STUDIES ON IMMUNITY IN EXPERIMENTAL SYPHILIS*

I. IMMUNOLOGIC RESPONSE OF RABBITS IMMUNIZED WITH REITER PROTEIN ANTIGEN AND CHALLENGED WITH VIRULENT *TREPONEMA PALLIDUM*†

BY

JAMES N. MILLER, SUKOO JACK WHANG, AND FRANK P. FAZZAN

From the Treponemal Research Laboratory, Department of Medical Microbiology and Immunology, School of Medicine, University of California, Los Angeles.

Numerous attempts to immunize rabbits against syphilitic infection with killed, virulent *Treponema pallidum* have proven unsuccessful. Consistent failure has resulted from the use of virulent spirochaetes inactivated by heat (Eagle and Fleischman, 1948; McLeod and Magnuson, 1953), by Mapharsen (McLeod and Magnuson, 1953), and by lyophilization (Magnuson, Halbert, and Rosenau, 1947; Waring and Fleming, 1951). More recently, McLeod (1962) has shown that a protein-like antigen derived from virulent *T. pallidum* (TPCF antigen) is ineffective as a vaccine.

Many investigators have reported the failure of non-pathogenic treponemes to confer protection against experimental luetic infection (Kolmer, Kast, and Lynch, 1941; Eagle and Germuth, 1948; Khan, Nelson, and Turner, 1951). However, Gelperin (1951) found that active immunization of rabbits with the avirulent Reiter strain of *T. pallidum* resulted in an extension of the incubation period and a reduction in the size of the lesions compared with control animals after intradermal challenge with 200 *T. pallidum*. This degree of relative immunity suggested that a serologically-active protein (RP) antigen extracted from the Reiter strain (D'Alessandro and Dardanoni, 1953) and antigenically related to a protein fraction of virulent *T. pallidum* (Dardanoni and Censuales, 1957; Cannefax and Garson, 1959) might be capable of protecting rabbits.

The present study was designed to determine the clinical and serological response of rabbits immunized with RP antigen and challenged intratesticularly with virulent *T. pallidum*. Further attempts were made to relate the Reiter protein complement-fixation (RPCF) antibody response following immu-

nization both to the results of challenge and to various antibodies directed against non-treponemal and treponemal antigens employed in this study.

Material

Forty normal, adult, Dutch male rabbits with non-reactive *Treponema pallidum* immobilization (TPI), RPCF, and VDRL tests were selected for the study. The animals were housed in individual cages at an environmental temperature of 68-70° F.

Method

Each of twenty rabbits was immunized over a 5-week period with a total of 3.25 ml. sterile, serologically-active, RP antigen*† prepared according to the method of D'Alessandro and Dardanoni (1953) (Table I).

*6.7 mg. total protein.

†This antigen was kindly supplied by the Sylvania Chemical Company.

TABLE I
SCHEDULE FOR IMMUNIZATION OF RABBITS WITH REITER PROTEIN ANTIGEN

Week	Amount of Antigen* (ml.)	Route of Injection
1	0.1 0.1 0.1	Subcutaneous Intravenous Intravenous
2	0.15 0.15 0.15	Subcutaneous Intravenous Intravenous
3	0.2 0.2 0.2	Subcutaneous Intravenous Intravenous
4	0.3 0.3 0.3	Subcutaneous Intravenous Intravenous
5	0.5 0.5	Subcutaneous Intravenous
Total	3.25 (6.7 mg.)	

*Injections given on alternate days

*This study was supported, in part, by Contract No. Saph. 69590 from the U.S. Public Health Service.

†Presented in part, at the XI Annual Symposium on Recent Advances in the Study of Venereal Diseases, April 1960.

One animal died during the immunization from causes unknown.

