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Leptospiral Selection, Growth, and Virulence in Synthetic Medium[1]

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ABSTRACT

STALHEIM, O. H. V. (National Animal Disease Laboratory, Ames, Iowa). Leptospiral selection, growth, and virulence in synthetic medium. J. Bacteriol. **92**:946–951. 1966.—The need for protein in leptospiral cultural medium may be circumvented by the use of strains which tolerate the lytic activity of polyoxyethylene sorbitan monooleate (Tween 80), a relatively nonlytic source of essential fatty acids. In an otherwise adequate medium, the primary function of a serum protein (bovine albumin fraction V) in the cultivation of *Leptospira pomona* was detoxification of fatty acids. Treatment to destroy or block end groups (amino, sulfhydryl, or hydroxyl) did not impair this function, but, after treatment with trypsin, albumin was inactive. Synthetic and derived peptides or polyvinylpyrrolidone did not substitute for albumin. *L. pomona* grew in medium with surface tension values of 44 to 58 dynes/cm^2; after growth, the values were increased slightly (5 to 8). The growth responses did not correlate with the surface tension of the medium, but they were in proportion to the concentration of Tween 80. Of six strains of *L. pomona*, five were transferred from medium containing rabbit serum and were subcultured in Tween synthetic medium (TSM) containing low, nonlytic concentrations (0.002%) of Tween 80. The poor antigenicity of *L. pomona* in carbon-limited TSM was associated with a deficiency of those carbonaceous cellular components which were extractable with 50% ethyl alcohol. After as few as four subcultures in TSM, *L. pomona* tolerated higher concentrations of Tween 80 (0.06% was optimal; MTSM). If grown on a shaker, the rate and amount of growth and the antigenicity of *L. pomona* in MTSM equaled that in medium supplemented with rabbit serum. After cultivation in MTSM, all of the five strains were avirulent when administered to hamsters, guinea pigs, and swine. They were still avirulent after three subcultures in complex media or after two serial passages in hamsters.

During earlier studies by Stalheim and Wilson on active immunization against leptospirosis, *Leptospira pomona* was propagated in a synthetic medium containing very low concentrations of essential long-chain fatty acids. The amount of growth was only one-tenth that in medium supplemented with rabbit serum or bovine serum albumin (24, 25). In addition, the leptospires were poorly agglutinated by homologous antiserum and did not adequately protect guinea pigs against infection with *L. pomona* (26). The objective of the research reported herein was to achieve good growth of fully antigenic *L. pomona* in a medium free from extraneous antigens. Since the antigenicity and immunogenicity of *L. pomona* apparently depended upon good growth, and since good growth apparently required serum protein in the cultural medium, the initial part of the current study was devoted to investigating the role of bovine serum-albumin in the growth and antigenicity of *L. pomona*. In the course of these studies, further selection of two *L. pomona* strains occurred, so that good growth of fully antigenic leptospires was achieved in a synthetic medium containing adequate amounts of fatty acids. Some factors of the selective procedure and its effect on virulence are described.

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MATERIALS AND METHODS

Role of albumin. L. pomona strains Johnson and pomona were used for this portion of the study. They had been selected in boiled serum medium (30) and

were maintained for more than 18 months in Tween synthetic medium (TSM) containing polyoxyethylene sorbitan mono-oleate (Tween 80) and polyoxyethylene sorbitan monostearate (Tween 60) with subcultures at intervals of 1 or 2 weeks (26).

Fatty acid-poor bovine albumin and the preparations of fraction V albumin from different species of animals and man were obtained from Pentex, Inc., Kankakee, Ill., and were dissolved in 0.02 M phosphate buffer (*p*H 7.2) at a concentration of 5%. Bovine albumin was treated with trypsin, as described by Dunn (8).

To destroy or block end groups, fatty acid-poor bovine albumin was altered as follows: amino groups by reaction with 1-fluoro-2,4-dinitrobenzene (14) and the sodium salt of 2,4-dinitrobenzene sulfonate (10), by acetylation (9), and by reaction with carbobenzoxychloride (1); sulfhydryl groups by iodination (18) and by reaction with *p*-chloromercuribenzoate (2); and hydroxyl groups by periodate oxidation (6). After dialysis against sterile phosphate buffer at 5 C for 24 to 48 hr, solutions of the altered albumins were adjusted to the original volume and were sterilized by Seitz filtration.

