Broadly Reacting Precipitating and Agglutinating Antigen of *Leptospirae*

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A saprophytic *Leptospira biflexa* strain of equine origin was found which cross-reacts with immune rabbit antisera to 14 pathogenic *Leptospira interrogans* serotypes. Sera from goats experimentally inoculated with the saprophyte showed multiple low-level cross-agglutination reactions against a battery of live *L. interrogans* serotypes. Sonically treated and saline-extracted suspensions of the *L. biflexa* strain and serotypes *canicola*, *icterohaemorrhagiae*, and *pomona* yielded a common precipitating protein antigen that was detected by immunodiffusion and immunoelectrophoresis with all of the antileptospiral sera examined. In cross-absorption and gel diffusion tests, the precipitinogen from each of the strains was shown to be identical. Formaldehyde treatments and heating at 100°C suggest that the cellular location of the common antigen is either somatic or subsurface, and Sephadex G-200 gel filtration enabled the isolation of the active fraction of the *L. biflexa* antigen. Monoprecipitin sera against the common antigen of *L. biflexa* were produced by immunizing rabbits with specific precipitates in agar. In gel diffusion and immunoelectrophoresis tests the antisera with each of the soluble antigens developed a single precipitin formation, and the antisera agglutinated formalized and heated whole-cell suspensions of serotypes *canicola*, *icterohaemorrhagiae*, and *pomona* at low dilutions. The soluble *L. biflexa* antigen was evaluated as an immunogen and in passive immunity tests for protection against death and kidney infection in hamsters. No cross-protection occurred when the hamsters were challenged with virulent leptospires. In contrast, the animals vaccinated or administered hamster immune serum before challenge died earlier than the control animals.

In a previous study with isolates of *Leptospira biflexa* obtained from renal tissue of horses (15), a strain was examined that was agglutinated by horse sera containing naturally occurring agglutinins to diverse and multiple pathogenic *Leptospira interrogans* serotypes. Absorption of the horse sera with the saprophytic strain removed the agglutinins to the pathogenic strains. Depending upon the growth medium employed for preparing antigens, the *L. biflexa* strain was also agglutinated by most rabbit immune antileptospiral reference sera to pathogenic serotypes. These findings suggested that persistence of certain *L. biflexa* strains in the kidney provides a continuing antigenic stimulus. Such a stimulus may be responsible for some of the cross-reactivity frequently encountered with mammalian sera in diagnostic agglutination tests.

The present immunological study was initiated to provide further evidence that this cross-reactive response is most likely due to a common broadly reacting antigen of *L. biflexa* and path-

ogenic serotypes, and to report on its antigenic character.

MATERIALS AND METHODS

Cultures. *L. biflexa*, designated as H 48, was obtained from renal tissue of a horse by Myers (15). The 14 pathogenic *L. interrogans* reference strains were selected from the culture collection of this center and included the following serotypes: *pomona* strain Pomona, *australis* strain Ballico, *ballum* strain Castellon 3, *grippotyphosa* strain Moskva V, *tarassovi* strain Perepelicin, *hebdomadis* strain Hebdomadis, *bataviae* strain Van Tienen, *canicola* strain Hond Utrecht IV, *hardjo* strain Hardjoprajitno, *wolffi* strain 3705, *pyrogenes* strain Salinem, *sejroe* strain M 84, *icterohaemorrhagiae* strain RGA, and *autumnalis* strain Akiyami A. All of the serotypes were maintained in tubes containing 5.0 ml of Fletcher (Difco) semisolid medium and in tubes containing 10 ml of Stuart (Difco) liquid medium (with 10% pooled normal rabbit serum). Where indicated, cultures were also maintained in tubes of bovine albumin polysorbate 80 (BA-P80) semisolid and liquid media containing fraction V bovine albumin (Pentex) prepared as described by John-

son and others (12, 13). Subcultures were routinely made by using a 1:10 ratio for inoculating fresh media at 3-month intervals if the organisms were growing in semisolid media and weekly if in liquid media. Cultures were incubated at 30°C.

