

SELECTED ASPECTS OF SYPHILIS AND GONORRHOEA RESEARCH IN THE UNITED STATES, 1967*†

BY

LESLIE C. NORINS

*Venereal Disease Research Laboratory, Venereal Disease Program, National Communicable Disease Center,
Bureau of Disease Prevention and Environmental Control, Public Health Service,
U.S. Department of Health, Education, and Welfare
Atlanta, Georgia 30333,
U.S.A.*

In recent years programmes concerned with the control of syphilis and gonorrhoea have depended largely on the use of penicillin and on epidemiological methods, but many venereologists and other physicians concerned with public health problems have indicated the need for more laboratory research to provide additional means of controlling and eventually eradicating these diseases. It has therefore seemed timely to take stock of relevant research programmes being conducted in the United States, selecting for discussion those in which progress is already being made or is particularly desirable. It is hoped that this composite description of various researches, some of which are still preliminary, will stimulate investigators with similar interests to contact each other, and will contribute to international research awareness and co-operation.

I. Syphilis Research

Most people are aware of the decline of syphilis after the widespread introduction of penicillin therapy, and of the subsequent resurgence of the disease during a period of public indifference. However, it is not so widely known that the rising incidence of syphilis has not brought about a corresponding increase of research interest in the disease.

Three of the major ways in which present research might contribute to the control and eradication of syphilis are the development of a vaccine, the improvement of serological tests, and the development of a skin test.

(A) Vaccine

Success in the elaboration of a syphilis vaccine has eluded competent workers for several decades (Turner and Hollander, 1957). Nevertheless, the

problem is at present being re-examined in several laboratories. Miller, in Los Angeles, is utilizing gamma-irradiated *Treponema pallidum* organisms as a type of "attenuated" immunizing agent. His early studies, partly in collaboration with Bekker and de Bruijn in Utrecht, showed that the injection of such organisms into rabbits could stimulate the production of *T. pallidum* immobilizing antibodies without producing overt lesions at the site of inoculation. There had been earlier reports that, of all the antibodies measured in the usual serological tests for syphilis, the treponemal-immobilizing antibodies were possibly the best correlated with immunity.

In his more recent experiments, Miller "vaccinated" rabbits with intracutaneous injections of irradiated but motile *T. pallidum*. Although the results of the subsequent challenge are not clear-cut, Miller feels that the data are encouraging.

The gamma-irradiated treponemes have been found to cause cardiolipin-reagin reactivity in the rabbits vaccinated with them, and a few comments regarding sero-reactivity and vaccines seem pertinent.

Because non-treponemal cardiolipin-reagin tests are so widely used as screening tests for the detection of syphilis, serious problems could arise if widespread use were made of a vaccine that triggered conversion to reactivity in such tests, especially if the reaction produced were more than transient. This problem might be circumvented by developing a more sophisticated serological test which could differentiate antibodies to a vaccine from those produced against an active infection. Alternatively, serial testing of sera might reveal stabilization or decline of the reactivity triggered by vaccine, in contrast to the rising titre indicating active infection. However, a rising titre might also result from a vaccine that was replicating and thereby increasing its antigenic mass. Naturally, the administration of

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a vaccine that caused sero-conversion would hamper clinical work and disturb epidemiological surveys that utilize the present tests on a single-specimen basis to ascertain the level of treponemal disease in a given population.

In Houston, Knox and Dacres have immunized rabbits with cultivable Nichols treponemes and bacterial adjuvants, and may have conferred at least some resistance to subsequent challenge. They feel that their method circumvents the problem of sero-conversion.

Another point to be considered is that, even if treponemal vaccines did not cause conversion of the reagin tests, they might stimulate the appearance of treponemal antibodies of the type detected in the *Treponema pallidum* immobilization (TPI) and fluorescent treponemal antibody-absorption (FTA-ABS) tests. In such circumstances, it would not be possible to use the TPI and FTA-ABS tests as diagnostic aids if a vaccinated person developed reagin test reactivity for reasons other than syphilis, *i.e.* a biological false positive reaction. Furthermore, the appearance of treponemal antibodies after vaccination would complicate screening the population with an automated test that was specific for treponemal antibodies; for example, an automated FTA-ABS test.