Cultivation in modified TSM. Strains of *L. pomona* capable of good growth in synthetic medium were derived by the following procedure. Two-day-old cultures of *L. pomona* strains Johnson, pomona, Ohio, Wickard, DM_2, and MLS in Stuart's medium (27) were inoculated (10%) into TSM. When evidence of growth appeared, 3, 5, or 10 ml was added to 10 ml of synthetic medium containing different concentrations of Tween 80 (0.002, 0.005, 0.007, 0.02, and 0.04%). Observations of the rate and amount of growth were recorded, and the agglutinability of the leptospires was determined as previously described (23, 26).

Cultures of derived strains of *L. pomona* were incubated at 29 C with shaking [110 strokes per min with a 1-inch (2.54-cm) excursion] in Erlenmeyer flasks (1 liter) containing 500 ml of TSM modified by increasing the concentration of Tween 80 to 0.06% (MTSM). The amount of growth was estimated nephelometrically until it was maximal; then the bacterial density was determined by microscopic counts (23).

Studies of antigenicity. The antigenicity of the organisms was determined by the microscopic agglutination (MA) test (26) and the agglutinin absorption test (23) with two different homologous rabbit antisera. The first serum had a titer of 1:10,000; the second serum, which was used for the reciprocal absorption studies, had a titer of 1:1,000,000. To compare their ability to stimulate the production of specific antibodies, cultures of *L. pomona* grown in TSM and MTSM were killed by heat (56 C for 30 min) and were administered intravenously to rabbits at intervals of 4 or 5 days. Five doses of 1, 2, 3, 4, and 5 ml were given. The serum obtained 10 days after the last injection was stored at 5 C and was tested by the MA test.

Comparisons were made of the content of fraction A (carbohydrate) and fraction S (protein) in *L. pomona*, when grown in 20 liters of TSM and in 10 liters of MTSM as described by Schricker and Hanson (20).

Estimation of virulence. The virulence of *L. pomona* strains Johnson, pomona, Ohio, DM_2, and Wickard was estimated by the febrile responses of guinea pigs after the intraperitoneal administration of ca. 3×10^8 organisms (26) and by determinations of the LD_{100} and ID_{100} values for hamsters (22). Estimations were made for strains in Stuart's medium, after adaptation to growth in MTSM, and after three subcultures of the selected strains in complex media. In addition, *L. pomona* strain Ohio in MTSM was passed through hamsters. Approximately 5×10^8 organisms were administered by intraperitoneal injection to two hamsters. After 48 hr, the hamsters were anesthetized with ether and were bled from the heart. Part of the blood from each hamster (0.1 ml) was inoculated into each of two tubes of Stuart's medium (10 ml) and was decimally diluted through six more tubes; the rest (at least 0.2 ml) was administered to two different hamsters. This procedure was repeated twice. After hemoculture, the first two hamsters only were killed with ether, and the liver and kidney were cultured for leptospires; the kidneys of the other hamsters were cultured 3 to 4 weeks after exposure, as previously described (21). Similarly, attempts were also made to pass this strain through swine, except that 6×10^9 organisms were administered, and that, in addition to cultural procedures (Stalheim, Am. J. Vet. Res., *in press*), sera were obtained at the time of autopsy and were tested for agglutination of *L. pomona*. To observe their colonial morphology, virulent and avirulent leptospires were grown as isolated colonies in the solidified medium of Cox and Larson (4), as previously described (23).

RESULTS

Role of protein. As shown in Table 1, the growth of *L. pomona* in TSM was greatly increased by supplementation with serum albumin from the cow, horse, pig, rabbit, or man. Cultures in supplemented media were agglutinated by homologous antiserum at a final dilution of 1:10,000, whereas TSM-grown cultures were incompletely agglutinated only in low (1:10 and 1:100) dilutions. Trypsinized bovine albumin inhibited growth, as did fatty acid-poor albumin, unless additional Tween 80 was added to the medium.

When albumin was added to cultures of *L. pomona* in TSM and was incubated at 5 C for 48 hr, the MA titer was not increased. Full agglutinability was restored after as few as seven generations in Stuart's medium.

Table 2 shows the growth responses of *L. pomona* in medium supplemented with bovine albumin which had been treated with reagents to block or destroy different end groups of the protein molecule. Treatment to block or destroy amino, sulfhydryl, or hydroxyl groups did not abolish or seriously impair the growth-supporting

activity of bovine albumin with either sodium oleate or Tween 80 as a source of fatty acid. After treatment with 1-fluoro-2,4-dinitrobenzene, gross distortion of the electrophoretic pattern of albumin was observed (model R microzone system; Beckman Instruments, Inc., Fullerton, Calif.). The patterns of the other preparations were essentially normal.

