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13. ABSTRACT

I. Development of an Effective and Practical Vaccine Against Experimental and Human Syphilis

A. Rabbits were vaccinated intramuscularly at 3-week intervals with a total of 3 vaccine doses, each containing an equal volume of 4×10^9 γ-irradiated, liquid nitrogen-preserved T. pallidum and an alginate-gluconate adjuvant.

1. Intradermal challenge revealed enhanced partial immunity compared to that achieved without adjuvants.
2. One animal developed latent syphilis following vaccination, indicating the presence of infectious treponemes in the vaccine.
3. Each of the vaccinated animals developed VDRL antibody; in contrast, none of those tested to date have developed TPI antibody.

B. Preliminary studies indicate that Treponema pallidum, Nichols strain, suspensions can be purified by both multiple centrifugation and discontinuous Ficoll density gradient techniques.

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Abstract (continued)

II. Mechanism(s) of Immune Response in Experimental and Human Syphilis

A. The Role of Humoral Antibody

It has now been possible to demonstrate a humoral mechanism operative during the immune process in experimental syphilis. Passive protection experiments utilizing the continuous daily injections of immune sera into normal recipient rabbits following challenge has resulted in significant delays in the incubation period and/or the development of flat erythematous, atypical lesions compared to control animals. Further, preliminary neutralization experiments have shown that rabbits inoculated intradermally with treponeme-immune serum mixtures incubated at 34°C for 16 hours fail to develop lesions during a 55-day observation period, as compared to treponeme-non-immune serum control sites where typical lesions occurred in 14-18 days. Studies to determine the role of humoral antibody in the human immune response have been planned.

B. The Role of Cell Mediated Phenomena

Preliminary studies have shown that peripheral lymphocytes prepared from patients with secondary and latent syphilis have a significant cytotoxic effect upon T. pallidum, Nichols strain, suggesting that cell-mediated phenomena play a role in the human syphilis immune process.

III. The "In Vitro" Cultivation of Treponema pallidum in Tissue Culture Monolayers

Treponemes inoculated into secondary monolayer cultures derived from uninoculated rabbit testicular tissue were shown to have taken up an intracellular residence within 30 minutes after inoculation. Preliminary results indicate that the virulence of the intracellular organisms persist for at least 24 hours.

14. KEY WORDS	LINK A		LINK B		LINK C	
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Immunity- Experimental and Human Syphilis A. Vaccine 1. γ-irradiated Treponema pallidum, liquid nitrogen preservation. 2. Adjuvant 3. Homologous Acquired Resistance 4. Antibody Response 5. Purification - Treponema pallidum B. Mechanisms of Immunity 1. Humoral Antibody a. "In Vitro" - "In Vivo" Neutralization b. Passive Protection 2. Cell-Mediated Phenomena a. Direct Cytotoxicity b. Anti-Rabbit Thymocyte Globulin C. "In Vitro" Cultivation in Tissue Culture Monolayers						

ANNUAL REPORT NO. 2

BACKGROUND AND RESEARCH ACCOMPLISHMENTS

I. DEVELOPMENT OF AN EFFECTIVE AND PRACTICAL VACCINE AGAINST EXPERIMENTAL AND HUMAN SYPHILIS (OBJECTIVE NO. 1)

A. Immunization of Rabbits with Liquid Nitrogen-Preserved, γ-Irradiated Treponema pallidum, Nichols Strain, in a Reduced Injection Schedule (With and Without Adjuvants)

1. Degree of Homologous Acquired Resistance

The basis for continued studies toward the development of a practical vaccine has now been firmly established (Miller, 1973). Briefly, it has been shown that rabbits immunized intravenously over a 37-week period with a total of 3.71×10^9 freshly isolated, γ-irradiated T. pallidum, Nichols strain, are completely immune to intradermal challenge with homologous treponemes for at least one year following the completion of vaccination. However, as indicated in Annual Report No. 1 and Renewal Application 4/18/73, the use of 60 intravenous injections over a 37-week period is far from practical and points to the necessity for studies designed to determine immunogenicity with reduced numbers of immunizing injections, with or without adjuvants. Such reduced vaccination schedules require the use of individual doses containing significantly larger numbers of attenuated treponemes than those used to achieve a total dose of 3.71×10^9 organisms. Additionally, if the development of complete resistance is dependent upon the intact immunological integrity provided by freshly-isolated, γ-irradiated T. pallidum as postulated, then it also becomes essential to preserve them in this state over relatively long periods of time rather than relying upon the impractical method of vaccination the day of extraction and irradiation. The most satisfactory and practical procedure for preservation was formulated after a year of exhaustive investigation. Treponema pallidum, Nichols strain, is extracted from infected rabbit testes in 70 or 140 ml of 50% serum saline equilibrated with 95% N_2 - 5% CO_2, depending upon the availability of rabbits with a suitable orchitis. The suspension is centrifuged at low speed to remove gross tissue debris, adjusted to contain not more than 6.5×10^7 organisms per ml, then observed for motility. The suspension is then dispensed in 11 ml amounts into each of 6 or 12 tubes (depending upon whether extraction was carried out in 70 or 140 ml). Six tubes are placed in specially-designed 6-tube plastic holders and equilibrated with 95%N_2-5%CO_2(if 12 tubes of suspension