Another vaccine strategy that aims in a different way at the modification of *T. pallidum* is being conducted at the Venereal Disease Research Laboratory (VDRL) in Atlanta. Thomas, Russell, and others are chemically treating *T. pallidum* organisms harvested from rabbits in the hope of enhancing the immunogenicity of these organisms. In the initial series of experiments, the organisms have been coupled through methylation or diazotization with each other or with bovine serum albumin. At the present time, rabbits have been injected with these killed and modified *T. pallidum* organisms, the sero-conversion of these animals in both non-treponemal and treponemal tests is being assessed, and animals are being challenged by intracutaneous inoculation to find out whether the injection of these modified organisms has conferred any protection.

Early in the course of this work it became apparent that chemical modification and detailed studies of antigenic composition called for large numbers of purified *T. pallidum*. These organisms should be free of the rabbit testicular tissue proteins and debris that are usually present after conventional extraction from rabbit testicular syphilomata. Rathlev and Pfau (1965) have accomplished rather successful purification of small numbers of *T. pallidum* by using density gradient ultracentrifuga-

tion; however, with conventional rotors, it is not possible to use this method on large volumes of the crude material.

Accordingly, Russell and Thomas at the VDRL have undertaken collaborative studies with Anderson and Cline at the Oak Ridge National Laboratory in Tennessee to utilize the newer techniques and equipment of zonal ultracentrifugation to accomplish the large-scale purification of *T. pallidum* organisms. By these means they have thus far been able to process, in one batch, 13 litres of extract from rabbit testicular syphilomata, and from this to obtain fractions that are rich in *T. pallidum* organisms, but free, to the limits of present measurement, from rabbit testicular proteins and debris. Although these organisms were no longer infectious, they did retain both their antigenicity, as evidenced by their ability to react with syphilitic human and rabbit sera, and their immunogenicity, as evidenced by their ability to incite the formation of anti-treponemal antibodies when injected into rabbits. This zonal-ultracentrifugation technique will make it possible for the first time to obtain large quantities of purified *T. pallidum* organisms; fortunately, the process does not itself produce too deleterious an effect upon the treponemes. This ability to obtain large numbers of purified organisms will allow better defined studies on the antigenic composition of *T. pallidum*. It may also provide a plentiful source of purified antigen for serological tests using *T. pallidum*. Also, if rabbit-grown organisms are to be used as a human syphilis vaccine, it would appear advantageous to have such organisms free of rabbit testicular materials lest the vaccine trigger some kind of anti-testicular autoimmune process when it is injected into human subjects.

Another way of acquiring large numbers of *T. pallidum* organisms would be successful cultivation *in vitro*, a long-elusive goal. Knox, Dacres, Wende, and others, in Houston, are attempting to cultivate the organism in cell culture, and a few pharmaceutical manufacturers with vaccine experience have expressed interest in similar approaches. I think it fair to say that only a few of the newer cell lines that are available to-day have been examined in attempts to propagate *T. pallidum* in cell culture. Because anaerobiosis recurs as a persistent theme in reports of cultivation attempts in earlier decades, it will no doubt be well to include in the selection of cell lines various tumour cells or other cells that tend towards anaerobic conditions. However, this traditional emphasis on anaerobic conditions is made in the face of the observation that *T. pallidum* thrives in the rabbit testis, a richly oxygenated tissue. Hardy, in Baltimore, is continuing his studies

on the growth requirements of both *T. pallidum* and the cultivable treponemes.

In other experiments at the VDRL, Cannefax and Hanson have begun attempts to produce a hybrid treponeme that is both virulent and cultivable. The general approach has been to try to achieve genetic transformation of the virulent organisms to a cultivable state, or *vice versa*, through the use of nucleic acids from treponemes having the desired properties. Cannefax and associates have been utilizing an *in vivo* transformation system, modelled on an early report by Zaffiro (1962) that Reiter organisms persisted in the rabbit testes in the presence of *T. pallidum* infection. A genetic transformation *in vivo* between the cultivable Reiter treponemes and the virulent *T. pallidum* would make an interesting parallel to the classic demonstration of the transformation *in vivo* of pneumococci from avirulence to virulence. To date, none of these initial attempts at transformation have proved successful.

In other experiments aimed towards a vaccine, Knox and Dacres are attempting to immunize rabbits with the cultivable Nichols strain of treponeme, sometimes injected with adjuvants, especially a lipopolysaccharide from Gram-negative bacteria. Although they feel their preliminary results are encouraging, the situation is not yet clear-cut.