In addition, a recently isolated virulent strain of serotype *icterohaemorrhagiae* from a fatal human infection and a freshly isolated strain of serotype *hardjo* of equine origin were also used as hamster challenge inoculum. Virulence of these two cultures were maintained by alternately passing in hamsters and subcultured once before use. Experimental inoculations of 0.5 ml of the *icterohaemorrhagiae* serotype (approximately 3.8×10^6 organisms per ml) produced death within 5 days, whereas 0.5 ml of a 7-day-old culture of the nonlethal *hardjo* serotype grown in BA-P80 medium produced renal infection in hamsters for at least 13 weeks after intraperitoneal inoculation.

Serological tests. Microscopic agglutination (MA) tests were performed with antigens of 4- to 7-day-old live cultures grown in Stuart or BA-P80 liquid media and compared with washed and killed cells from the same cultures that were either formalized to contain 0.5% (vol/vol) formaldehyde or formalized and heated at 100°C for 30 min. For all agglutination tests, 0.2-ml volumes of diluted sera in Sorensen phosphate-buffered saline (pH 7.2) (to give final twofold serum dilutions ranging from 1:10 to 1:40, 960), were mixed with equal volumes of standardized antigen suspension (9). The highest serum dilution showing 50% or more agglutination was considered the end point titer. Titrations with live antigens were read after 2 h of incubation at room temperature (about 25 to 27°C), and titrations with formalized or formalized and heated suspensions were read at 4 and 24 h (4).

Preparation of soluble antigens. Log-phase cells from actively growing 5- to 7-day-old stationary cultures of serotypes *canicola*, *icterohaemorrhagiae*, *pomona*, and *biflexa* H 48 grown from a 10% inoculum in 2 liters of liquid Stuart or BA-P80 media were formalized (0.5% vol/vol) and centrifuged in an International model V centrifuge at $1,500 \times g$ for 30 min to remove extraneous material. Cultures were then harvested by centrifugation in a SS-1 Sorvall super-speed angle centrifuge at $15,000 \times g$ for 15 min. The supernatant was discarded and the leptospiral pellet resuspended in 200 ml of 0.5% formalized phosphate-buffered saline (pH 7.2), and the centrifugation process was repeated once more. The resulting pellet was suspended in buffered saline to approximate 1/200th of the original culture volume. The dense leptospiral suspension was subjected to sonic vibration in a Raytheon 10-kc magnetostriction oscillator for 10 min and then examined by dark-field microscopy to assure that complete disintegration of the leptospirae had occurred. The sonically treated antigen was then centrifuged ($1,500 \times g$ for 30 min) to remove the insoluble cellular debris. The supernatant fluid was frozen at -25°C and on the next day, centrifuged again after thawing to remove a further precipitate that had formed which proved to contain 40 mg% of lipid material (19). The clear, opalescent antigen was dialyzed for 72 h against cold phosphate-buffered saline and stored frozen at -25°C or lyophilized and used in gel diffusion (GD) tests, immunoelectrophoresis (IEP),

serum absorptions, and animal inoculations. The completed antigen was stable for at least 6 months, had a protein content of 250 mg% (biuret) (5), and was negative in tests for polysaccharides (20, 22).

Gel filtration of soluble *L. biflexa* antigen on Sephadex G-200. The *L. biflexa* H 48 soluble antigen was fractionated by passing 1.0 ml of the antigen through a column of Sephadex G-200 (2 by 90 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0) in 0.15 M sodium chloride containing 0.02% sodium azide. The absorption of the effluent at 280 nm was continuously recorded with an LKB 8300 Uvicord II absorptimeter and a LKB 6520 Chapparr bar recorder. Column eluates of 3.0 ml each were collected on a LKB 7000 Ultrarac collector equipment. Selected fractions were pooled, dialyzed against distilled water for 72 h, concentrated to 0.3 ml by freeze-drying, and examined for antigenic activity by GD and IEP tests.

Immunodiffusion and IEP. The microslide double GD method of Ouchterlony (16) was used. The agar gel consisted of 8.0 ml of 1.25% melted agar (Difco) in phosphate-buffered saline (pH 7.2) with 1:10,000 merthiolate to prevent contamination. Wells were cut using a Feinberg agar gel cutter (Consolidated Laboratories, Inc., Chicago, Ill.), or 3-mm-diameter wells were cut with a punch and placed 6 mm apart. Antigens were loaded in the central wells, and sera were placed in the peripheral wells. The slides were incubated at room temperature in individual petri dishes. Humidity was maintained by the addition of saline to the bottom of each dish. The slides were examined daily for 72 h for precipitin formation.