are prepared, a second plastic holder is used to accomodate the remaining 6 tubes). The plastic holder(s) is placed in the central well of a Co^{60} source, and exposed to 650,000 rads. Thus, the irradiation process is completed in one or two short exposure times which vary monthly depending upon the decay rate (presently it is 25 minutes,39 seconds). The 6 or 12 tubes of treponemal suspension are pooled, motility is determined, and glycerol is added to a final concentration of 15%. The irradiated suspension is then aliquoted into 2 or 4 tubes, and sedimented by centrifugation at 19,000 X g for 30 min. The supernatant is completely removed from 1 or 3 tubes, decanted to 1 ml in the 2nd or 4th tube, and stored at -20°C. The sedimented treponemes in the tube(s) containing 1 ml of serum-saline-glycerol are resuspended, then transferred to the remaining 1 or 3 tubes (one at a time, in the case of the latter), for resuspension of the sedimented organisms. The final suspension, which contains from 0.82 to 6.5 X 10^9 γ-irradiated T. pallidum, Nichols strain, is dispensed into sterile 2-ml-capacity vials, placed in the Revco (-76°C) for 30 minutes, then stored in liquid nitrogen at -200°C. Morphology, as determined by darkfield examination, is excellent. Further, the degree of motility after irradiation and just prior to storage has always been essentially the same.

Preparation and storage in liquid nitrogen are carried out periodically (depending upon rabbit orchitis availibility and necessity for immunoserologic testing) and have been continuing for the past 3 years.

In March, 1972, enough vaccine doses were accumulated to initiate experiments utilizing preserved, irradiated treponemes in both a reduced intravenous and reduced intramuscular infection schedule without adjuvants. As described in Annual Report No. 1, the 1 ml vials of vaccine were thawed the day of innoculation and pooled to give 27 to 50 ml of a treponemal suspension containing approximately 2 to 4 X 10^9 organisms per ml. The morphology by darkfield was excellent. Further, no change in the degree of motility was noted after thawing just prior to inoculation. Each of 12 rabbits was immunized intravenously and each of 12 intramuscularly at 3-week intervals with a total of 3 vaccine doses, each containing approximately 4 X 10^9 treponemes (total of 12 X 10^9); one animal died during the immunization procedure. No significant weight loss was observed among any of the vaccinated animals during or after immunization. Darkfield examination of the testes of each rabbit 2-1/2 weeks after the first 2

injections and one week after the last injection was negative, suggesting the non-infectivity of the vaccine doses. As a further check on infectivity, a single popliteal node and testis were removed from each of 22 surviving animals one week after the last immunizing injection and inoculated into the tests of serologically non-reactive, normal rabbits; 2 intravenously immunized and 4 intramuscularly immunized donor rabbits died as a result of the surgical procedure. None of the recipient animals showed evidence of infection during the 6 months of observation, as measured by negative darkfield examination and TPI tests. Three weeks after the last immunizing injection, the 9 animals vaccinated intravenously, the 8 vaccinated intramuscularly, and the 12 non-immunized controls were challenged intradermally at each of 4 sites with 1500 virulent T. pallidum, Nichols strain, per site. 8 of the 9 animals immunized by the intravenous route showed a significant delay in the development of lesions, ranging from 6 to 9 days after the average incubation period of 11 days in the non-immunized control rabbits; one animal failed to develop lesions during the 88-day observation period and was considered immune on the basis of negative darkfield, FTA-ABS, and TPI tests obtained during 6 months of observation of the normal recipient injected intratesticularly with the right node and testis of the vaccinated animals. Each of the 8 animals immunized by the intramuscular route also exhibited a significant delay in the development of lesions, ranging from 5 to 7 days after the average incubation period in the non-immunized control rabbits; one animal developed atypical, flat, darkfield negative lesions which healed 34 days after challenge, at a time when the non-immune control rabbits were exhibiting indurated and/or ulcerative lesions; right node and testis transfer into the testes of a normal recipient after 88 days of observation resulted in no evidence of infection, as measured by negative darkfield examination, FTA-ABS, and TPI tests carried out during the 6-month observation period.

Thus the experiment clearly shows that some degree of homologous acquired resistance develops as a result of intravenous or intra muscular vaccination with liquid nitrogen-preserved T. pallidum, Nichols strain, employing a practical time schedule, and provided a sound rationale for experiments utilizing adjuvants.

The use of a sodium alginate-calcium gluconate adjuvant to enhance the immune response was predicated upon its successful use with particulate S. typhi vaccines and the possibility of its utilization in humans without harmful effects (Shapiro, Modai, and Kohn, 1967). As in the previous experiment without adjuvants, the 1 ml vials of preserved irradiated treponemes were thawed the day of inoculation. They were then pooled to give 18 to 19 ml of a treponemal suspension containing ~4 X 10^9 organisms per ml, and combined with an equal volume of 4% sodium alginate-0.67% calcium gluconate adjuvant containing 0.4% phenol as a presevative. The morphology of the treponemes in adjuvant by darkfield microscopy was excellent, and no change in the degree of motility was noted just prior to inoculation. In an effort to employ the same injection schedule as in the non-adjuvant experiment already described, each of 15 serologically non-reactive rabbits was immunized by the intramuscular route at 3-week intervals with a total of 3 vaccine doses (2 ml per dose), each containing an equal volume of the preserved, irradiated treponeme- adjuvant mixture; each animal thus received a total of ~12X 10^9 organisms; one animal died during the immunization process. No significant weight loss was observed among any of the vaccinated animals during or after immunization. Darkfield examination of the testes of each rabbit 2 1/2 weeks after each of the first 2 injections and one week after the last injection was negative suggesting the non-infectivity of the vaccine doses. As a further check on infectivity, a single popliteal node and testis were removed from each of the 14 surviving animals one week after the last immunizing injection and inoculated into the testes of serologically non-reactive, normal rabbits; 2 of the immunized donor rabbits died of unknown causes following the surgical procedure and prior to challenge. Although negative by testicular darkfield examination during 6 months of observation, recently completed findings showed that one recipient animal developed both FTA-ABS and TPI antibody within 1 month and 2 months, respectively, after node and testes transfer, indicating the presence of viable, infectious treponemes in the vaccine dose(s) received by the immunized donor rabbit. None of the remaining recipient animals showed evidence of infection during the 6 months of observation, as measured by negative darkfield examinations and TPI tests (FTA-ABS tests have been completed on 4 1/2 month specimens and are negative). The present of infectious treponemes within a vaccine consisting of γ-irradiated, liquid nitrogen-preserved organisms, together with the fact that freshly isolated, γ-irradiated T. pallidum has never been shown to