Albumin treated with *p*-chloromercuribenzoate was toxic. After inoculation, leptospires rapidly lost motility, became shrunken, and lysed within 6 hr. The toxicity could not be reversed either by dialysis of the albumin preparation against TSM plus cystine (0.005 mg/ml) for 72 hr or by the addition of cysteine (1.8 mg/ml).

TABLE 1. *Effects of albumin on growth and agglutinability of Leptospira pomona in synthetic medium*[a]

Cultural medium	Growth (organisms/ml × 10^6)	Agglutinability (MA titer)
Synthetic medium	24	10^{-2}
Synthetic medium plus		
Bovine albumin	385	10^{-4}
Equine albumin	120	10^{-4}
Porcine albumin	224	10^{-4}
Rabbit albumin	276	10^{-4}
Human albumin	284	10^{-4}
Trypsinized bovine albumin	<1	Not done
Fatty acid-poor bovine albumin	<1	Not done
Fatty acid-poor albumin plus Tween 80, 0.004%	285	10^{-4}

[a] Duplicate tubes of synthetic medium (TSM) were supplemented (26) with 1% of the different albumins and were inoculated with *L. pomona* to an initial concentration of 10^6 per milliliter. After 7 days of incubation at 29 C, the cultures were counted and used as antigen in the microscopic agglutination (MA) test.

Attempts were made to replace the function of albumin in TSM with several materials. Glycerol (0.025 to 1.0%), glucose (0.025 to 1.0%), insulin (0.0008 to 0.8 units/ml), thiamine pyrophosphate (10, 20, or 50 μg/ml), coenzyme A (10, 20, or 50 μg/ml), or short-chain fatty acids (*n*-valerate, isovalerate, isobutyrate, and 2-methyl butyrate; 0.02 to 1 μmole/ml) did not stimulate growth; neither did any one of 41 different synthetic peptides (0.01 and 0.05%), supplied and described by Nutritional Biochemicals Corp., Cleveland, Ohio, Difco tryptose phosphate (0.05 to 0.5%), Georgia bulgaricus factor (29), the dialysate of normal rabbit serum (17), or a cellophane sac containing normal rabbit serum suspended in a flask of synthetic medium.

The possibility that the growth-supporting activity of serum protein could be equated with surface activity was investigated with a Du Noüy tensiometer. The surface tension values of different cultural media were: Stuart's, 53.7; Fletcher's (13), 52.6; Ellinghausen and McCullough's (11), 47.6; TSM plus bovine albumin (1%), 58.0; TSM with bovine albumin plus Tween 80 (0.1%), 47.6; TSM with bovine albumin plus sodium oleate (0.01%), 55.0; and TSM with 0.05% of Tween 60, 47.6; Tween 40, 44.6; or Tween 20, 45.0. Except for the latter medium, all supported moderate to good growth of *L. pomona* strains Johnson and pomona. After leptospiral growth, the surface tension values of the media were 5 to 8 points higher.

The surface tension and growth-supporting activity of TSM with different concentrations of Tween 80 were determined (Fig. 1). The surface tension values decreased with increasing amounts of Tween 80, but the growth-responses of *L. pomona* strains Johnson and pomona were in proportion to a concentration of Tween 80; growth did not occur in medium with 0.5% or more of Tween 80. When tested in MA tests, leptospires in MTSM containing 0.06% of Tween 80 were

TABLE 2. *Effects of end-group destruction on ability of albumin to support the growth of Leptospira pomona*[a]

Reagent	End group	Growth response (organisms/ml × 10^6)	Agglutinability (MA titer)
1-Fluoro-2,4-dinitrobenzene	$-NH_2$	<1	Not done
Na-2,4-dinitrobenzene sulfonate	$-NH_2$	220	10^{-4}
Acetic anhydride	$-NH_2$	186	10^{-4}
Carbobenzoxy chloride	$-NH_2$	194	10^{-4}
Sodium periodate	—OH	204	10^{-4}
Potassium iodide	—SH(—OH)	188	10^{-4}
p-Chloromercuribenzoate	—SH	<1	Not done
None, control		248	10^{-4}

[a] Fatty acid-poor bovine albumin was treated as described in the text, was added to synthetic medium at a concentration of 1% with either sodium oleate (0.01%) or Tween 80 (0.1%), and was tested as described in Table 1.

agglutinated by a 1:10,000 dilution of antiserum. In view of the range of surface tension among the several media listed above, all of which supported good growth, it is improbable that the surface tension effect of Tween 80 influenced either the growth or agglutinability of *L. pomona*.