IEP was performed by the method of Scheidegger (21) with microslides (50 by 75 mm) coated with 8.0 ml of 0.9% agarose (Seakem Biomedical, Rockland, Maine) in 0.05 M barbital buffer (pH 8.2). The size of the antigen well was modified to contain approximately 0.02 ml of the antigen sample, and the serum trenches contained approximately 0.5 ml. Electrophoresis of the antigens were carried out in the barbital buffer for 45 min while maintaining a potential difference of 28 V between the both extremes of the slide. Arcs of precipitate developed after the addition of the serum and incubation of the slides in humid petri dishes from 24 to 48 h at room temperature.

GD and IEP slides were examined before and after treatment for antigen/antibody specificity (23) with a 5.0% solution of sodium citrate and stained by methods described by Crowle (7). Some slides were stained with oil red O and alcian blue for an indication of lipoproteins and polysaccharides, respectively, and others were stained with amidoschwarz and photographed.

Sera. Hyperimmune antisera for each of the above-mentioned 14 pathogenic *L. interrogans* serotypes were prepared in rabbits by the method of Galton et al. (9) with 4 weekly increasing intravenous injections of 1.0- to 6.0-ml amounts of 7-day-old heat-killed (56°C for 10 min) Fletcher semisolid cultures. Antiserum to the *L. biflexa* H 48 strain was similarly prepared with semisolid cultures grown in both Fletcher and BA-P80 media as inocula.

Sera were also obtained from three 8-week-old male goats that were inoculated subcutaneously with 5.0 ml of the *L. biflexa* H 48 culture grown for 5 days in Fletcher semisolid medium. The inocula contained

approximately 9×10^8 viable leptospire per ml (1). For 2 weeks before inoculation, serological examinations were negative by MA tests for leptospiral agglutinins to the battery of pathogenic *L. interrogans* serotypes and to the *L. biflexa* H 48 strain. The inoculated goats were kept in isolation at all times and bled biweekly for serological tests during a period of 6 months.

In addition, goat sera collected 6 months post-inoculation were also absorbed with whole-cell suspensions of formalized *L. biflexa* H 48 by the technique described by Kmety et al. (14). In other serum absorptions for IEP tests, the soluble antigens were freeze-dried in 1.0-ml amounts, and 0.5 ml of the serum to be absorbed was added to the dried antigens. The mixtures were allowed to stand at room temperature for 1 h before placing them into the serum trench. Some sera were also treated with equal volumes of 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) at final concentrations of 0.1 and 0.05 M for 2 h at 37°C for an indication of the class of antibody immunoglobulin. For use in MA tests the initial serum dilution was adjusted by using 2 volumes of the mixture or concentrated in IEP tests ($\times 2$) by filling the serum trench twice (23).

Production of monoprecipitin antisera. Two rabbits were used to prepare specific immune sera against the common broadly reacting precipitating antigenic component (CA). For the inoculum, the specific arc precipitate produced in IEP with the soluble *L. biflexa* H 48 antigen and serotype *icterohaemorrhagiae* RGA antiserum was removed from several slides. They were then washed in saline solution for 4 days to remove non-precipitated material. The agar precipitates were homogenized in saline and mixed with an equal volume of Freund complete adjuvant. A 1-ml amount of the mixtures was injected intradermally into approximately 20 sites on the shaved back of each rabbit. On the next day, 1.0 ml of *Bordetella pertussis* vaccine (Lemos Laboratories, Buenos Aires, Argentina) containing 200,000 organisms per ml without adjuvant was similarly administered. Two weeks later, the rabbits were reimmunized with 1.0 ml of the same agar precipitate and adjuvant mixture intramuscularly into the hind legs. Blood was obtained by cardiac puncture 2, 4, and 6 weeks later and examined directly for agglutinin and precipitin activity. The immunoglobulin class of antibody activity was determined by gel-filtration on Sephadex G-200, isolation of the gamma globulins with 50% ammonium sulfate, and treatment of the sera with 2-mercaptoethanol.