contain infectious organisms, suggests the interesting possibility that DNA repair has occurred during storage of the treponemal suspensions, thus pointing to the necessity for increasing the total γ-irradiation dosage to that which will result in irreversible DNA damage.

Four weeks after the last immunizing injection the 12 surviving vaccinated rabbits and 12 non-immunized control animals were challenged intradermally at each of 4 sites with 2360 T. pallidum, Nichols strain, per site. Evidence of partial immunity among the 11 animals receiving non-infectious vaccine was demonstrated by the occurrence of a significant delay in the development of lesions, ranging from 3 to 14 days in each of the immunized rabbits, compared to the non-immunized controls. Further, 8 of the 11 vaccinated animals developed relatively flat, erythematous, atypical lesions which healed at a time when 10 of the 12 control rabbits were exhibiting ulcerative lesions. It is interesting to note that the animal immunized with vaccine containing infectious treponemes did not develop a greater immunity than the animals which were partially protected by means of completely inactivated organisms; atypical lesions developed after a delay in the incubation period of 7 days compared to the control animal and persisted longer than the atypical lesions which occurred in the other "test" animals.

The enhancing effect of the adjuvant was suggested by a comparison of these results with those obtained in the previously described intramuscular experiments in which the vaccine contained no adjuvant and the immunized animals were challenged with significantly fewer treponemes (1500 per site). In the latter experiment, none of the 8 vaccinated rabbits exhibited a delay of more than 7 days in the appearance of lesions compared to the non-immunized controls. Additionally, in contrast to 8 of the 11 animals vaccinated with the treponeme-adjuvant mixture, only 1 of the 8 rabbits developed flat, erythematous, atypical lesions.

Thus, the experiments show some degree of enhanced homologous acquired resistance resulting from the use of an alginate-gluconate adjuvant with irradiated, liquid nitrogen-preserved T. pallidum, Nichols strain, as a vaccine utilized in a practical, reduced time and injection schedule, and points to the necessity for further experiments employing modifications in the spacing and numbers of vaccine injections as well as smaller challenge inocula in order to clearly demonstrate low level but significant resistance. The finding of infectious treponemes in the preserved vaccine also indicates

the necessity for exposing the treponemes to a greater total γ-irradiation dosage prior to processing and storage in liquid nitrogen.

2. Antibody Response

Inasmuch as VDRL, TPI, and FTA-ABS antibodies are important in the diagnosis and control of syphilis, the finding that they develop during the 37-week intravenous immunization process and persist in some animals for at least one year after vaccination is of great significance, and points to the serious restrictions which could be imposed upon a human vaccine with the same antibody-producing capacity (Miller, 1973). Thus, as described in Annual Report No. 1 and Renewal Application 4/18/73, it is interesting to note that two weeks after completion of immunization and just prior to challenge, each of the animals vaccinated by the intravenous or intramuscular route with liquid nitrogen-preserved treponemes without adjuvants in the previously described experiment had developed both VDRL and FTA-ABS antibodies. The VDRL antibody titers ranged from 1:64 to 1:256 among the intravenous-immunized and from 1:2 to 1:8 among the intramuscular-immunized rabbits, while FTA-ABS titers ranged from 1:160 to 1:2560 among the intravenous-immunized and from 1:160 to 1:1280 among the intramuscular-immunized animals. In contrast, only 3 of the 9 intravenous-vaccinated animals. In contrast, only 3 of the 9 intravenous-vaccinated animals exhibited TPI titers, ranging from 1:11 to 1:26, while none of those vaccinated by the intramuscular route showed evidence of immobilizing antibody.

Similarly, each of the animals vaccinated intramuscularly with preserved treponemes and alginate-gluconate adjuvant had developed VDRL antibody titers, ranging from 1:2 to 1:4 within 4 weeks after completion of immunization and just prior to challenge, while none of the 5 animals tested to date had developed TPI antibody (FTA-ABS determinations have not as yet been carried out). It is interesting to speculate concerning the possibility that VDRL antibody may have developed in response to rabbit tissue present in the vaccine rather than to the treponemes per se, particularly in light of recent findings of Smibert that cardiolipin is present in rabbit testicular tissue and absent from T. pallidum, Nichols strain (Personal Communication). Such findings lend further impetus to the necessity for studies relating to the purification of T. pallidum.