(B) Serological Tests

In the area of improving serological tests for syphilis (STS), we at the VDRL have three main goals: to find a test that will recognize syphilis very soon after invasion by spirochaetes; to differentiate early from late syphilis and treated from inadequately treated or untreated syphilis; and to automate serological tests for syphilis.

(i) Our main strategy for developing a serum test for incubating syphilis is based on the observation that in many experimental antigen-antibody systems there is a prompt recognition of foreign antigen by the body. Failure to detect this presumed recognition would then result from not using the correct antigen, or from using an assay system that is insensitive to the interaction of our antigen with the early antibody. Of course, one might always argue that there is no early recognition of *T. pallidum*, but this suggestion is not heuristically useful. Tringali (1965) in Italy and Julian and colleagues at the VDRL have found that, in experimental rabbit syphilis, the earliest non-treponemal test reactivity is localized to the IgM antibodies; later in the infection the activity includes or is restricted to the IgG antibodies. Unfortunately, both Tringali and Julian have found that serum

obtained from human subjects with primary syphilis does not show that the cardiolipin-reagin activity is confined to the IgM molecules; instead, it is present in both IgM and IgG. Thus, at least superficially, the human situation does not follow the simple rabbit model. However, it is necessary to keep in mind the fact that what is clinically "primary" or "early" syphilis may, immunologically, represent a host response that is already well advanced. To examine this point, one needs serum from patients at the very earliest time that the non-treponemal tests become reactive. This will present practical difficulties, since in many cases the patient may not yet have developed the clinical symptoms that will bring him to the attention of a physician.

However, Kuhn and colleagues may shed valuable light upon this point by their studies of experimental syphilis infection in chimpanzees maintained at the VDRL. Although larger subhuman primates were used for experimental syphilis by some of the very earliest investigators, it was not possible at that time to maintain them in a state of good health and under controlled conditions. Kuhn has demonstrated that the chimpanzee responds to experimental infection with *T. pallidum* in much the same way as does the human; early lesions develop, as do reactive non-treponemal and treponemal serological tests. Thus, sequential serum samples from the chimpanzees can be used to examine the very important interval between the time of infection and the development of reactivity in the present conventional STS. To try to show that reactivity is present in early serum samples that appear to lack reactivity in the current conventional tests, newer treponemal antigens are being used. These are being prepared from the larger supply of purified *T. pallidum* that is now available through the zonal-ultracentrifugation techniques described earlier.

An unexpected observation in the course of these studies was that a significant proportion of "normal" chimpanzees give reactive STS, both in the non-treponemal screening test and in the confirmatory treponemal tests, such as the TPI and FTA-ABS. The nature of the organism (if indeed there is an organism) that causes this serological reactivity, and the possible relevance of such an organism to a syphilis vaccine are subjects of considerable interest. Certainly it is relevant to inquire whether or not there is an endemic treponematoses, perhaps something like yaws, among chimpanzees in their native habitat.

(ii) The studies regarding the differentiation of adequately treated from untreated syphilis are still in the preliminary stages. In a collaborative study

with Atwood and Miller in New York, we have recently found that reactivity in the TPI and FTA-ABS tests persists for 13 years or longer in patients treated in the later stages of syphilis. Thus, it will not be possible to use either of these two treponemal tests, as performed at present, as an index of therapeutic response.

At this point a few remarks are appropriate on the use of immunofluorescent techniques as a means of identifying *T. pallidum* and other spiral organisms. The early interest in identification by immunofluorescent techniques was based on the value of the procedure for finding *T. pallidum* organisms in materials from clinical lesions [the fluorescent antibody darkfield (FADF), Kellogg and Deacon, 1965) and rapid immunofluorescence staining (RIS) procedures (Kellogg and Deacon, 1964)] and on its usefulness in identifying *T. pallidum* in tissues of experimentally infected animals (Yobs, Brown, and Hunter, 1964). The early applications of these techniques were fairly straightforward, but interest in them was considerably heightened after the stimulating reports by Collart (1964) that *T. pallidum* organisms might persist in rabbits and humans even after penicillin therapy of syphilis infection. More recently, wider interest in similar questions has been provoked by the reports of Smith and Israel (1967a, 1967b) and Goldman and Girard (1967) that spiral organisms, which are sometimes motile, can be demonstrated in the aqueous humour and cerebrospinal fluid of treated syphilitics. Both Smith and Goldman have strongly suggested that this spiral organism is *T. pallidum*.