Immunogenicity of *L. biflexa* H 48 soluble antigen in hamsters. The *L. biflexa* H 48 soluble antigen was evaluated for possible protective effects in hamsters. For these tests, groups of 10 to 16 21-day-old weanling hamsters were immunized by intraperitoneal inoculation with 0.5 ml (equivalent to 1.25 mg of protein) of the soluble antigen. One of the immunized groups and a group of uninoculated animals of the same age were used for each experiment as controls. At 21 days postvaccination, one group of immunized hamsters and a group of uninoculated control animals were challenged intraperitoneally with 0.25 ml of a virulent strain of serotype *icterohaemorrhagiae* suspension containing approximately 3.8×10^6

organisms per ml. The challenge dose of leptospire was expressed in terms of the organisms administered rather than as a 50% lethal dose or 50% infectious dose because of the variations in the stability of virulence known to exist among the different leptospiral serotypes as a result of culture medium and/or the experimental animal used.

With other groups of hamsters, either pooled sera obtained from hamsters that were vaccinated 3 weeks earlier were mixed in equal volumes (0.25 ml) with the challenge strain before infecting with the mixture, or 0.5 ml of the pooled hamsters immune serum was administered subcutaneously to each animal the day before intraperitoneal challenge with the virulent *icterohaemorrhagiae* serotype.

Another group of hamsters which were immunized with the soluble *L. biflexa* H 48 antigen and a group of control animals were used to determine whether a protection against kidney infection occurs in hamsters. These animals received an intraperitoneal inoculation with 0.5 ml of a 7-day-old culture of serotype *hardjo* grown in semisolid BA-P80 medium.

At 21 days after challenge, surviving animals from each of the different groups were sacrificed. Kidneys were removed aseptically and cultured in tubes containing 5.0 ml of Fletcher and BA-P80 semisolid media as described previously (15). Cultures were incubated at 30°C and examined weekly by dark-field microscopy for 8 weeks to detect leptospire.

RESULTS

Agglutination occurred when live *L. biflexa* H 48 organisms grown in BA-P80 liquid medium were employed as antigen in MA tests against antisera prepared to the above-mentioned 14 *L. interrogans* pathogenic collection serotypes. Serum titers ranged from 1:80 to 1:640. In contrast, no agglutination resulted with rabbit antisera prepared to whole cells of *L. biflexa* H 48 when examined in MA tests with the live pathogenic strains grown in either Stuart or BA-P80 liquid media as antigens. As shown in Table 1, washed and formalized antigens of serotypes *canicola*, *icterohaemorrhagiae*, *pomona*, and *L. biflexa* H 48 examined against their corresponding antisera showed a decline in homologous titers in MA tests when compared with titers obtained by employing living antigens. Homologous agglutinin titers were decreased further by heating the formalized antigens to 100°C for 30 min. In contrast, heterologous agglutinin titers became positive or showed increased cross-reactivity after formalization.

The results obtained with three goats experimentally inoculated with the *L. biflexa* H 48 strain and examined periodically by MA tests against the battery of live *L. interrogans* antigens are shown in Table 2. No serological cross-reactivity with the pathogenic serotypes was evident during the first 8 weeks post-inoculation, although increasing antibody levels to the inoc-

TABLE 1. Comparison of cross-reacting agglutinin titers with leptospiral antisera to serotypes *canicola*, *icterohaemorrhagiae*, and *pomona* and the *L. biflexa* H 48 strain against living, formolized and formolized and heated antigens

Antigen	Antiserum	Agglutination titer with antigen ^a		
		Live	Formolized (0.5%)	Formolized and heated (100°C, 30 min)
<i>canicola</i> Hond Utrecht IV	<i>canicola</i>	40,960	5,120	1,280
	<i>icterohaemorrhagiae</i>	640	1,280	160
	<i>pomona</i>	20	2,560	160
	<i>biflexa</i> H 48	—	320	160
<i>icterohaemorrhagiae</i> RGA	<i>canicola</i>	640	1,280	640
	<i>icterohaemorrhagiae</i>	40,960	10,240	2,560
	<i>pomona</i>	80	320	320
	<i>biflexa</i> H 48	—	80	160
<i>pomona</i> Pomona	<i>canicola</i>	—	1,280	640
	<i>icterohaemorrhagiae</i>	—	640	640
	<i>pomona</i>	10,240	5,120	1,280
	<i>biflexa</i> H 48	—	320	640
<i>biflexa</i> H 48	<i>canicola</i>	320	2,560	80
	<i>icterohaemorrhagiae</i>	640	2,560	1,280
	<i>pomona</i>	160	2,560	1,280
	<i>biflexa</i> H 48	40,960	5,120	5,120

^a Titer expressed as reciprocals of the highest dilution showing 50% or more agglutination; — indicates absence of agglutination at the lowest serum dilution of 1:10.