Cultivation in MTSM. In the first subculture, good growth of *L. pomona* in synthetic medium occurred; it was attributable to the presence of serum from the inocula. In the second transfer, there was a lag of 7 to 27 days before evidence of leptospiral growth was observed. Two further subcultures of five strains (MLS died) were made in medium containing very low amounts (0.002%) of Tween 80; growth did not occur in medium containing 0.005% or more of Tween 80, and a concentration of 0.1% was extremely lytic. After the fourth subculture, but not before, the amount of growth of all strains was proportional to the amount of Tween 80 in the medium as shown in Fig. 1. During subsequent subcultures, growth was observed in medium containing 0.1% and even 0.2% Tween 80, but the amount and rate of growth did not exceed that in MTSM. Treatment to remove free oleic acid (5) did not change the nutritive character of Tween 80.

The five adapted strains of *L. pomona* were transferred from MTSM to Stuart's medium. After four subcultures (about 28 generations), washed leptospires still had the ability to grow when transferred back to MTSM; they were subcultured four times.

Antigenicity. L. pomona grown in MTSM was indistinguishable from *L. pomona* grown in Stuart's medium by MA tests or agglutinin absorption tests (Table 3). After three subcultures in medium lacking adequate amounts of Tween 80 (TSM), *L. pomona* organisms incompletely absorbed serum agglutinins and were poorly agglutinated in low dilutions only of homologous antiserum (1:100). When inactivated by heat and injected into rabbits, cultures grown in MTSM, but not in TSM, stimulated the production of serum agglutinins in titers of 1:100,000.

Strains of *L. canicola*, *L. autumnalis*, and *L. grippotyphosa* capable of good growth in MTSM were selected also. When tested, their agglutinability equaled that of leptospires grown in Stuart's medium.

The yields of cellular fractions of *L. pomona* were calculated as percentages of the dry weight of the whole cells. Cells grown in TSM or MTSM, respectively, yielded 0.06 and 1.12% as the A fraction, and 1.6 and 1.04% as the S fraction.

Virulence estimations. Only the wild type of *L. pomona* strain Ohio in Stuart's medium caused fevers in guinea pigs. Leptospires were not isolated from the kidneys of guinea pigs exposed with selected strains of *L. pomona* in MTSM.

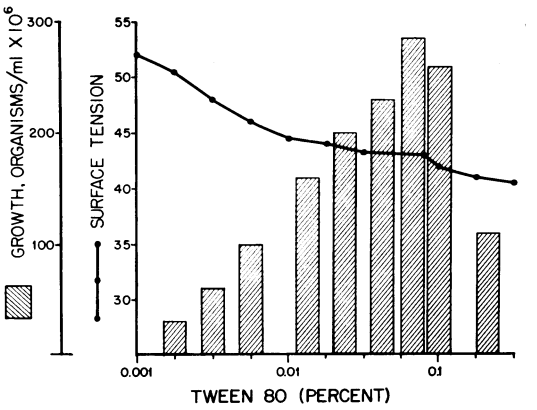

FIG. 1. *Surface tension in dynes per square centimeter and growth responses of Leptospira pomona in synthetic medium containing different amounts of Tween 80.*

Before cultivation in MTSM, wild type *L. pomona* strain Ohio had an LD_{100} value for hamsters of less than 100 organisms. After cultivation in MTSM, more than 5×10^8 organisms caused neither acute leptospirosis and death, nor persisting renal infections. The strain was still avirulent after three subcultures in Stuart's medium, and virulence was not increased by passage through hamsters. Leptospires were isolated from the liver, kidney, and blood of both of two hamsters, 48 hr after exposure. The organs contained 2×10^3 to 2×10^4 leptospires per gram of tissue. When passed in blood to two more hamsters, leptospires were again isolated from the blood of both hamsters, but isolations were not made after a third hamster passage. A culture of *L. pomona* in Stuart's medium from the second hamster passage was administered to three hamsters. Approximately 3×10^8 organisms caused neither death nor renal leptospirosis.

Four other strains of *L. pomona*, which originally were capable of initiating renal infections in hamsters (ID_{100} was less than 10,000 organisms), were also avirulent for hamsters after growth in MTSM and did not regain virulence during three transfers in Ellinghausen and McCullough's medium.

Leptospires were not isolated from the liver, kidney, or blood of two pigs, when cultured 48 hr after exposure with *L. pomona* in MTSM. In addition, the blood did not cause infections in other pigs, since all sera failed to agglutinate *L. pomona*, and since leptospires were not isolated from renal tissue.

Avirulent *L. pomona* strain Ohio had well-developed hooks at cell termini. Colonies in solidified medium were large and translucent, with distinct borders (LTD).