For the investigation of these phenomena it seemed desirable to produce a fluorescein-conjugated anti-*T. pallidum* antiserum of rather well-defined properties and specificities. This project has recently been completed by Mothershed, who utilized sequential bleedings from syphilitic rabbits. After conjugation with fluorescein, the immune globulins from each bleeding were examined for reactivity against a variety of spiral organisms. After ascertaining which of the sequential bleedings contained the greatest reactivity against *T. pallidum* (in these experiments it was the 6-week bleeding), the fluorescein-conjugated immune globulins from that bleeding were made more specific for *T. pallidum* by a combination of various absorptions and dilutions. A detailed description of this conjugate and a standardized technique for its use are available from the VDRL. It is hoped that this conjugate will also be available commercially in the near future. For the present, we prefer to interpret with caution the results obtained through the use of this conjugate, since it is always possible that it

might stain some unusual but nonpathogenic spiral organisms with which it has not been specifically absorbed.

(iii) Another area of current research activity is the automation of serological tests for syphilis. This would offer several advantages in certain circumstances:

- (a) Help cope with the estimated 38 million STS being performed at present in the United States;
- (b) Help alleviate the shortage of trained serologists;
- (c) Encourage hospitals to re-institute routine STS on all patients;
- (d) Allow STS to be included in automated multiphasic screening programmes, including those for blood-typing;
- (e) Improve reproducibility through reducing variations inherent in manual tests.

Two of the various systems under development may be described as examples. An automated cardiolipin-reagin test was worked out in a collaborative study by McGrew at the VDRL and DuCros at the Technicon Instruments Corporation (Ardsley, N.Y.). The instrument is a Technicon AutoAnalyzer machine and the antigen is rapid plasma reagin (RPR) card test antigen obtained from Hyson, Westcott, and Dunning, Inc. (Baltimore, Md.). This antigen—a modified VDRL antigen suspension containing choline chloride—also contains specially-prepared carbon particles as a visualization agent. In brief, serum or plasma samples are tested at the rate of 100 per hour. Reactions are seen grossly as black agglutinates deposited on a moving white filter-paper strip. Although with a little experience a serologist can easily judge the degree of reactivity on the tape, an instrumented assessment, perhaps with a densitometer, will probably be a necessity for large-scale use of this test. Both in trials at the VDRL, and in the field, the results of the automated test so far approximately parallel the performance of the manual VDRL slide and RPR (Circle) card tests.

In other studies, the Space-General Corporation (El Monte, Calif.) has under development a system aimed towards the automated performance of the FTA-ABS test. If it were possible to automate a specific and sensitive treponemal test at a relatively low cost, then it might be possible to use it for screening purposes in preference to reagin tests and thus circumvent the problem of false positive reactors. VDRL staff members and Dr James N. Miller are among the serologists who have cooperated on various aspects of this system.

(C) Skin Tests

An immediate hypersensitivity skin test, even if it were presumptive and not definitive, would be very useful for certain purposes, such as screening in the clinic and in field studies. Ideally, such a test would be reactive even during the incubation stage of syphilis, so that it might also be useful in epidemiological investigations. Recent basic immunological studies have shown that there are two kinds of antibodies, gamma-1 and gamma-2, that can stimulate the skin for immediate hypersensitivity reactions. However, of these, only the gamma-1 antibodies can sensitize homologous skin, and yet these same gamma-1 antibodies do not fix complement in tests *in vitro* (Ovary, 1965). With respect to a syphilis skin test, this property of gamma-1 antibodies has the important implication that skin-test reactivity in syphilis need not correlate with the humoral reactivity demonstrated in the conventional tests *in vitro*. Thus, although the present serological tests do not become reactive until some time after infection, skin tests of the immediate hypersensitivity type may follow a different pattern. At the VDRL this new knowledge about skin-sensitizing antibodies is being applied in studies in guinea-pigs, rabbits, monkeys, and chimpanzees by Cohen, Bullard, Logan, and Kuhn. One important aspect is that emphasis is being given to assay in the homologous system (guinea-pig antibodies assayed in guinea-pigs; rabbit antibodies in rabbits; and human, chimpanzee, and monkey antibodies in chimpanzees and monkeys). At this time, research is centred on exploring various antigenic extracts of *T. pallidum* and other treponemes to discover promising skin-test materials.

