>
<td>6</td>
<td>26/26 (100)</td>
<td>17.4 ± 2.1</td>
<td></td>
<td>27/30 (90) 16.9 ± 1.6</td>
</tr>
<tr>
<td>4 wk basal</td>
<td>6</td>
<td>30/30 (100)</td>
<td>16.4 ± 1.8</td>
<td></td>
<td>28/30 (93) 18.5 ± 2.1</td>
</tr>
<tr>
<td>&gt;6 mo NRS</td>
<td>3</td>
<td>11/15 (73)</td>
<td>18.6 ± 4.4</td>
<td></td>
<td>15/15 (100) 17.6 ± 2.7</td>
</tr>
<tr>
<td>&gt;6 mo IRS</td>
<td>3</td>
<td>0/15 (0)</td>
<td></td>
<td></td>
<td>7/15 (46) 27.6 ± 2.0</td>
</tr>
</tbody>
</table>

a

b
e

f
g

h
Table 1. Continued.

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
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<tr>
<td>a</td>
<td>Summation of three experiments. See Materials and Methods for details of micro-NZ assay.</td>
</tr>
<tr>
<td>b</td>
<td>56°C for 30 minutes.</td>
</tr>
<tr>
<td>c</td>
<td>Inoculum from each serum was injected into five sites for unheated and heat-inactivated sera (exception: unheated test serum from one 3 week old neonate was inoculated into only one site due to lost inoculum).</td>
</tr>
<tr>
<td>d</td>
<td>Number of days from inoculation to first appearance of erythema and induration. Values are mean ± one standard deviation.</td>
</tr>
<tr>
<td>e</td>
<td>$P &gt; 0.05$ Student's t Test; comparison among neonatal sera, neonatal sera and NRS, and unheated and heat-inactivated samples.</td>
</tr>
<tr>
<td>f</td>
<td>Normal rabbit serum obtained from non-infected VDRL non-reactive adult rabbits susceptible to symptomatic infection with <em>Treponema pallidum</em>.</td>
</tr>
<tr>
<td>g</td>
<td>Immune rabbit serum obtained from infected adult rabbits immune to symptomatic reinfection upon challenge with <em>T. pallidum</em>.</td>
</tr>
<tr>
<td>h</td>
<td>$P &lt; 0.05$ comparison with neonate and IRS control sera.</td>
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TABLE 2. Comparison of basal serum neutralizing activity and *Treponema pallidum* lesion development among neonates 2, 3, and 4 weeks of age.

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>No. of Animals</th>
<th>Primary Lesion Development</th>
<th>Serum Neutralizing Activity</th>
<th>Incubation Period (Days) Mean ± SD</th>
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<tbody>
<tr>
<td>2 wks</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>17.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>16.6 ± 2.8</td>
</tr>
<tr>
<td>3 wks</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>17.3 ± 2.4</td>
</tr>
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<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>17.6 ± 1.7</td>
</tr>
<tr>
<td>4 wks</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>16.4 ± 1.8</td>
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*a* Neonatal rabbits 2, 3, and 4 weeks of age were inoculated with $1 \times 10^6$ *T. pallidum* at each of two sites (Gamboa and Miller 1984). One day prior to inoculation the same animals were bled for basal serum samples which were stored at $-76^\circ$C until needed for these micro-NZ assays.

*b* Results of tests performed by Gamboa and Miller (personal communication). Neonate lesions, where developed (+), were atypical, indicative of resistance.

*c* Results from the present neutralization study which tested basal serum samples from 18 neonates.
Figs. 14-24. Representative examples of post-inoculation lesion development on rabbit backs, demonstrating the results of micro-NZ assays for *Treponema pallidum* neutralizing activity by basal sera from 2, 3, and 4 week old neonatal rabbits.
Figs. 14-15. Day 15. — 14. Rabbit no. 3. Erythema clearly demonstrable at most test sites, except at 3C-HI and 4E-HI. Development is absent at IRS and IRS-HI sites as predicted. — 15. Rabbit no. 4. Erythema development replicates that seen in rabbit no. 3.
Figs. 16-17. Day 22.--16. Rabbit no. 3. Test sites are consistently erythematous and indurated, except 3C-HI and 4E-HI. Controls NRS, NRS-HI, IRS, IRS-HI, VC and VC are as predicted.--17. Rabbit no. 4. Lesion development closely replicates rabbit no. 3. Note site VC is near ulceration.
3C-HI and Figs. 18-19. Day 32.--18. Rabbit no. 3. 3C-HI and 4E-HI sites are erythematous, indurated; other test sites and, NRS and NRS-HI are near ulceration; IRS and IRS-HI sites remain lesion free.--19. Rabbit no. 4. Test sites are ulcerated, except 3C-HI and 4E-HI; NRS and NRS-HI are near ulceration; and IRS and IRS-HI remain lesion free.
Figs. 20–21. Day 43.--20. Rabbit no. 3. All sites ulcerated, except positive NZ control sites, IRS and IRS-HI.--21. Rabbit no. 4. Most lesions are beginning to heal.
DISCUSSION

