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EDITORIALS

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THE DOUBLE-DIFFUSION AGAR GEL TESTS

In the early days of the study of serology at the turn of the century, leprosy was given more attention than was commensurate with its importance as a disease in Europe. The basic complement-fixation reaction of Bordet and Gengou (1901) was soon applied to leprosy by Eitner (1906), and mention of his reaction appears frequently in literature of that earlier period. The reason for special interest, however, was the fact that, because of the "panreactivity" of sera of lepromatous cases, leprosy was found to be the only nontreponematous disease that quite regularly gave false positive results with the complement-fixation test for syphilis of Wassermann, Neisser and Brück (1906), which observation led Wassermann to send Meier to Bergen to study the matter comparatively in Hansen's place.

In the years since then tremendous amounts of time and energy have been applied to work on the serology of leprosy, and yet the status of the matter is such that most of the available texts on leprosy have little or nothing to say about it. At first Chaussinand[1a] simply said that no serologic reaction has proved useful in the diagnosis of leprosy, but later[1b] he discussed the hemagglutination and conditioned

[1] CHAUSSINAND, R. La Lèpre. Paris: L'Expansion Scientifique Française (*a*) First edition, 1950, p. 130; (*b*) second edition, 1955, pp. 149-155.

hemolysis reactions as applied in leprosy. So, more briefly, did Carpenter and Naylor-Foote in Cochrane's new book.[2]

The situation is no better in tuberculosis with respect to a useful serologic test; although, since the tuberculin reaction can be diagnostic, the need is not so great. Of the methods which have been employed for the detection of antibody (say Parlett *et al.*[3]), including the agglutination, complement-fixation, precipitation, and hemagglutination tests, none has proved to be sufficiently reliable or valid for the serologic diagnosis of tuberculosis.

A new avenue of approach to the study of relationships of and cross reactions between bacteria was opened by the advent of an entirely new approach, the gel-diffusion precipitation technique. This method, devised by Oudin[4] in 1948, is a precipitin test in which the reacting antigen and antibody meet in an agar gel menstruum and produce visible bands or zones of precipitate. No attempt will be made the review the literature of work done by this method and its variants, only its application to the mycobacteria—first done by Parlett and Youmans in 1956.[5] The principal variations of technique will, however, be noted.

Oudin's[4] method was a tube one, in which the antibody was in the agar gel in the lower part of the tube; the antigen solution was layered over that; and, when the reactive elements of the antigen diffused into the antibody-agar column, bands of precipitate were produced there. Ouchterlony, also in 1948,[6] devised a petri-dish modification of the test in which antigen and antibody are placed in different neighboring depressions, or wells, cut or cast in a layer of neutral agar, to produce bands of precipitate where they meet in diffusing. This method is the one principally used.

Oakley and Fulthorpe[7] later reported an improved tube method. As applied by Seibert and Soto-Figueroa,[8] in a small tube lined with a thin layer of dried agar, the two columns of fluid reagents (serum below and antigen above) were separated by a 1 cm. column of neutral agar in which the reaction took place. Parlett and Youmans, who at first worked with a modified Ouchterlony plate technique,[5, 9] have recently been working

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[2]Cochrane, R. G., Ed. Leprosy in Theory and Practice. Bristol: John Wright & Sons, Ltd.; Baltimore: Williams & Wilkins Company; 1959, pp. 7-21.

[3]Parlett, R. C., Youmans, G. P., Rehr, C. and Lester, W. The detection of antibodies of tuberculous patients by an agar double-diffusion precipitation technique. American Rev. Tuberc. & Pulmon. Dis. **77** (1958) 462-472.

[4]Oudin, J. L'analyse immuno-chimique qualitative: Méthode par diffusion des antigènes au sein de l'immunsérum précipitant gélosé. Ann. Inst. Pasteur **75** (1948) 30-51; *also* Homogeneity of proteins and polysaccharides; agar diffusion techniques, *in* Methods of Medical Research, Vol. 5; Chicago, Year Book Publishers, Inc., 1952, pp. 360-375.