TABLE 3. *Antigenicity of Leptospira pomona when grown in Stuart's medium and in synthetic medium with adequate (MTSM) or inadequate (TSM) amounts of Tween 80*[a]

Antigens	Nonabsorbed antiserum	Antiserum absorbed with *L. pomona* grown in		
		TSM	MTSM	Stuart's
L. pomona grown in TSM.....	100[b]	100	0	0
L. pomona grown in MTSM.....	1,000,000	1,000	10	10
L. pomona grown in Stuart's.....	1,000,000	1,000	10	10

[a] *L. pomona* cells were grown in three media, adjusted to 10^7 organisms per ml, and were tested for agglutinability by the MA test with nonabsorbed and absorbed, homologous antiserum.
[b] Reciprocal of highest, final, serum dilution showing at least 50% clearing with agglutination.

DISCUSSION

The poor growth and antigenicity of *L. pomona* in TSM prompted Stalheim and Wilson to suggest a protein requirement for the synthesis of leptospiral surface antigens by means of a "lipid-feeding mechanism" (25). On the contrary, the present study demonstrates that, in a nutritionally adequate medium, the primary if not sole role of a serum protein such as albumin is to detoxify fatty acids by neutralizing their lytic activity; the source of the albumin is not critical. Albumin binds fatty acids by three classes of binding sites (15), which apparently are not the end groups, since they are not essential for this function of albumin.

The earlier reports of differences in susceptibility (25) and adaptability (30) to lytic conditions in certain cultural media, together with the current observations, suggest a unifying concept: namely, that different growth characteristics among pathogenic leptospires are due to differences either in susceptibility or adaptability, or both, to unfavorable in vitro environments rather than due to different nutritional or biochemical characteristics as proposed by Yanagawa and Wilson (30). Such a concept suggests that the considerable body of data on leptospiral nutrition is valid for all pathogenic leptospires.

When grown in TSM, *L. pomona* was poorly agglutinated by homologous antiserum although it absorbed agglutinins. Instead of a lack of protein per se, the poor antigenicity is associated with a relative deficiency of extractable cellular carbohydrates (fraction A). TSM differs from MTSM in that it lacks an adequate concentration of fatty acids, the major source of carbon and energy for leptospires (16). Limitation of the amount of available carbohydrate in the medium significantly influences the synthesis of non - nitrogenous components of *Aerobacter aerogenes* (7) and *Mycobacterium phlei* (29). The antigenic deficiency in TSM-grown leptospires may be qualitative as well as quantitative, since Schricker and Hanson (20) found six to eight precipitinogens in the carbohydrate fraction of *L. pomona*.

Attempts to improve the growth of leptospires in a growth-limiting medium, TSM, by adding various growth factors have always failed. However, during studies on the effect of surface tension with different amounts of Tween 80, good growth occurred unexpectedly with two strains of *L. pomona* which had been maintained in TSM for 18 months. Strains capable of growth in MTSM may be selected in as few as four subcultures. The essential features of the procedure described herein are: (i) the use of Tween 80 as a relatively nonlytic source of fatty acids at a low concentration (0.002%); (ii) massive inocula and prolonged periods of incubation (up to 28 days); and (ii) the occurrence of growth, after four or more transfers in TSM, at successively higher concentrations of Tween 80, with synthesis of the usual complement of surface antigens. The genetic nature of these events has not been investigated.

When a synthetic medium (TSM), which was devised for *L. canicola*, was modified by increasing the amount of Tween 80 to adequate levels (MTSM), the growth and antigenicity of selected strains of four pathogenic leptospiral serotypes equaled that in Stuart's medium. Thus, the requirement for serum or serum albumin in leptospiral growth media may be circumvented by the use of strains of leptospires which are capable of tolerating an adequate concentration of Tween 80 as a source of fatty acids.

Contrary to previous reports (3, 19) of wide fluctuations in leptospiral virulence during in vivo or in vitro growth, the virulence of a particular strain may be maintained relatively constant for the same animal species (22). However, strains selected in MTSM are avirulent for hamsters, guinea pigs, and swine; they cause neither clinical nor renal leptospirosis. Although avirulent *L. pomona* persisted in hamsters for at least 48 hr during "blind passages" in hamsters, virulence for the hamster was not regained in this study. Concurrent changes in cellular and colonial morphology, as described for *L. icterohaemorrhagiae* (12), were not observed.

These results justify a comparison of wild-type,

virulent leptospires, and their avirulent progeny, grown in synthetic medium, to find biochemical differences which may correlate with the virulence of the organisms or with some mechanism of pathogenesis. Strains of avirulent leptospires may be useful as immunizing agents. The immunogenicity of avirulent *L. pomona* has been investigated in hamsters and swine (*unpublished data*).

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