TABLE 2. Agglutinin titers to live antigens with sera of three goats examined periodically after subcutaneous inoculation with living *Leptospira biflexa* H 48 organisms

<i>Leptospira</i> serotype live antigen	Serum agglutinin titer with antigen ^a at week post-inoculation:										
	2	4	6	8	10	12	14	17	19	21	24
<i>pomona</i>	—	—	—	—	—	—	—	—	20	—	10
<i>australis</i>	—	—	—	—	—	—	—	10	20	10	10
<i>ballum</i>	—	—	—	—	20	32	20	25	32	40	32
<i>grippityphosa</i>	—	—	—	—	—	13	<10	10	25	10	10
<i>tarassovi</i>	—	—	—	—	—	—	10	20	25	20	20
<i>hebdomadis</i>	—	—	—	—	—	—	—	—	10	—	—
<i>bataviae</i>	—	—	—	—	—	—	<10	20	20	20	40
<i>canicola</i>	—	—	—	—	—	—	—	—	10	—	—
<i>hardjo</i>	—	—	—	—	—	—	—	20	14	20	10
<i>wolffi</i>	—	—	—	—	—	—	—	—	10	—	—
<i>pyrogenes</i>	—	—	—	—	—	—	—	10	—	10	—
<i>icterohaemorrhagiae</i>	—	—	—	—	—	<10	<10	10	40	—	—
<i>sejroe</i>	—	—	—	—	10	13	—	13	32	16	10
<i>autumnalis</i>	—	—	—	—	—	—	<10	—	16	20	14
<i>biflexa</i> H 48	640	403	508	1,280	1,280	1,613	2,032	1,613	1,280	640	403

^a Reciprocal of serum dilution, expressed as the geometric mean of positive reactions; — indicates negative reactions.

ulating strain were detected. From week 10 to month 6 postexposure, all three animals showed consistent low-level and increasing multiple agglutination titers which ranged from 1:10 to 1:80 to the pathogenic serotypes. Goat sera collected at 6 months postexposure were also examined by employing formolized antigens, after absorbing the sera with formolized *L. biflexa* H 48 cells and after treatment with 0.05 M 2-mercaptoethanol. These results are shown in Table 3. In the comparative MA tests, the expected higher se-

rum agglutinin titers obtained with the formolized antigens were removed by either absorbing with the *L. biflexa* strain or by 2-mercaptoethanol treatment. These results indicate that the cross-reactivity of the goat sera to pathogenic serotypes is due to a common antigenic factor present in the saprophytic strain and is apparently released in vivo from viable organisms or by eventual lysis of intact leptospiral cells. The removal of agglutination by 2-mercaptoethanol treatment also shows that this serological activ-

TABLE 3. Comparative results of agglutination tests with formalized antigens on absorbed and 2-mercaptoethanol-treated sera from three goats taken 6 months after subcutaneous inoculation with live *L. biflexa* H 48 organisms

<i>Leptospira</i> serotype formolized antigen	Serum agglutinating titer with antigen ^a		
	Un- treated	Absorbed with for- molized <i>L.</i> <i>biflexa</i> H 48 cells	Treated with 2-mer- capto- ethanol 0.05 M
<i>pomona</i>	127	20	—
<i>australis</i>	16	—	—
<i>ballum</i>	10	10	—
<i>grippotyphosa</i>	25	10	—
<i>tarassovi</i>	—	—	—
<i>hebdomadis</i>	10	—	—
<i>bataviae</i>	40	10	—
<i>canicola</i>	403	20	<10
<i>hardjo</i>	10	—	—
<i>wolffi</i>	—	—	—
<i>pyrogenes</i>	160	10	—
<i>icterohaemorrhag-</i> <i>iae</i>	25	<10	—
<i>sejroe</i>	64	—	—
<i>autumnalis</i>	40	—	—
<i>biflexa</i> H 48	508	—	—

^a Reciprocal of serum dilution expressed as the geometric mean of positive reactions; — indicates negative reactions.

ity is restricted to the immunoglobulin M class of proteins and is a nonspecific reaction.