The challenge inoculum was prepared as follows:

Ten normal, adult, Dutch male rabbits were infected intratesticularly with approximately 10^7 *T. pallidum*, Nichols strain; 12 days after inoculation, at a time when maximum syphilomata had developed, the testes were removed aseptically, minced with scissors, and extracted for 5 minutes at room temperature in normal rabbit serum diluted 50 per cent. with physiological saline. The suspension was then centrifuged for 10 minutes at 1,000 r.p.m. to remove gross tissue particles. The supernatant containing the treponemes was removed and adjusted with the serum-saline solution to contain 7.0×10^6 treponemes per ml. suspension, according to the quantitative method of Magnuson, Eagle, and Fleischman (1948). Of 200 treponemes examined in the residual suspension after inoculation, 198, or 99 per cent. were actively motile.

One week after completion of the immunization procedure, each of the twenty immunized rabbits was challenged intratesticularly with 2.1×10^6 *T. pallidum* per testis or a total of 4.2×10^6 treponemes; this is equivalent to four treponemes per high dry dark-field utilizing $15 \times$ oculars and a $40 \times$ objective. At the same time, twenty normal rabbits were injected intratesticularly with the same number of treponemes from the same suspension as a control on the degree of infectivity. The use of a smaller inoculum was considered unsatisfactory for intratesticular challenge because of the senior author's previous experience of inconsistent syphiloma formation (unpublished data). Further, the intratesticular route was chosen rather than a series of intradermal injections in an effort to demonstrate relative immunity in the form of asymptomatic infection, if present.

Both groups of animals were observed daily for 12-weeks after the injection for the appearance of dark-field positive lesions. Animals developing such lesions were considered to have symptomatic infection. Rabbits failing to develop lesions at the end of this time were killed, and the testes and popliteal lymph nodes removed. Following trituration, as previously described, in separate 1 ml. amounts of 50 per cent. normal rabbit serum and saline, the testicular suspension was injected into the right testis, and the popliteal lymph-node suspension into the left testis of a normal rabbit. Animals in which the injected testes and/or popliteal lymph nodes developed dark-field positive lesions during a 12-week observation period were considered to have had asymptomatic infection. Rabbits in which the testes and nodes were not infective were considered to have been immune to the challenge.

Blood samples for serological testing were obtained by alternately bleeding one-half of the rabbits in the immunized group *via* cardiac puncture at 2-week intervals during the immunization period and after the challenge; all animals were bled just before the challenge. The same procedure was followed in obtaining blood samples from the control group. Following removal of the serum, the samples were stored at -20°C . until tested.

The VDRL slide flocculation tests were performed as described in the "Manual of Serologic Tests for Syphilis" (1959). The TPI test was carried out according to the method of Nelson and Mayer (1949) with modifications as described by Magnuson and Thompson (1949), Thompson and Magnuson (1951), and Boak and Miller (1954). The RPCF and *Treponema pallidum* complement-fixation (TPCF) tests were performed according to the method described by Portnoy and Magnuson (1955) for the TPCF test, using the 1/5 volume Kolmer technique.*

Results

Clinical Response

Eighteen of the nineteen rabbits immunized with RP antigen developed dark-field positive lesions upon challenge and only one was considered immune. Of the twenty normal rabbits infected intratesticularly with the same suspension of *T. pallidum*, nineteen developed symptomatic and one asymptomatic infection. No significant difference between the immunized and control rabbits was observed in the size of the lesions. Furthermore, the average time for the development of dark-field positive lesions after infection was 24.3 days for the immunized group and 24.6 days for the control group (Table II). Thus, under the conditions of the experiment, immunization with RP antigen conferred no significant immunity as measured by the development, size, and time of appearance of dark-field positive lesions when compared to the non-immunized control group.

TABLE II

CLINICAL RESPONSE OF REITER PROTEIN ANTIGEN-IMMUNIZED AND CONTROL RABBITS TO CHALLENGE WITH 4.2×10^6 VIRULENT *T. PALLIDUM*.

Classification of Rabbits	Total Number of Rabbits	Clinical Response to Challenge			Average Time for Development of Lesions* (days)
		Symp-tomatic	Asymp-tomatic	Immune	
Immunized	19	18	0	1	24.3
Control	20	20	1	0	24.6

* No significant difference in the size of lesions among the immunized and control rabbits.