[5]Parlett, R. and Youmans, G. P. Antigenic relationships between mycobacteria, as determined by agar diffusion techniques. American Rev. Tuberc. & Pulmon. Dis. **73** (1956) 637-649.

[6]Ouchterlony, Ö. Antigen-antibody reactions in gels. Arkiv. Kemi, Minerol. Geol. (B) **26** (1948) 1-9; *also: Idem.* IV. Types of reactions in coordinated systems of diffusion. Acta path. et microbiol. Scandinavica **32** (1953) 231-240. [The former reference has been seen in only one place (Burrell and Rheins); the second reference is the one usually used.—Editor]

[7]Oakley, C. L. and Fulthorpe, A. J. Antigenic analysis by diffusion. J. Path. & Bact. **65** (1953) 49-60.

[8]Seibert, F. B. and Soto-Figueroa, E. Study of tuberculin protein and polysaccharide antigens by gel-diffusion technique. American Rev. Tuberc. & Pulmon. Dis. **75** (1957) 601-607.

[9]Parlett, R. C. and Youmans, G. P. Antigenic relationships between ninety-eight strains of mycobacteria using gel-diffusion precipitation techniques. American Rev. Tuberc. & Pulmon. Dis. **77** (1958) 450-461.

out a modified Oakley tube test.[3, 10, 11] They incorporate one of the reagents (e.g., the antigen) in agar in the lower part of the tube, layer over that a neutral agar reaction column, and fill the rest of the tube with the other reagent in fluid form. At first the neutral agar column was 3 cm. long,[3] then 0.75 cm.,[10] and now 0.5 cm.[7]

So far as we are aware, the agar diffusion method has been applied to leprosy material only three times, two of these times more or less incidentally, and not once by a leprologist.

Burrell and Rheins[12] investigated the antigenicity of lepromin, using the Ouchterlony method, and got some interesting results which should have been followed up long before now. For example, of 44 sera from nontuberculous Philippine schoolchildren 6-9 years old (children who give very high rates of lepromin positivity[13]) practically all (43, or 98%) showed agar positivity with lepromin, whereas of 44 comparable children in Columbus, Ohio, only 7 (or 16%) did so. There was also an apparent difference in the nature of the antibodies involved, because the reactivity of all the Philippine sera was blocked by OT, but not that of the Ohio sera.

Pepys *et al.*,[14] also using the Ouchterlony technique, tested many antigenic substances against a single selected antituberculosis rabbit serum. The antigens included three supposed lepromin-type preparations, none being of the regular Mitsuda-Hayashi kind. (See letter from Pepys in the *Correspondence* section of this issue.)

In their tests for tuberculosis antibodies in sera in the tube method, Parlett *et al.*[5] included 16 sera from the Carville leprosarium, 13 from patients and 3 normals. None gave an antibody reaction (i.e., for tuberculosis), and this result was considered as significant with respect to specificity of the reaction.

Are leprologists missing a bet—overlooking a line of research that might be very rewarding to follow up? Reviewing the situation as we have, the possibilities seem many, and exciting. Somewhere it has been said, expressing very well an unfortunate truth, that "because of the relative isolation of many of the leprosy investigators, many scientific techniques employed in the investigation of other diseases await application in leprosy." With respect to the present matter there are few leprosy workers in circumstances which would permit carrying on such work. Then there is the negative factor of discouragement and disinterest in the serology of leprosy as such (against which should be counterbalanced present interest in questions of the antigenicity of the leprosy bacillus); and also a negative factor of the apparent complexities of the generally-used Ouchterlony technique.

Parlett et al.[3] pointing out the need of a simple and reliable technique for the study of antibodies in tuberculosis, say that the

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[10]PARLETT, R. C. and REHR, C. A. Further studies on gel diffusion tests in tuberculosis. I. Methods for standardization of the antigen and detection of small amounts of antibody. American Rev. Resp. Dis. **80** (1959) 886-894.