Sonically treated soluble antigens in immunodiffusion and IEP. In GD tests a sharply defined connective precipitating line showing antigenic identity was produced with rabbit antileptospiral sera to pathogenic serotypes and with the soluble *L. biflexa* H 48 antigen (Fig. 1). The same precipitin line was also produced with the sera and sonically treated soluble antigens of serotypes *canicola*, *icterohaemorrhagiae*, and *pomona*. The line was located centrally between the antigen and serum wells. In selective staining, the line stained positively for protein and negatively with oil red O and alcian blue stains for lipids and polysaccharides. The precipitin was not removed by treatment with 5.0% sodium citrate and was not produced in control tests with normal rabbit and goat sera, uninoculated culture media, or physiological saline. With some of the positively reacting sera, one or two additional diffuse precipitin bands were formed depending upon the antigen preparation used. These were, however, located either adjacent to the serum well or close to the antigen well.

To determine the specificity and electrophoretic mobility of the broadly reacting common

antigen, IEP of *L. biflexa* H 48 and serotypes *canicola* and *icterohaemorrhagiae* soluble antigens were performed and examined against each of the three corresponding antisera. Each antigen revealed a single fine arc for each of the three antisera. This common arc showed slight anodic mobility with antigenic identity and was located just outside the antigen well in the zone corresponding to β -migrating proteins (Fig. 2). The characteristic arc was absent after absorbing each of the three antisera with homologous or heterologous soluble antigens and was not affected after absorbing the sera with live leptospirae, treating the sera with 2-mercaptoethanol (0.1 M and 0.05 M), or heating the antigens and sera to 56°C for 1 h. Variation in intensity of the precipitin reaction of the common arc was observed with the *L. biflexa* H 48 antisera. Rabbit immune sera prepared with cultures grown in Fletcher medium as inocula required concentration ($\times 3$) to show precipitin activity at the same intensity as antiserum prepared by employing BA-P80-grown cultures. The *L. biflexa* soluble antigen, however, was equally sensitive when prepared with cells grown in either BA-P80 or Stuart liquid media.

Gel filtration of *L. biflexa* H 48 soluble antigen on Sephadex G-200. Gel filtration of the *L. biflexa* H 48 soluble antigen on a column of Sephadex G-200 enabled isolation of the CA produced in GD and IEP with antileptospiral sera. The CA was eluted from the column in the exclusion volume corresponding to a molecular weight of approximately 140,000. A single precipitin formation was observed when the active pooled fractions were dialyzed, concentrated, and tested in GD and IEP tests with sera from rabbits immunized with the *L. biflexa* strain and pathogenic serotypes (Fig. 3). Similarly, when the globulin of the three goat sera obtained 6 months after exposure to *L. biflexa* H 48 organisms was precipitated at 50% saturation with ammonium sulfate, dialyzed, concentrated, and tested, the same single precipitin line developed with the active antigen fraction.

Immunogenicity of common antigens. The CA precipitate produced in agar by IEP with *L. biflexa* H 48 soluble antigen after the addition of the serotype *icterohaemorrhagiae* antiserum was antigenic for the two immunized rabbits. Precipitating 2-mercaptoethanol-resistant antibodies were demonstrated in GD tests with homologous and heterologous soluble antigens 2 weeks after the last immunization (Fig. 4). Sera of both rabbits agglutinated formalized or formalized and heated suspensions of *L. biflexa* H 48 and serotypes *canicola*, *icterohaemorrhagiae*, and *pomona* with titers as high as

FIG. 1. Immunodiffusion pattern with soluble *L. biflexa* H48 antigen (inner wells) and the following antisera to serotypes (clockwise in the peripheral wells): 1, *pomona*; 2, *australis*; 3, *ballum*; 4, *grippotyphosa*; 5, *tarassovi*; 6, *bataviae*; 7, *canicola*; 8, *hardjo*; 9, *pyrogenes*; 10, *icterohaemorrhagiae*; 11, *autumnalis*; and 12, *L. biflexa* H 48.

1:80, whereas only the *L. biflexa* strain was agglutinated by employing live antigens and at titers from 1:320 to 1:640. When one of the immune rabbit anti-CA sera was gel filtered on a column of Sephadex G-200, the active fractions in GD and IEP were eluted in the exclusion volume corresponding to immunoglobulin G.