3. Degree of Heterologous Acquired Resistance

As indicated in Renewal Application 4/18/73, the data of Turner and Hollander (1957) suggest that rabbits infected with the Nichols strain of T. pallidum exhibit a lesser degree of immunity to re-infection with some heterologous rabbit-adapted human strains as compared with the homologous strain. Further evidence for possible protective antigenic dissimilarity among strains emanates from studies in this laboratory in which it has been shown that (a) a lipopolysaccharide antigen from the avirulent cultivatable T. reiteri is shared by the rabbit-adapted Nichols strain of T. pallidum but not by human strains (Miller, Debruijn, Bekker and Onvlee, 1966), (b) an ultracentrifugally homogeneous polysaccharide isolated from the Nichols strain reacts with homologous rabbit but not human anti-syphilitic sera (Miller, Bekker, DeBruijn and Onvlee, 1969), and (c) rabbit anti-syphilitic sera prepared against the rabbit-adapted Utrecht strain of T. pallidum and absorbed with an ultrasonic lysate prepared from the homologous strain, show reactivity in the presence of Nichols strain lysate antigen (Miller, Bekker, Onvlee and Debruijn- unpublished data). Of critical importance, then, is the necessity for determining whether animals immunized with and resistant to the Nichols strain of T. pallidum are also immune to heterologous rabbit-adapted human strains. Such studies are predicated upon the successful achievement of acquired resistance employing adjuvants and liquid nitrogen-preserved T. pallidum, Nichols strain, as an intramuscular vaccine in a reduced injection and time schedule.

B. Purification of T. pallidum, Nichol's Strain

The preparation of pure treponemal suspensions is essential not only to the development of a practical experimental and human vaccine utilizing intact T. pallidum, but also as a prelude to the isolation and characterization of protein and polysaccharides from the Nichols and other rabbit-adapted strains of T. pallidum which conceivable could be employed as either immunogens for vaccination or as antigens for specific serologic diagnosis. Speculatively, the administration of rabbit tissue-contaminated vaccines to humans could lead to hypersensitivity reactions related to foreign protein sensitization as well as to testicular damage resulting from an immune response to testes-specific antigens (Miller, 1973).

As described in Annual Report No. 1 and Renewal Application 4/18/73, "clean" suspensions with excellent morphology when examined under the darkfield

microscope have been prepared by multiple centrifugation techniques. Briefly, rabbits were infected intratesticularly with T. pallidum, Nichols strain, and injected with cortisone in order to produce maximal yields of treponemes. The testes were aseptically removed at the height of lesion development, carefully segmented, washed, suspended in phosphate buffered saline and extracted by shaking under 95%N_2 - 5%CO_2 equilibration for 1-1/2 hours in the cold. Additional medium was added and extraction allowed to continue overnight. The suspensions were removed after each extraction and centrifuged four times at 2000 RPM for 10 minutes. After the final centrifugation, the supernatant was removed, centrifuged at 19,000 X g for 30 minutes, washed four times with phosphate buffered saline, and resuspended in approximately 1 ml of the last wash. The suspensions were examined by darkfield microscopy and frozen at -20°C. Portions of the "clean" suspensions are awaiting further analysis (a) for tissue components by electron microscopy and gel diffusion utilizing anti-serum prepared against rabbit testicular homogenate and (b) for serological activity. Additionally, as described in Renewal Application 4/18/73, purification experiments utilizing discontinuous "Ficoll" density gradients for separation have been initiated at the California Hospital Medical Center in cooperation with Dr. John Sykes, who has successfully employed this technique for the separation of tumor cells from fibroblasts (Sykes, Whitescarver, Briggs and Anson, 1970). Preliminary studies already carried out have shown that "clean" actively motile preparations of T. pallidum, Nichols strain, can be obtained. Intratesticularly-infected rabbits were sacrificed at the height of orchitis development and the testes aseptically removed and segmented. Extraction was carried out in 50% heat-inactivated rabbit serum-saline medium under 95% N_2 - 5% CO_2 equilibration for 1 hour at room temperature. In order to minimize the interfering effects of the fibrinous exudate, the resulting treponemal suspension was diluted 10 times with Eagles minimal essential medium without calcium (MEM) and containing 50% heat-inactivated fetal calf serum. The diluted extract was then strained through 4 layers of gauze to remove gross tissue particles, then centrifuged at 900 X g for 10 minutes to remove further small-particle debris. The supernatant was centrifuged at 19,000 X g for 20 minutes, the resultant pellet re-suspended in 10 ml of the MEM-fetal calf serum solution, and additional clarification carried out by centrifugation at 900 X g for 10 minutes. The suspension was again centrifuged at 19,000 X g for 20 minutes, the supernatant discarded, and the pellet re-suspended in MEM containing Ficoll(density + 1.030). The MEM-Ficoll suspension was layered onto various discontinuous gradients of Ficoll dissolved

in MEM and centrifugation carried out at 8,923 X g for 10 minutes at 25°C in a Spinco L-2 HV preparative ultracentrifuge using an SW-50 swinging bucket rotor. Following centrifugation, the various interdensity zones were collected by bottom puncture under direct visual observation and examined by darkfield and electron microscopy; additionally, the treponemes from each interface were examined in the FTA-ABS test for antigenic integrity and purity. The cleanest preparations were obtained at the 1.050 - 1.065 gms/ml interface. Both darkfield and electron microscopy failed to reveal evidence of tissue contamination; FTA-ABS tests employing the gradient treponemes as antigen not only revealed reactivity at the same level as standard, non-purified antigen preparations, but also appeared free of any tissue debris. Of important significance was the finding that gradient treponemes retained their motility, thus lending hope to the possibility that immunogenicity and virulence has also been retained.

II. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL SYPHILIS (OBJECTIVE NO. 2)

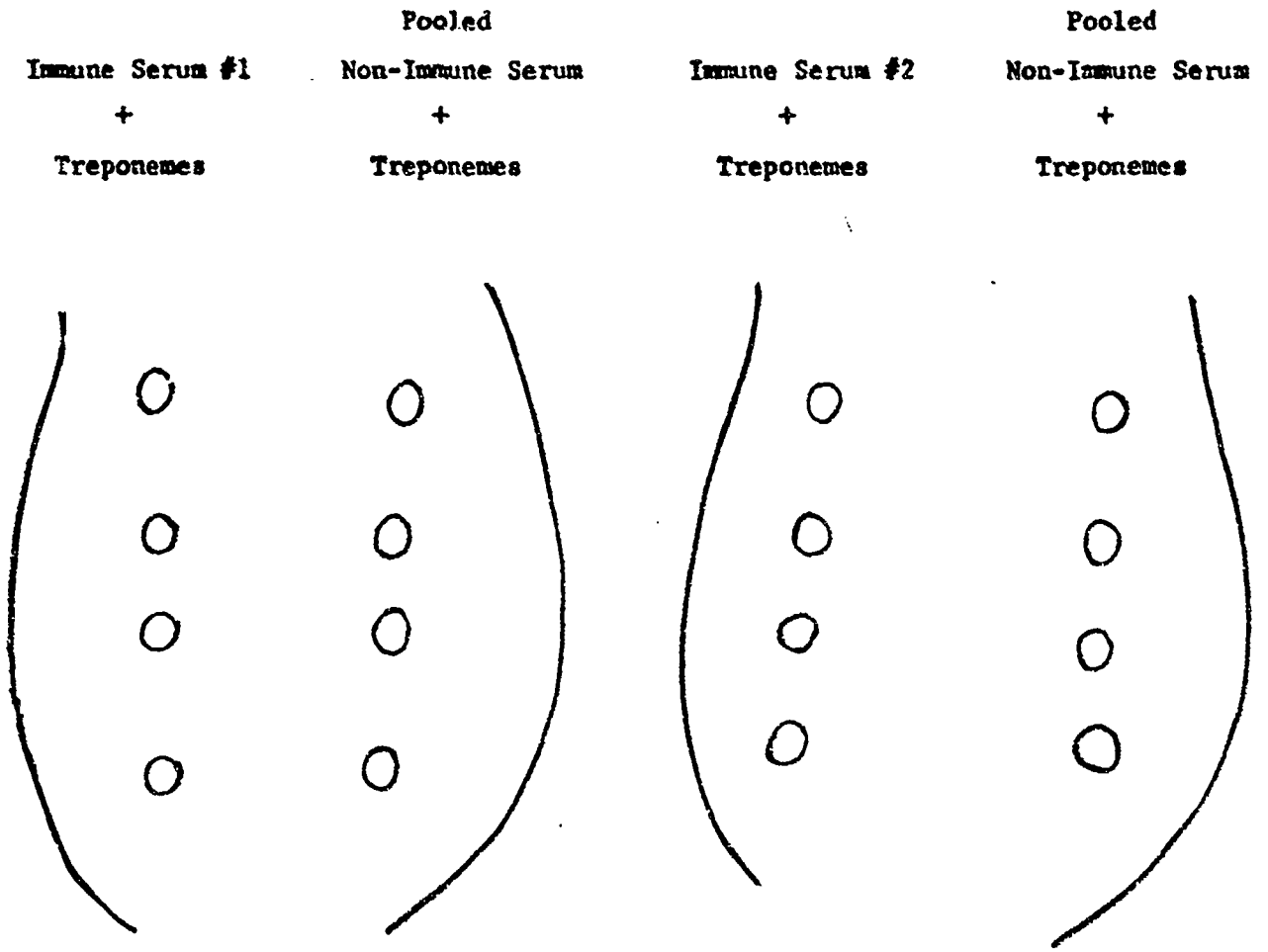
A. The Role of Humoral Antibody

1. Experimental Syphilis

As described in Annual Report No. 1, Renewal Application 4/18/73, and Miller (1973), it had not been possible to demonstrate, by "in vitro" or "in vivo" methods, a role for humoral antibody in the immune response. The finding that TPI antibody is not associated with acquired resistance together with the inability to demonstrate evidence of passive protection in rabbits injected with a single dose of immune serum or immune globulin, seemed to support the conclusion that immunoglobulins are not involved.

a. Combined "In Vitro" - "In Vivo" Netralization

As described in Annual Report No.1 and Renewal Application 4/18/73, preliminary experiments employing a combined "in vitro" - "in vivo" neutralization system have suggested that humoral antibody is operative. Sera were obtained from 2 infected-immune and from normal (non-immune) rabbits on the day of the experiment. A suspension of T. pallidum, Nichols strain, containing 10^4 treponemes was prepared and added, in equal volumes, to tubes containing each of the immune and the pooled non-immune sera. The tubes were then equilibrated with 95% N_2 - 5% CO_2 and placed in the 34°C incubator. After 4 and 16 hours, respectively, 0.2 ml of each of the mixtures (containing 1000 treponemes) were inoculated intradermally into the shaved backs of 2 normal recipient rabbits for each time schedule according to the following protocol:



Those sites which received the immune sera-treponeme mixtures incubated for 4 hours developed generally smaller lesions after a 2 to 4 day longer incubation period as compared to those sites injected with the non-immune sera treponeme suspension. Those sites which received immune sera-treponeme mixtures incubated for 16 hours failed to develop lesions during the 55-56 day observation period as compared to the non-immune-treponeme control sites, where lesion development occurred in 14-18 days.