Serological Response

2 weeks after the immunization procedure was initiated, an average RPCF antibody titre of 1:448 was obtained for the immunized rabbits. The average titre reached a maximum of 1:1280 during the fourth week, declining slightly to 1:885 on the day of

*TPCF antigen was kindly supplied by Cappel Laboratories.

challenge. Four weeks after challenge, an average peak titre of 1:2240 consistent with an anamnestic response was reached which steadily declined to 1:500 8 weeks later.

The control rabbits developed a maximum titre 6 weeks after infection of 1:425, declined slightly during the next 2 weeks to 1:205, and remained at this approximate level for the next 4 weeks (Figure).

FIGURE.—RPCF antibody titres of immunized and control rabbits.

Both TPI antibody and reagin failed to develop during immunization of the animals with RP antigen.

Qualitative TPCF tests were carried out on the basic sera which were obtained from the rabbits before starting this study and had exhibited non-reactive VDRL, RPCF, and TPI tests. It was of interest to note that 38 of 39 basal sera showed reactivity ranging from 1+ to 4+ with TPCF antigen; the serum exhibiting the non-reactive result was obtained from a control rabbit. Subsequent quantitative TPCF testing of sera from two immunized animals failed to show the occurrence of a significant anamnestic response during the immunization period, thereby suggesting the failure of serologically active RP to stimulate TPCF antibody.

Relationship of RPCF Antibody to the Clinical Response

As shown in Table I, 4 weeks after the RP antigen-immunized rabbits were challenged, at a time approximating to that of the development of symptomatic infection, an average peak titre of 1:2240 consistent with an anamnestic response was observed. Previous studies by Magnuson and Rosenau (1948) indicated that rabbits infected with *T. pallidum* develop a high degree of immunity to challenge 12 weeks later. Thus, the RPCF antibody levels obtained on the sera from immunized animals during the course of this investigation were considered to be of sufficiently high titre to determine its relationship to immunity. The failure of RPCF antibody to confer protection against the challenge inoculum is apparent.

Discussion

The inability of RP antigen to confer protection against challenge with 4.2×10^6 *T. pallidum* does not imply absence of acquired resistance to a smaller inoculum. However, the results strongly suggest that serologically-active RP antigen would not be suitable for use as a vaccine in human or experimental syphilis acquired under the usual conditions of transmission. The failure of protein-extracted virulent and avirulent *T. pallidum* to produce acquired immunity suggests that antigenic cell components are incapable of affording protection *per se*. Moreover the unsuccessful use of killed, virulent *T. pallidum* may be attributed to an alteration in antigenicity caused by artificial inactivation. In the light of our present knowledge, it would appear that the effectiveness of an immunizing agent is dependent upon the use of virulent treponemes rendered avirulent but retaining intact and complete antigenicity.

Our findings that neither reagin, TPI, nor TPCF antibody develop after immunization with RP antigen agree with those of other investigators (Portella, 1957; Dardanoni and Censuales, 1957; Cannefax and Garson, 1959). These data not only imply the dissimilarity of these antibodies from RPCF antibody, but also suggest that the "ubiquitous" lipid in RP antigen described by De Bruijn (1958) is present in amounts insufficient to produce a reagin response. When considered together with the failure of whole Reiter organisms to stimulate TPI antibody, the results also imply that TPI antibody, in the strictly immunological sense, may be regarded as an antibody to virulent treponemata only.

The presence of TPCF antibody in 98 per cent. of the "normal" rabbit sera tested is consistent with the observation that many syphilitic antigens react

with animal sera. Over eighty rabbits were screened by the RPCF procedure until forty exhibiting non-reactivity were found; similar results were reported by Wheeler (1960). The mechanism whereby these antigens react with components of animal sera is poorly understood.