The whole soluble antigen of *L. biflexa* H 48 was evaluated for possible protective effects in hamsters. The results of these experiments are presented in Table 4. As shown, 16 hamsters vaccinated with the saprophyte soluble antigen and then challenged with a virulent strain of serotype *icterohaemorrhagiae* 3 weeks later produced more deaths at 7 to 9 days postchallenge than the 16 control hamsters that were infected only with the challenge culture. The infecting strain was recovered from all seven surviving animals of both groups from kidney cultures performed 3 weeks after challenge.

Similarly, the lethal enhancing effect was further demonstrated when hamster GD-reacting immune serum to the *L. biflexa* H 48 soluble antigen was either subcutaneously administered to 10 animals on the day before challenge or after mixing the immune serum with the *icterohaemorrhagiae* serotype at the time of challenging 10 other animals. In both experiments there were no surviving animals beyond day 9 postchallenge. No deaths occurred in any of the immunized groups of control hamsters that received only the *L. biflexa* soluble antigen or hamster immune serum.

In a complementary experiment, 10 hamsters that were immunized with the *L. biflexa* H 48 soluble antigen were experimentally infected with a nonlethal *hardjo* serotype for possible protection against renal leptospirosis. Kidney cultures performed on these animals 3 weeks later showed that leptospires were easily re-

FIG. 2. IEP comparison of *L. biflexa* H 48 antigen (center well A) showing one precipitin arc migrating slightly towards the anode with homologous immune rabbit serum (upper trench) and serotype icterohaemorrhagiae antiserum (low trench). IEP of the soluble antigen of serotype icterohaemorrhagiae (center well B) with *L. biflexa* H 48 antiserum (upper trench) and canicola antiserum (lower trench). Anode to the right, cathode to the left.

FIG. 3. Precipitin lines produced by rabbit hyperimmune sera to *L. biflexa* H 48 (well 1) and serotypes icterohaemorrhagiae (well 2), and canicola (well 3) by GD against the active fraction of the soluble *L. biflexa* H 48 antigen obtained by Sephadex G-200 chromatography (center well). Well 4 contained normal rabbit serum.

covered from renal tissue of all of the hamsters in BA-P80 semisolid media, and no cross-protection occurred.

DISCUSSION

In a previous paper (15) it was shown that a *L. biflexa* isolate obtained from the renal tissue of a horse was agglutinated by most equine sera containing antibody to pathogenic *L. interrogans* serotypes. Horse sera showing 2-mercaptoethanol-sensitive MA agglutinins to as many as 13 antigenically distinct pathogenic serotypes were removed by absorbing the sera with this saprophyte. The present studies were designed to further elucidate the cause of persistent, multiple nonspecific MA agglutinins that may be encountered in diagnostic tests with mammalian sera. An attempt was made to isolate a common antigen of saprophytic and pathogenic leptospires and to determine some of its serological and chemical properties.

Although the goat is susceptible to experimental infection with leptospires, naturally occurring caprine leptospirosis is fairly uncommon. This animal species was selected as a model to show the development of serological cross-reactivity among pathogenic *L. interrogans* serotypes after inoculation with the *L. biflexa* H 48 strain. Beginning at 10 weeks post-inoculation and continuing throughout the 6-month study period, multiple cross-agglutination reactions occurred to the battery of live pathogenic leptospiral antigens. The late appearance of cross-reactive agglutinins apparently results from the establishment of the saprophyte in the host with the eventual release of CA from viable organisms or during lysis and degradation of old cells. At 6 months post-inoculation the agglutinins were 2-mercaptoethanol sensitive and were removed by absorption with the *L. biflexa* strain. Precipitated and concentrated gamma globulin of the goat sera also produced precipitin activity.

Preliminary separation procedures to isolate

FIG. 4. Production of antibodies by rabbits immunized with precipitate formed in agar by serotype *icterohaemorrhagiae* antiserum after IEP of the *L. biflexa* H 48 antigen (wells 1 and 3). Wells 2 and 4 contained rabbit antisera to serotypes *icterohaemorrhagiae* and *canicola*, respectively. The active Sephadex G-200 fraction of *L. biflexa* H 48 antigen was placed into the center well.