Results of this study show an absence of detectable neutralizing activity in the sera of 2, 3, and 4 week old rabbits. A correlation between neutralizing activity of neonatal basal sera and resistance to symptomatic infection was suggested in a previous study by Gamboa and Miller (1984). Their suggestion was based on 1) the presence of neutralizing activity in basal sera from one week old rabbits and their resistance to symptomatic infection following intradermal inoculation with *Treponema pallidum* and 2) the absence of neutralizing activity in basal sera and the waning resistance to *T. pallidum* infection of five week old neonates. The resistance to symptomatic infection following intradermal inoculation with *T. pallidum* in the same animals from which our sera is derived throws suspicion on the influence of a neutralizing factor(s) on their resistance. Apparently, serum neutralizing activity 1) may contribute nothing to the resistance demonstrated by neonatal animals, 2) may only partially contribute, or 3) the sensitivity of the micro-neutralization assay is insufficient to detect the full in vivo potential of serum as concentrations of its activity begin to drop in neonates after one week of age.

The last option is of particular interest. As mentioned in the results, there appears to be a hint of residual
neutralizing activity in the unheated serum of two neonatal samples (5 of 6 inoculation sites developed lesions following approximately a 21 day incubation period). Heat-inactivation of the neonatal basal serum seems to slightly enhance this neutralizing activity (5 of 10 lesions were absent, the remaining were notably delayed). This heat-inactivation enhancement is a consistent finding, also seen in previous studies (Gamboa and Miller, personal communication). These results are not surprising in light of the fact that we are examining an age frame in which declining neutralizing activity can be expected. Therefore, though the numbers are small and only suggestive of residual activity, perhaps they should not be ignored. Possibly, the concentrations of the neutralizing factor(s) present in the serum may have diminished to levels such that the assay sensitivity may not be capable of detecting it. Once heated, however, we may be enhancing that minimal amount of neutralizing factor(s), and we are able to pick it up in some individual cases. It is also possible that the reduction of T. pallidum over the 16 hour in vitro incubation period of the micro-NZ assay is masked in the in vivo portion of the assay. Fitzgerald (1981) inoculated rabbits with divergent ranges of T. pallidum. Sites receiving 10 viable organisms demonstrated an accelerated incubation period of approximately 15 days when the rabbits had been additionally
injected with 10 viable organisms at other sites. (The normal incubation period for 10 organisms averages approximately 24 days.). The delayed incubation periods indicative of partial neutralizing activity may have been masked by sites on our animals receiving the full viable inoculum of 3 10 T. pallidum; therefore, partial neutralization, which could be expected as the factor declines in concentrations, is not detected in our assay.

The delayed incubation periods and absence of lesions at sites receiving HI-NRS is a consistent finding (Bishop and Miller 1976, Blanco et al. 1984, Gamboa and Miller 1984). Blanco et al. (1984) have demonstrated that the IgG nature of neutralizing activity in immune rabbit serum, and its largely abrogated activity upon heating, is most likely due to the elimination of complement. It has been suggested that endogenous complement from the extraction of T. pallidum from rabbit testicles may account for the residual neutralization seen in these suspensions (Bishop and Miller 1976). On the other hand, it is possible that some neutralizing activity in IRS is independent of complement. It has been demonstrated that HI-NRS enhances phagocytosis of T. pallidum by proteose peptone-induced rabbit peritoneal macrophages (Lukehart and Miller 1978). Therefore it is feasible that opsonization may account for the residual neutralization seen in these suspensions.
Interest was focused on the fetal disease as early as 1913; Uhlenhuth and Mulzer (1913) set up studies on the inheritance of syphilis in rabbits, controlling the experimental conditions to resemble human syphilis whenever possible. Unfortunately, their methods were not sufficiently outlined to permit comparisons with later studies. Other research groups followed. Grigoriew (1929) described a single experimental case of congenital transmission of syphilis from one doe to her offspring. In yet another study, Bessemans and Van Canneyt (1932) concluded that, although many suggestions of congenital infection resulted, they could not prove conclusively the existence of congenital syphilis in 34 rabbits born from parents having ocular syphilitic lesions. Seiffert (1934) briefly described eight experiments dealing with infection with *T. pallidum* as a result of cohabitation or cross-placental transmission but failed to describe the route of infection of his experimental animals (mice and rabbits). Kemp and Rosahn (1937) did not sufficiently describe their experimental methods, making questionable their conclusions that a placental barrier prevented the spread of infection from doe to offspring, or the existence of a treponemical factor(s) in the fetus. In addition, and in rather forceful terms, Kemp and Fitzgerald (1938) concluded that syphilis is not transmitted from an infected doe to her offspring. In 1957, Pautrizel et al. concluded that 1) the
rabbit fetus possesses a natural immunity to infection by
*T. pallidum* and 2) maternal antibodies play only a sec-
ondary role in the prevention of transmission. Interest­
gly, nearly twenty years elapsed after the results of Kemp
and Fitzgerald (1938) were published before additional work
using the rabbit as a possible model for congenital syphilis
was again presented. Festenstein and Bokkenheuser (1961) and
Festenstein et al. (1967) attempted to tolerize neonatal
rabbits to *T. pallidum* and found an increased suscep­
tibility as defined by the appearance of a runting syndrome.