Definitive experiments are now underway to 1) confirm the suggested role of humoral antibody utilizing this technique, 2) determine whether neutralization can be used to assess the "immune status" of the rabbit host during the development of resistance, and 3) determine the location of neutralizing antibody during the development of resistance. Fifty-one rabbits have been given an "immunizing infection" intratesticularly with T. pallidum, Nichols strain. The developing immune response has been determined by intradermal

challenge with 1000 homologous treponemes at 10 days, 1 month, 2 months, 4-1/2 months, 6 months and 8 months after infection. Sera have been obtained just prior to each challenge, pooled, and frozen. Quantitative neutralization tests will be carried out on each sequential set of pooled sera according to the method already described but utilizing only the 16-hour "in vitro" incubation period prior to injection of the serum-treponeme mixture into normal recipients. An attempt will then be made to correlate the neutralization titers with the immune status of the animals at the spaced intervals of time following infection. Quantitative neutralization tests will also be carried out on (1) the sera obtained from rabbits vaccinated with γ-irradiated, preserved treponemes and alginate-gluconate adjuvant in an attempt to correlate titers with the immune response to challenge and (2) the IgG and IgM fractions of the infected-immune and vaccinated-immune sera in an attempt to determine the immunoglobulin location of "neutralizing" antibody during the development and persistance of resistance. Appropriate controls will be included during each experiment.

b. Passive Protection

Further evidence for a humoral antibody mechanism has come from both preliminary and definitive (in progress) passive protection experiments. As noted in Annual Report No. 1 and Renewal Application 4/10/73, based upon the presumption that previous failure may have been due to the relatively rapid disappearance of antibody from the rabbit host following a single injection of immune serum or globulin, a pilot study was designed in which 2 normal rabbits were injected intravenously with 5 ml of immune serum, challenged intradermally at 4 sites with 1000 T. pallidum, Nichols strain, 2 hours later, then injected daily with 4.5 to 5 ml of immune serum for a total of 25 days. As controls, 2 additional rabbits were similarly injected with 5 ml of normal (non-immune) serum and 2 rabbits with 5 ml of saline. The 2 saline-injected animals developed typical lesions in 15 and 18 days, while the 2 non-immune-injected rabbits developed typical lesions in 13 and 17 days after challenge. In contrast, one of the animals that received immune serum developed atypical, flat, erythematous lesions (characteristic of partial resistance) 12 days after challenge with

complete healing occurring at day 40. The second rabbit recipient of immune serum failed to develop lesions for 35 days after challenge, thereby providing additional evidence for a humoral antibody mechanism. The delay in the incubation period could signify the presence of insufficient amount of antibody necessary to destroy all treponemes in the challenge inoculum. However, it was interesting to note that typical lesions developed in this animal 10 days after the cessation of immun serum injections, suggesting the rather interesting hypothesis that the challenge treponemes may have taken up an intracellular residence upon introduction into the host, with continual humoral suppression by antibody occurring until the discontinuance of immune serum injections. Evidence of protection was again noted in an additional preliminary experiment in which 2 rabbits that had received daily intravenous injections of 5 ml of immune serum and were challenged intradermally with 2000 T. pallidum at each of 4 sites 2 hours after the initial injection, developed atypical, flat, erythematous lesions, while 2 similarly injected and challenged developed small, flat lesions compared to the typical lesions which occurred in the non-immune serum-and saline-injected controls. Further, the "test animal" lesions remained as described up to the 75 days of observation in contrast to the progressive development of indurated and ulcerative lesions in the control animals. An attempt to obtain a similar protective effect was unsuccessful with 5 ml of immune serum administered intravenously every 3 days or every 5 days as well as with a single intraperitoneal injection of either 10 ml or 40 ml of immune serum.

Thus, the evidence appeared to indicate that passive protection could be convincingly demonstrated only by continuous daily intravenous injections of immune serum utilizing up to twice that amount employed in the previous experiments. A definitive experiment now in progress was designed in which each of 5 serologically non-reactive, normal rabbits were inoculated intravenously with 3 ml per Kg body weight (~10ml) pooled immune sera freshly obtained from rabbits with a T. pallidum infection of 13 months duration; the pooled sera were injected at 4-hours prior to intradermal challenge with 1000 T. pallidum at each of 4 sites, and daily thereafter for 37 days. At the same time, fresh normal rabbit serum and saline were injected into

each of 5 rabbits under the same conditions and for the same length of time. The protection afforded by the immune sera was unequivocal. While typical lesions developed in the normal serum and saline recipients in 10 to 14 days, each of the 5 animals receiving immune sera developed only a faint erythema at 1 to 4 sites in 13 to 54 days (average 35) after challenge and from 1 day before to 40 days after the appearance of the last lesion in the control animal; further, the redness disappeared in 6 to 26 days after its appearance from all but the 2 animals in whom the erythema developed 50 to 54 days after challenge or 13 to 17 days after the last injection of immune sera. (The latter have shown the appearance of induration at day 54.) Reappearance of the erythema occurred in 1 animal 13 days after the final immune sera administration and has progressed to an irregular induration up to the present time (54 days). The results would seem to suggest that the use of twice the amount of immune sera, compared to the preliminary experiments, produced an initial and continued antibody level which allowed a greater number of treponemes to be destroyed, thereby eliciting a clearer demonstration of protection. The re-appearance of erythema in 1 animal 13 days following cessation of immune sera injections, together with the development of initial lesions in 2 animals 13 to 17 days after immune sera injections were terminated, suggests the possibility of an intracellular location for treponemes surviving in the challenge inoculum. Node and testes transfer of the "test" rabbits to normal recipients is planned after 3 months of observation in order to ascertain intracellular location and whether dissemination has occurred from the local sites of challenge. Separate experiments to determine the immunoglobulin location of "protective" antibody have also been planned.

2. Human Syphilis

Although the mechanism(s) whereby immunity develops in human syphilis is unknown, the recent success in elucidating a role for humoral antibody in experimental syphilis has prompted the application of the same neutralization and passive protection techniques to the human disease in an effort to reveal an operative antibody mechanism. Blood specimens from patients with primary, secondary, latent, and tertiary syphilis are being obtained from the Los Angeles County Health Department (Hollywood-Wilshire V.D. Clinic).

