Complement Fixation with Leptospiral Antigens. (20708)

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Although there have been indications for many years that a complement fixation test may be a practical procedure in the diagnosis of leptospirosis*(1-5), it has not received general acceptance. Current interest in complement fixation in this country was first stimulated by the report of Randall *et al.*(6) which described the preparation of an antigen by means of sonic disintegration of washed leptospirae. Such antigens have been shown to have diagnostic value but tend to cross react with sera for heterologous types(7,8). The supernatant fluid of leptospiral cultures was later shown to contain a relatively type-specific soluble antigen that fixed complement in the presence of hyperimmune rabbit sera(9). More recently Schneider(10,11) has obtained by chemical fractionation of washed leptospirae a complement fixing antigen which shows a wide range of cross reaction with heterologous leptospiral antisera. In a complement fixation test for leptospirosis in cattle York(12) has used an antigen consisting of a heavy suspension of *Leptospira pomona* cultivated in chick embryos.

In the present investigation we have attempted to compare the antigenicity of several leptospiral preparations as determined by complement fixation tests with sera of immunized rabbits.

Materials and methods. Stock cultures of leptospirae† were maintained in Korthof's medium(13) at room temperature with transfers every 2 to 3 weeks. In some instances the leptospiral concentration in cultures and allantoic fluid was determined by direct microscopic count using a Petroff-Hauser counting chamber. Rabbits were immunized with live cultures giving intravenous injections at 5-day intervals starting with a dose of 1 ml and increasing to 5 ml for the last injection. *The agglutination-lysis test* was performed by adding 0.1 ml of culture to 0.1 ml of each serum dilution. Serial doubled dilutions of serum were made in saline. If necessary, cultures were diluted with Korthof's medium to contain about 50 organisms per high dry darkfield. After incubation of the mixtures for 2 hours at 37°C they were examined under the darkfield microscope. The highest dilution of serum showing definite clumping was recorded as the titer. *Complement fixing* activity of antigens was determined by combining 0.2 ml of antigen dilution, 0.2 ml of a 1:20 dilution of immune serum inactivated at 62°C for 40 minutes and 0.2 ml of fresh guinea pig serum dilution containing 2 units of complement. After fixation overnight at 6-8°C, 0.4 ml of hemolytic system containing 2 units of hemolysin was added and the tests were read after 30 minutes incubation at 37°C. The reciprocal of the highest dilution of antigen showing complete fixation was recorded as the titer.

Results. Culture fluid antigens. Numerous 14- and 28-day cultures of 8 types of leptospirae in Korthof's medium were killed by the addition of merthiolate, 1:10000, and were centrifuged at 15000 r.p.m. (Spinco) for 30 minutes. The majority of these undiluted supernatant fluids completely fixed complement in the presence of homologous immune serum; a few gave titers of 2 but none showed a titer greater than 4. These fluids were frequently anticomplementary in a dose of 2 antigenic units even after heating at 62°C for 40 minutes. The supernates of cultures grown for 28 days tended to be more antigenic than those from 14-day cultures suggesting that the autolysis which occurs as cultures age

* The early literature is reviewed in the monograph by Borgen(1).

† Strains of *L. icterohemorrhagiae, L. canicola, L. pomona,* and *L. grippotyphosa* were kindly supplied by Dr. P. B. Beeson, Emory University Medical School, Atlanta, Ga. *L. autumnalis,* type AB (*L. akiyama A*) and *L. bataviae* (Swart v. Tienen) were obtained from Dr. E. H. Thomas, National Microbiological Institute, Bethesda, Md.

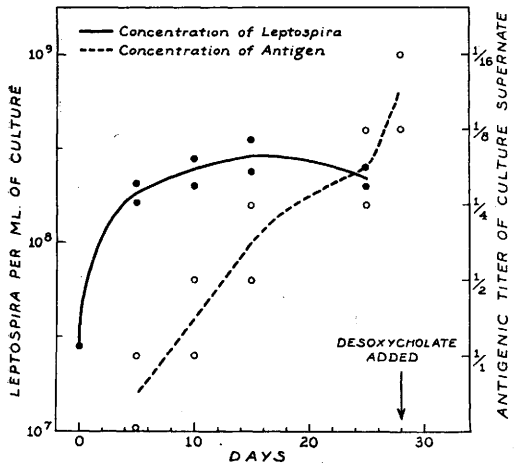

FIG. 1. Antigen concentration in culture supernate in relation to growth of *Leptospira pomona.*

may contribute to the antigenicity. In 3 experiments in which duplicate cultures of *L. icterohemorrhagiae* and *L. pomona* were grown at 28° and 37°C for 14 days the antigenic titer of the supernatant fluid of the 37°C culture was twice that of the 28°C culture in spite of the higher concentration of leptospirae at the lower temperature as demonstrated by actual count. This difference was presumably due to the increased autolysis at 37°C(14). We were not able, however, consistently to prepare sufficiently antigenic supernates for practical use even when a long period of incubation at 37°C was employed. To explore further the relation of soluble antigen concentration to the concentration of organisms leptospiral counts and soluble antigen determinations were made on samples removed at intervals from duplicate cultures of *L. pomona* grown at 28°C (Fig. 1). The number of leptospirae per ml of culture reached a maximum in about 15 days and then declined. The antigenic titer of this supernate continued to increase while the concentration of organisms was decreasing, again suggesting the liberation of antigens as a result of autolysis. In order to bring about further lysis sodium desoxycholate(10) to give a concentration of 0.2% was added to the culture after 28 days of incubation. After 1 hour at 37°C and 18 hours in the refrigerator the cultures were centrifuged and the supernatant fluid dialysed against isotonic phosphate saline solution(15). This treatment resulted in a still further increase in antigenic titer of the culture fluid.