TABLE 4. Summary of immunization trials in hamsters with *L. biflexa* H 48 soluble antigen and antiserum

Immunization procedure	Mortality (no. dead/no. alive) by day postchallenge:					
	5	7	9	11	13	15
<i>L. biflexa</i> H 48 soluble antigen administered intraperitoneally 2 weeks before challenge (1.25 mg/animal) ^a	0/16	5/11	6/5	2/3	0/3	0/3
Hamster immune <i>L. biflexa</i> H 48 serum administered subcutaneously (0.5 ml) day before challenge	0/10	7/3	3/0	0/0	0/0	0/0
Hamster immune <i>L. biflexa</i> H 48 serum administered intraperitoneally (0.25 ml) at time of challenge	0/10	9/1	1/0	0/0	0/0	0/0
None	0/16	1/15	5/10	3/7	3/4	0/4

^a Animals were challenged intraperitoneally with 0.25 ml of a virulent suspension of serotype *icterohaemorrhagiae* containing approximately 3.8×10^6 organisms per ml.

a CA involved extractions of washed leptospiral cells with sodium dodecyl sulfate by using methods described by Yanagawa et al. (25). When it became apparent that non-immunological precipitin lines in GD occurred with normal control

sera, similar to those reported by Cho and Feng (6) and Palmer et al. (17), the technique was abandoned. Subsequently, a broadly reacting soluble antigen was obtained from the *L. biflexa* strain with formaldehyde treatments and sonic

disruption which stimulated the production of both agglutinins and precipitins in rabbits. In chemical tests the sonically treated antigen is mainly protein, negative for lipids or polysaccharides and is relatively heat stable. In GD tests with antileptospiral sera the precipitinogen is not affected by dialysis. In IEP, before and after gel filtration, the CA activity appears in the zone corresponding to β -migrating proteins. Similar sonically treated preparations of serotypes *canicola*, *icterohaemorrhagiae*, and *pomona* showed in GD and IEP tests that each antigen contained a precipitinogen identical with one of the precipitinogens of the soluble *L. biflexa* antigen. With homologous or heterologous rabbit hyperimmune antileptospiral sera, a single connecting precipitin line is formed that is 2-mercaptoethanol resistant. The precipitin line is absent after absorbing the sera with each of the soluble antigens.

The anatomical location of the CA appears to be either somatic or subsurface in that Formalin treatments to leptospires remove the enveloping sheath and damage the protoplasmic spiral (24) and heating at 100°C destroys the axial filament as well as the outer envelope (8). The chemical and serological properties of the CA obtained from leptospires in this study suggest its possible relationship to a subsurface protein "d" antigen of undetermined serospecificity that was isolated from a strain of *L. biflexa* by Graves and Faine (10, 11). The CA protein preparation differs from other broadly reacting antigens known to be present in leptospires in that seroreactivity of the latter are attributed to polysaccharides (8).

In view of the broad antigenic cross-reactivity observed in the current study with the *L. biflexa* H 48 strain and pathogenic *L. interrogans* serotypes, the potential of the CA of *L. biflexa* as a polyvalent vaccine for protection against death or protection from kidney infection was evaluated in hamsters. Although immunity to infection is regarded as being serotype specific, there is evidence that a cross-immunity mechanism exists (18). Most leptospiral vaccines in use today are made with whole, killed virulent leptospires or live avirulent organisms of which the antigenic determinants involved in protection are unknown. Recent studies by Auran et al. (2) and Bey et al. (3) have shown that the leptospiral outer envelope is a potent immunogen in hamsters. Faine (8) has also reported that immunity could be transferred passively with antiserum prepared from an externally located lipopolysaccharide somatic antigen.

Immunization of hamsters with the CA of *L. biflexa* H 48 and challenge with a virulent strain of serotype *icterohaemorrhagiae* and a nonlethal strain of serotype *hardjo* showed that pro-

tection against death and kidney infection did not occur. In contrast, the findings that more of the hamsters immunized with the CA died earlier than the unvaccinated controls was unexpected. When hamster anti-CA antibodies were used in passive transfer of immunity experiments against the challenge infection, the lethal capacity of the serotype *icterohaemorrhagiae* challenge strain was also increased. Similarly, when the hamster anti-CA immune serum was mixed with the challenge strain in vitro before infecting, identical results were obtained. These findings suggest the possibility that the phenomena associated with immunological enhancement may be involved. Further studies, however, are required to clarify this point.

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