Serum is removed at the U.C.L.A. laboratory and frozen for future use in planned neutralization experiments analagous to those already described for the experimental syphilis system. Passive protection experiments with human syphilitic sera necessitate the use of recipient rabbits made tolerant at birth to human globulin. These studies are being planned.

B. The Role of Cell-Mediated Phenomena In Experimental and Human Syphilis

As indicated in Annual Report No. 1 and Renewal Application 4/18/73, the demonstration that cell-mediated phenomena may participate as a mediator of acquired resistance in experimental and/or human syphilis has been hampered for the most part by the lack of satisfactory methodology which would allow the performance of experiments from which definitive and unequivocal conclusions could be drawn. This laboratory is now concerned with the development and application of techniques which could be utilized in experiments relating to (a) the direct cytotoxic action of "immune lymphocytes" upon T. pallidum and (b) alteration of the immune response with goat anti-rabbit thymocyte globulin.

1. Direct Cytotoxicity of "Immune Lymphocytes" Upon T. Pallidum, Nichols Strain

Determination of the cytotoxic effect of immune lymphocytes upon T. pallidum is predicated upon the preparation of pure lymphocytes which will remain viable during their interaction with suspensions of virulent and motile T. pallidum without exerting a non-specific harmful effect upon the treponemes. Conversely, the treponeme suspension must allow the survival of lymphocytes during the interacting period. Previous experiments have been based upon the hypothesis that an atmosphere of 95% N_2 - 5% CO_2 is essential for these experiments, inasmuch as this environment is necessary for T. pallidum survival for 40 or more hours. However, recent findings by Fathlev at the State Serum Institute in Copenhagen and in this laboratory have suggested that the use of McCoy's 5a medium allows both lymphocytes (as measured by trypan blue exclusion) and treponemes to survive under aerobic conditions of incubation at 35°C for approximately 40 hours.

In preliminary experiments, relatively pure lymphocytes were prepared from 12 patients with latent syphilis, 3 patients with secondary syphilis, and normal control donors as follows:

a. Heparinized blood was layered onto a 9% Ficoll-hypaque gradient and centrifuged at 400 X g for 20 minutes.

b. The lymphocytes separated at the interface were washed several times with

Hank's balanced solution. Each washing was followed by centrifugation at 400 X g for 10 minutes and resuspension of the pellet in the Hank's solution.

c. After the final washing, the lymphocytes were suspended in McCoy's 5a medium to give a concentration of approximately 10^6 lymphocytes per ml.

Treponemal suspensions in McCoy's 5a medium were adjusted to contain 1-2 organisms per field, combined with the lymphocyte preparations, and incubated aerobically at 35°C. Motility was determined by observing 25 to 50 treponemes under darkfield microscopy. After 5 to 10 hours of incubation, all treponemes in the presence of the "test" lymphocytes were rendered non-motile as compared to the 50% - 80% motility observed in the presence of "normal control" lymphocytes. These exciting results suggest that cell-mediated phenomena play a role in the human syphilis immune process and are being continued. Heparinized specimens from primary, secondary, latent, and tertiary patients are being collected from the L.A. County Health Department (Hollywood-Wilshire V.D. Clinic) for use in direct cytotoxicity studies designed to correlate the appearance and persistence of cell-mediated phenomena with the development of the human syphilitic immune process. Additionally, the methodology is being extended to the study of cell-mediated immunity in experimental syphilis. Rabbits solidly immune as a result of infection with T. pallidum, Nichols strain, will be tested for evidence of lymphocyte cytotoxicity. If successful, studies will be designed to determine whether the development and persistence of immunity can be correlated with the degree of lymphocyte cytotoxicity.

2. Alteration of Immunity and/or Enhancement of Susceptibility Experiments Utilizing Goat Anti-Rabbit Thymocyte Globulin (ATG)

As described in Renewal Application 4/18/73, rabbit thymuses have been collected, frozen and transported to Hyland Laboratories, where goat immunization was carried out. A total of approximately 550 ml has been provided for this study, which has already been designed and is underway. Fifty rabbits have been given an "immunizing infection" intratesticularly with T. pallidum, Nichols strain. At the end of 3 to 6 months, when a relatively solid immunity should be manifest, ATG, normal goat globulin (NGG) and saline will be administered intravenously in 0.6 ml/kg body weight amounts at days -7 (pre-challenge), -4, -1, 3(post-challenge) 7, 11, 15, and 19, as follows:

Immune Rabbits	20 - ATG 10 - NGG 20 - Saline
Non-Immune Rabbits	10 - ATG 10 - NGG 10 - Saline

At day -1, lymphocyte depletion or alteration will be determined on selected animals employing rabbit skin allografts. Ten rabbits in each group will be challenged intradermally at each of 4 sites with 1000 T. pallidum and examined for lesion development for 3 months. Additionally, each of 10 immune rabbits administered ATG and saline (controls) will not be challenged in an effort to ascertain whether ATG can cause the re-appearance of symptomatic disease (lesions) in animals with latent infection during a 3-month period. VDRL, FTA-ABS, and TPI determinations will be carried out on pre-and post-challenge blood samples.