[11]PARLETT, R. C. A modification of the Oakley tube method of the agar double-diffusion precipitation test for mycobacterial antibodies. Internat. J. Leprosy **28** (1960) 300-304.

[12]BURRELL, R. G. and RHEINS, M. S. Antigenic analysis of lepromin by agar-diffusion. Internat. J. Leprosy **25** (1957) 223-229.

[13]GUINTO, R. S. and WADE, H. W. Results of tests with serial dilutions of lepromin in separate groups of normal young children; with a comparison of two lepromins and the Dharmendra antigen. Internat. J. Leprosy **26** (1958) 328-345; Trans. VIIth Internat. Congr. Leprol., Tokyo, 1958; Tokyo, 1959, pp. 193-206.

[14]PEPYS, J., AUGUSTIN, R. and PATERSON, A. B. Common antigenic components of mycobacterial extracts. Tubercle (London) **40** (1959) 163-172.

tube method has those qualifications, and is readily carried out in hospital laboratories possessing a minimum of equipment. It is sensitive, and the results are free from nonspecific reactions so common in the complement-fixation and hemagglutinin tests; and it may have specificity.

Because of the possible usefulness of this method in studying the antigens and sera of leprosy there is presented in this issue, by special arrangement, a technical note by Parlett[11] based on a protocol of the most recent refinement of the method and including discussion of some of the points which may be of concern to workers who are not located in metropolitan centers. Doctor Parlett has kindly agreed to help with advice anyone in other countries who might find difficulties in undertaking such tests. Special attention to that article is invited.

It will be noted that almost nothing is said in it about the nature or preparation of antigens that might be used, nor is it indicated—as it is in one article[10]—that if called for by the experiment the set-up may be reversed, i.e., that a standard serum (antibody) may be used in the first lowermost agar column and varieties of antigen solutions in the third column. Much about the possibilities can be learned from the articles referred to.

Being concerned with leprosy, let us consider lepromin, which Burrell and Rheins used. First, to see if any results at all would be obtained with sera of patients, or contacts, or others, the whole suspension would be used, an aliquot of it being mixed with an equal quantity of the agar solution. (One advantage with lepromin is that it could if necessary be made in a greater concentration than the usual 1/20 or 1/30—e.g., 1/5 or 1/10.) Then, if positive reactions should be obtained, one would centrifuge the whole lepromin and filter the supernatant to see how much of the activity depended upon the dissolved elements; and, for comparison, the centrifuged deposit would be suspended in agar, perhaps after washing. A comparison of Dharmendra's antigen with lepromin would of course be in order. One might wish to break down the bacilli in the lepromin suspension, by ultrasonics or even prolonged grinding, to compare with the regular lepromin suspension or its supernatant. A comparison of a suitable preparation with Olmos Castro's leprolin would come in here.

In certain work with other mycobacteria the antigens used were culture filtrates,[5] but the living bacilli themselves have also been used.[9] For the latter effect a suitable fresh leproma could be ground up aseptically with sand in plain saline and filtered through nylon, one part to be used as "live" antigen and the rest to be autoclaved to serve as a fresh lepromin-like suspension for comparison.

What difference is there with respect to antigenicity between two lots of lepromin, one autoclaved and the other sterilized by boiling? Does any change occur on standing, i.e., does any difference develop

between a freshly-made lepromin and the same suspension six months or a year later?

Some of the exploratory work of this sort would be aimed to determination of what form of the antigen would be suitable for routine in clinical work. That decided, it would not take a highly developed imagination to set up an active program with leprosy cases, comparing the reactivity of the sera of the different types, forms, and stages, including reactional, and of contacts; and also of normal people of various ages. We say nothing of the problem of serologic relationship of leprosy to tuberculosis. Being interested in the problems of frequent low-grade reactivity to tuberculin in such regions as the Philippines, and the possibility that that condition may have some relationship to the almost universal lepromin positivity of young schoolchildren in the Philippines, we do, however, point out that the observations of Burrell and Rheins already mentioned suggest another line of investigation—perhaps with, besides lepromin, mycobacterial antigens of various other types.—H. W. WADE