In 1985 Fitzgerald listed four factors that select
against congenital syphilis in rabbits: 1) fetal damage
requires large numbers of *T. pallidum* that accumulate in
a short period of time (Magnuson et al. 1948, Fitzgerald et
al. 1982; 2) female steroids, which are elevated during
pregnancy (Tietz 1982), diminish rabbit lesion progression
(Frazier et al. 1935); 3) rabbit pregnancy results in multi­
ple births, further diluting the numbers of organisms per
fetus (Fitzgerald 1985); and 4) possibly, the heat-stable
treponemicidal factor found in the serum of 4 to 6 day old
rabbits (Gamboa and Miller 1984) begins killing *T.
pallidum* before birth. Taking these factors into account
Fitzgerald (1985) was successful in demonstrating the
passage of *T. pallidum* from infected does to fetal
rabbits, but only after multiple intravenous injections of
*T. pallidum* over a period of four weeks for a total of 7 $4 \times 10^7$ treponemes.

The large numbers of organisms necessary to demonstrate overt symptoms of transmission may be a reflection on the presence of additional resistance factors. As discussed by Gamboa and Miller (1984), resistance of 5 to 8 day old neonatal rabbits to dermal lesion development after intradermal inoculation with *T. pallidum* may be influenced by a number of factors. Group housing (nesting) could create unfavorable temperatures for the survival of *T. pallidum*. Experimental syphilis in rabbits requires that the animals be kept in cool quarters ($18-21^\circ C$) to allow for proper lesion development following intradermal challenge with *T. pallidum*. In addition, inoculation sites must be kept clipped. Therefore, higher temperatures due to huddling of neonates in a nest may contribute to their resistance, even though this influencing factor was shown not to be totally responsible for the absence of lesion development.

Nursing was also considered as a potential influencing factor (Brambell 1970a, b, Wilson and Miles 1975). Colostrum and milk of several mammals are known to contain factors which may influence resistance (Reiter and Oram 1967, Goldman and Smith 1973, Head and Beer 1979). Although there has been no evidence to substantiate a role for similar factors in rabbits, and several investigators have concluded
that the systemic protective factors transmitted in utero to the rabbit fetus are not supplemented by nursing after birth (Brambell et al. 1951, Kraehenbuhl and Campiche 1969, Brambell 1970c), conclusive evidence for or against this theory remains lacking.

The association of a "natural" antibody with innate resistance has been suggested as another factor possibly responsible for neonatal serum neutralizing activity. Several facts, however, have negated this as a possible explanation. Natural antibody has classically been associated with the IgM class of immunoglobulins (Solomon 1971) and in the rabbit, IgM is transmitted in utero (Hemmings and Jones 1962). Therefore, were natural antibody a participant in resistance, does' sera would also demonstrate neutralizing activity and this has not been the case (Gamboa and Miller 1984).

Gamboa and Miller (1984) also proposed that the absence of a nutritional factor(s) necessary for optimum survival and multiplication of the treponemes may influence resistance. The inability thus far to cultivate T. pallidum in pure culture makes the direct investigation of nutritional requirements difficult.

While several hypotheses have been advanced to explain the natural resistance of neonates to syphilitic infection, the definitive mechanism(s) has yet to be identified.
Studies on the isolation and identification of the neutralizing factor(s) of basal sera from one week old rabbits are ongoing (Gamboa, personal communication), but in light of this study, may still be only half the story.

Congenital and neonatal human syphilis is not a ghost of the past, but remains very much a disease of the present. A total of 159 cases of early congenital syphilis have been reported in the United States during 1982, an increase of 44 cases in four years (ASRMM 1983). These numbers "without doubt underestimate the true magnitude of the problem, because of misdiagnosis, and the occurrence of undocumented cases manifested by spontaneous abortion or stillbirth" (Hansfield and Lukehart 1984). Pregnant women and their health care providers need to be aware of the significance of the diagnosis of syphilis during pregnancy so that the truly innocent victims may be spared readily preventable suffering.
LITERATURE CITED