II. Gonorrhoea Research

To the best of my knowledge, most of the basic research on gonorrhoea, except for antibiotic trials, which is being conducted in the United States is taking place at the VDRL. At the present time, our chief aim is the development of a serological screening test, especially one that will detect the asymptomatic female carrier. The use of the selective culture media developed by Thayer and Martin (1966) indicates that there are many such carriers, but the acquisition of specimens is probably too cumbersome for mass screening. However, approximately 38 million blood specimens are already being examined for syphilis each year in the United States, and virtually all of these could also be screened with a serological test for gonorrhoea with the expenditure of relatively little extra effort outside the laboratory. I look forward, in fact, to the use of an automated system that will divide a blood

sample into two aliquots, and perform on one of these a serum test for syphilis and on the other a serum test for gonorrhoea.

The serological response to the gonococcus is at present being investigated at the VDRL. Cohen and Norins (1966), Cohen (1967), and Cohen and others (in press) have found that humans possess "natural" antibodies to the gonococcus in all three major classes of immunoglobulins: IgG, IgM, and IgA. The "natural" IgG antibodies in the sera of normal subjects were more reactive with heat-stable than with heat-labile gonococcal antigens, contrasting with the IgG antibodies in the sera of infected persons, which were more reactive with heat-labile surface antigens. Moreover, the immune IgG antibodies were more resistant to heating than were the "natural" IgG antibodies. In other studies on experimental gonorrhoea in male volunteers (Cohen and others, in press), the sequential humoral response to gonococcal antigens was studied. A few patients showed an increase of IgA or IgM antibodies to heat-labile gonococcal antigens but nine of ten patients showed an increased IgG titre to them. However, seven of ten patients showed an increased titre of IgA antibodies to heat-stable gonococcal antigens, but there was no increase of IgG or IgM antibodies. It was also found that the IgG antibodies that were present 14 days after inoculation showed a greater heat stability than did the IgG antibodies present just before inoculation.

The organisms which were infectious in these studies had maintained their virulence during 35 months of selective passage *in vitro*. Experimental studies by Kellogg, Peacock, Deacon, Brown, and Pirkle (1963) had shown that there were four distinct types of colonial morphology among the gonococci, which could be maintained and carried forward in culture by selective passage. Two of these known as Type 1 and Type 2, were virulent for male volunteers, while the two others, Type 3 and Type 4, were avirulent. If cultures of gonococci were carried forward in the laboratory by random unselected passages, then the avirulent types of gonococci came to predominate. (Many "laboratory" strains which have been passaged randomly for years may now be comprised of the avirulent colonial types of gonococci.) The ability to recognize virulent gonococci has an important bearing on the development of antigens for a serological test.

At the present time experiments are in progress (partly in co-operation with Dr Edgar Ribí and associates at the Rocky Mountain Laboratory of the National Institutes of Health) to characterize cell wall and other antigens of the gonococcus. However, despite the fact that these organisms can infect

human subjects, it has not so far proved possible to produce experimental infection in chimpanzees maintained at this laboratory.

Discussion

The research aspects of syphilis and gonorrhoea that have been mentioned do not, of course, include all work going on at present in the United States. On the other hand, it is important to realize that in the United States there are only about five places where research on syphilis is being conducted; and apparently there are only one or two places where any volume of research concerns gonorrhoea. Moreover, in these relatively few places, I know of only one or two instances in which a younger investigator has recently entered the field on any kind of a long-term basis. I have the impression that the situation is not very different in other countries. This research situation stands out rather sharply against the magnitude of the problem. In the United States, for the fiscal year 1967, there were 120,000 reported cases of syphilis and 400,000 of gonorrhoea. If one takes into account the under-reporting of cases, the contrast is even greater.

Perhaps one way in which an increase in research may come about is through an increase in collaborative projects between those having the newest basic tools and approaches for research in infectious disease and immunology and those with experience and knowledge concerning *T. pallidum* and the gonococcus.

Certainly the relatively small amount of research taking place makes it even more important for various investigators to keep in touch, and emphasizes the continuing value of international communication in syphilis and gonorrhoea research.

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