III. THE "IN VITRO" CULTIVATION OF T. PALLIDUM IN TISSUE CULTURE MONOLAYERS (OBJECTIVE NO.3)

The inability to culture T. pallidum "in vitro", either in artificial medium or in tissue culture, has hampered and made complex those studies relating to the biology and immunology of treponemes and treponemal disease. The demonstration by Sykes and Miller (1971) that T. pallidum takes up an intracellular location within the cells of rabbit testes following inoculation suggested an approach to tissue culture based upon our ability to locate the treponemes within cells. In cooperation with Dr. Sykes at the California Hospital Medical Center, studies were initiated in an effort to explore the fate of T. pallidum in tissue culture monolayers and possible factors which influence survival and/or growth. Secondary monolayer cultures derived from uninoculated rabbit testes were established utilizing Eagle's MEM medium without antibiotics, Hepes, and either 30% heat-inactivated fetal calf serum or 50% heat-inactivated normal rabbit serum. In the initial experiments, 2.5 to 3 ml of a T. pallidum suspension prepared in the tissue culture medium and containing 10 to 40 actively motile organisms per high dry darkfield was added to the monolayer cultures. Incubation was carried out at 35°C. Examination of the tissue culture fluid showed decreases in the number of treponemes with time, suggesting that the organisms may have taken up an intracellular residence. Electron micrographs confirmed the hypothesis and demonstrated entry into the monolayer cells within 30 minutes post-inoculation.

The development of lesions following the intradermal infection of rabbits with washed monolayer cultures at periods of time after treponeme introduction provided clear evidence for the presence and persistence of virulent organisms within the cells for at least 24 hours. Studies to determine how long they survive under these conditions are continuing. In addition, the following experiments have been planned to elucidate those factors which influence survival and/or growth.

1. The addition of superoxide dismutase (from E. coli) to the monolayers in and effort to neutralize possible oxygen toxicity for treponemes (McCord, Keele, and Fridovich, 1971).

2. The use of chambers separated by membrane filters, utilizing superoxide dismutase, reducing agents, or anti-oxidants on one side and the tissue culture monolayer on the other.

3. Pulse chasing the inoculated monolayers with short bursts of 95% N_2 - 5% CO_2.

4. Quantitation of the numbers of viable treponemes per individual cell at intervals of time after inoculation.

5. Passage of infected rabbit testes in monolayer to determine if the treponemes will reproduce within the new cells.

RESEARCH GOALS (JANUARY 1, 1974 - DECEMBER 31, 1974)

I. VACCINE DEVELOPMENT

A. Preparation and storage, in liquid nitrogen, of freshly isolated T. pallidum Nichols strain, which has been γ-irradiated with a total dosage of 1,000,000 rads, in an effort to effect complete treponemal inactivation without the possibility of DNA repair during storage.

B. Initiation of experiments designed to determine the degree of homologous resistance and antibody response of rabbits immunized intramuscularly with 4 liquid nitrogen-preserved, γ-irradiated (1,000,000 rads) T. pallidum vaccine doses administered at 4 week intervals with alginate-gluconate adjuvant. Each rabbit will receive $4X10^9$ treponemes per dose for a total of $16X10^9$ organisms.

C. Continuation of purification studies employing both multiple centrifugation and discontinuous Ficoll density gradient techniques as described in Background and Research Accomplishments. Suspensions will be analyzed for motility as well as for the presence of tissue components by darkfield and electron microscopy. Portions of each "clean" suspension will be lysed ultrasonically and tested for tissue contamination by gel diffusion utilizing antiserum prepared against rabbit testicular homogenate. Serological analysis will be carried out by (1) quantitative FTA-ABS tests utilizing "clean" vs standard, non-purified treponemes as antigen, and (2) qualitative C'F tests utilizing soluble ultrasonic lysates of "clean" vs standard, non-purified treponemes as antigen.

"Clean" as well as freshly extracted, non-purified suspensions which have been adjusted to contain identical numbers of organisms will be injected intratesticularly into normal rabbits in an attempt to detect differences in virulence. If no appreciable differences are noted in antigenicity and virulence of suspensions which have satisfied the criteria of purity, experiments will be initiated toward elucidating their efficacy as a source of γ-irradiated vaccine.

II. MECHANISM(S) OF THE IMMUNE RESPONSE IN EXPERIMENTAL AND HUMAN SYPHILIS

A. Completion of in-depth neutralization and passive protection experiments (described under Background and Research Accomplishments) designed to confirm preliminary findings which suggest a role for humoral antibody in the experimental immune response.

B. Initiation of in-depth neutralization and passive protection experiments similar to those referred to in A in an effort to demonstrate a role for humoral antibody in the human immune response.

C. Continuation of direct cytotoxicity studies (described under Background and Research Accomplishments) designed to determine whether cellular mechanisms are operative in experimental and/or human syphilis.

D. Continuation of studies (described under Background and Research Accomplishments) designed to alter the immune response and/or enhance susceptibility in experimental syphilis utilizing goat anti-rabbit thymocyte globulin.

III. "IN VITRO" CULTIVATION OF T. PALLIDUM IN TISSUE CULTURE MONOLAYERS

Continued efforts to explore the fate of T. pallidum in tissue culture monolayers and to elucidate those factors which influence survival and/or growth utilizing the approaches described under Background and Research Accomplishments.

REFERENCES

The following have been used as references under Background and Research Accomplishments in this report:

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3. Miller, J.N., Bekker, J.H., DeBruijn, J.H. and Onvlee, F C. "Antigenic Structure of Treponema pallidum, Nichols strain. II. Extraction of a Polysaccharide Antigen with 'Strain-Specific' Serological Activity." J. Bact. 99: 132-135, 1969.

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5. Sharpiro, A., Modai, Y., and Kohn, A. "Efficacies of Vaccines Containing Alginate Adjuvant," J. App. Bact., 30: 304-311, 1967.

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8. Turner, T.B., and Hollander, D.H. "Biology of the Treponematoses," WHO Monograph Series No. 35, 1957.

No ONR-sponsored research has been published as yet. However, it is anticipated that at least 2 papers will be published during 1974.