Summary

- (1) The immunization of rabbits with Reiter protein antigen failed to confer significant protection against "challenge" with 4.2×10^6 virulent *T. pallidum*, as measured by the development, size, and time of appearance of dark-field positive lesions.
- (2) The immunization of rabbits with Reiter protein antigen failed to produce either reagin, TPI, or TPCF antibody.
- (3) The presence of RPCF antibody after RP antigen immunization, did not protect rabbits against challenge.
- (4) Of the 39 "normal" rabbit sera with non-reactive VDRL, RPCF, and TPI tests utilized in this study, 38 (98 per cent.) exhibited TPCF reactivity ranging from 1+ to 4+.

REFERENCES

- Boak, R. A., and Miller, J. N. (1954). *Amer. J. Syph.*, **38**, 429.
- Cannefax, G. R., and Garson, W. (1959). *J. Immunol.*, **82**, 198.
- D'Alessandro, G., and Dardanoni, L. (1953). *Amer. J. Syph.*, **37**, 137.
- Dardanoni, L., and Censuales, S. (1957). *Riv. Ist. sieroter. Ital.*, **32**, 489.
- de Bruijn, J. H. (1958). *Antonie van Leeuwenhoek*, **24**, 253.
- Eagle, H., and Fleischman, R. (1948). *J. exp. Med.*, **87**, 369.
- and Germuth, F. G., Jr. (1948). *J. Immunol.*, **60**, 223.
- Gelperin, A., (1951). *Amer. J. Syph.*, **35**, 1.
- Khan, A. S., Nelson, R. A., Jr., and Turner, T. B. (1951). *Amer. J. Hyg.*, **53**, 296.
- Kolmer, J. A., Kast, C. C., and Lynch, E. R. (1941). *Amer. J. Syph.*, **25**, 412.
- Magnuson, H. J., Eagle, H., and Fleischman, R. (1948). *Ibid.*, **32**, 1.
- , Halbert, S. P., and Rosenau, B. J. (1947). *J. vener. Dis. Inform.*, **28**, 267.
- and Rosenau, B. J. (1948). *Amer. J. Syph.*, **32**, 418.
- and Thompson, F. A. (1949). *J. vener. Dis. Inform.*, **30**, 309.
- Manual of Serologic Tests for Syphilis. (1959). Public Health Service Publication No. 411, U.S. Govt. Printing Office, Wash., D.C.
- McLeod, C. P. (1962). *Publ. Hlth Rep.*, **77**, 431.
- and Magnuson, H. J. (1953). *Amer. J. Syph.*, **37**, 9.
- Nelson, R. A. and Mayer, M. M. (1949). *J. exp. Med.*, **89**, 369.
- Portella, O. B. (1957). In "Annual Report of Div. of Laboratories and Research", p.65, State Dept. of Health, Albany, N.Y.
- Portnoy, J., and Magnuson, H. J. (1955). *J. Immunol.*, **75**, 348.
- Thompson, F. A., and Magnuson, H. J. (1951). *Amer. J. Syph.*, **35**, 21.
- Waring, G. W., Jr., and Fleming, W. L. (1951). *Ibid.*, **35**, 568.
- Wheeler, A. H. (1960). Presented at XI Annual Symposium on Recent Advances in the Study of Venereal Diseases, Chicago, Ill.

Immunologie de la syphilis expérimentale

I. Réponse immunologique du lapin injecté par l'antigène protéique de Reiter à *T. pallidum* virulent.

RÉSUMÉ

- (1) L'immunisation par l'antigène protéique de Reiter ne protégea pas contre l'attaque du *T. pallidum* (4.2×10^6) des lapins dont on observa le développement, la grandeur, et le temps d'apparition des lésions positives à champ obscur.
- (2) L'immunisation par l'antigène protéique de Reiter ne produisit ni réagine, ni anticorps T.P.I., ni anticorps T.P.C.F.
- (3) L'anticorps R.P.C.F. (fixant le complément contre la protéine de Reiter) après immunisation par l'antigène protéique de Reiter ne protégea pas ces lapins contre l'attaque.
- (4) Sur 39 sérums "normaux" de lapin qui donnèrent des résultats négatifs aux tests V.D.R.L., R.P.C.F., et T.P.I., 38 (98%) montrèrent une réactivité de 1+ à 4+ contre le T.P.C.F.