Antigens from chick embryos. Six types of leptospirae were passed from 11 to 16 times through 8-day-old embryonated eggs. Allantoic fluid was harvested 5 days after inoculation and 0.5 ml transferred to the allantoic cavity of 8-day-old embryos. The concentration of organisms in the allantoic fluid reached a level comparable to that obtained in cultures, 100000000 to 200000000 per ml in most eggs. Since leptospirae failed to multiply in some eggs, however, it was necessary to check the allantoic fluid from each egg by darkfield. After high speed centrifugation, infected allantoic fluid fixed complement weakly if at all. This low concentration of soluble antigen was probably due to the fact that little autolysis took place in the relatively brief period of incubation. The isolation of a serologically active substance from typhus rickettsiae by boiling infected yolk-sac material in dilute alkali(15) suggested applying this procedure to leptospira-infected allantoic fluid. The pooled fluid from several eggs was centrifuged at low speed to sediment erythrocytes and debris. The supernatant fluid was made 5th normal with sodium hydroxide, placed in boiling water for 30 minutes, and neutralized by dialysis against buffered saline(15). The complement fixing titer of this dialysed material was determined in the presence of homologous immune rabbit serum. Of 13 preparations from 6 types of leptospirae, 6 had antigenic titers of 16, 1 had a titer of 8, 4 had titers of 4 and 2 had titers of 2. Only one of the preparations was anticomplementary in a dose of 2 units. There was a rough correlation between concentration of leptospirae in the fluid and the antigenic titer after treatment with alkali. Attempts to obtain agglutination of erythrocytes treated with these preparations have been completely unsuccessful.

Specificity of antigens. Table I shows the complement fixing titers of homologous and heterologous rabbit immune sera for 4 antigenic preparations from *L. pomona* as compared to the agglutinin titers of these sera. Each serum agglutinated its homologous type to a titer of at least 20480. The complement fixing antigens were used in a dose of 2 units provided by the dilutions indicated. The

TABLE I.
Specificity of *L. pomona* Complement Fixing Antigens as Compared with Agglutination.

Antigen	Titer of rabbit immune serum* 1	2	3	4	5	6	7	8
Whole culture (agglutination)	0†	0	81920	0	0	0	0	0
Culture supernatant fluid, undiluted	0‡	0	640	0	0	0	0	0
Supernatant fluid of culture treated with desoxycholate, 1:8	0‡	0	320	0	0	0	0	0
Allantoic fluid treated with NaOH, 1:8	0‡	0	160	0	0	0	0	0
Washed suspension of leptospirae from allantoic fluid, 1:16	0‡	0	640	0	0	0	0	0

* 1. *L. icterohemorrhagiae*, 2. *L. canicola*, 3. *L. pomona*, 4. *L. hebdomadis*, 5. *L. grippotyphosa*, 6. *L. mitis*, 7. *L. bataviae*, 8. *L. autumnalis*.
† No agglutination in a 1:80 dilution of serum.
‡ Incomplete complement fixation in a 1:20 dilution of serum.

washed suspension from allantoic fluid was prepared according to the method of York (12). Under these conditions none of the antigens showed complete fixation with any of the heterologous sera tested.

Discussion. Soluble antigens that fix complement have been prepared from leptospirae by several methods. Although there is as yet no proof of their identity, it is possible that the antigen present in untreated culture supernate, the antigen extracted from the organisms by treatment with desoxycholate, Fraction 1 of Schneider(10), and the antigen present in sodium hydroxide treated allantoic fluid are the same. The antigenic preparations from *L. pomona* studied here exhibited a high degree of type specificity. Antigens prepared by sonic disintegration(6,8) and by chemical fractionation(11), however, are reported to have a broad range of reactivity among the various types of leptospirae. In an extensive experience with leptospiral suspensions as complement fixing antigens Gaehtgens(4) observed cross reactions but he found the homologous titer always to be higher. Further work may reveal to what extent these apparent differences in type specificity may be due to quantitative differences in the reactivity of preparations or to variations in the cross reactivity of different strains.

Summary. The complement fixing activity of preparations from leptospiral cultures and from leptospira infected chick embryos has been studied in the presence of immune rabbit serum. The antigenicity of culture supernatant fluids was low but tended to increase as the temperature of incubation and the age of the cultures increased. The addition of sodium desoxycholate to cultures increased still further the ability of culture supernates to fix complement. Boiling infected allantoic fluids with N/5 NaOH yielded a soluble, non-dialyzable antigen. *L. pomona* culture supernates, alkali treated allantoic fluids and washed suspensions of leptospirae from allantoic fluids reacted specifically with *L. pomona* immune rabbit serum in the dilutions employed.

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