animals were injected into separate areas of the skin with 40 μg of cell walls and 40 μg of protoplasm of homologous organisms. At 48 hours postinjection, protoplasm had caused little reaction at the site of inoculation, whereas cell walls had produced severe reaction in the 6 animals that had received an initial injection of cell walls. The lesions were red and edematous and varied from 20 x 7 to 38 x 35 mm in diameter. The 3 animals that had received protoplasm as the initial inoculum developed flat lesions varying from 7 x 7 to 13 x 13 mm in diameter following the second inoculation of cell walls, although the 3 normal animals failed to respond. These results demonstrate that cell walls contain fractions capable of inducing hypersensitivity of the delayed type and of eliciting skin reactions in sensitized animals.

Initial lesions produced by mycobacteria injected into the skin of rabbits do not appear until after 4 or 5 days. In this respect these organisms differ from *B. tularense* and *Hemophilus pertussis* suspensions which produce visible effects in rabbits within 24 hours(10). Initial and sensitivity reactions produced by fractions of mycobacteria can be differentiated by their time of appearance. Details of these and other studies will be discussed later.

Summary. A technic was described for separation of cell wall and internal protoplasm from cells of *Mycobacteria*. Data are presented which demonstrated that cell walls produced lesions when injected intradermally into rabbits, whereas protoplasm failed to produce these lesions. Cell walls were also shown to be capable of inducing hypersensitivity of the delayed type. Separation of these morphological elements resulted in definite separation of one element possessing certain biological activities from material not possessing these characteristics. Such initial fractionation of the cell should facilitate purification and characterization of substances which elicit typical tissue responses to infection with tubercle bacilli.

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Serological Studies on Hemolytic Antigen from Leptospira. (24012)

C. D. COX, R. C. STOVER, AND R. W. TREICK

Department of Microbiology, School of Medicine, State University of South Dakota, Vermillion

A hemolytic (HL) procedure(1), which was a modification of the hemagglutination test of Chang and McComb(2), was reported from this laboratory. More recent reports (3,4) describe the preparation of this HL antigen from non-pathogenic *Leptospira biflexa*, its standardization and stabilization, and its evaluation in serodiagnosis of human leptospirosis. This evaluation demonstrated the generic specificity of the HL antigen, as well as its marked sensitivity with rabbit and human antisera, and suggested that currently used microscopic agglutination tests could be advantageously supplanted by the HL procedure in serodiagnosis of human leptospirosis. In view of its potential usefulness, further serological studies on HL antigen were carried out.

Materials and methods. HL antigen was prepared from *L. biflexa* as previously de-

scribed(3). Antisera were prepared in rabbits by immunizing them intravenously with suspensions of leptospires(3), suspensions of leptospires previously extracted with ethanol or ether, the HL antigen, or HL antigen adsorbed onto rabbit erythrocytes. A total of 10 ml of HL antigen, containing at least 1024 units/ml(3), was administered over 2 weeks. HL antigen was adsorbed onto sheep or rabbit rbc as previously described(3), and approximately 20 ml of 10% sensitized rabbit rbc (exposed to 640 units HL antigen/ml) were given for 2 weeks. Sheep and rabbit rbc were sensitized with HL antigen in like manner for use in adsorbing HL antibodies from sera. Pre-immunization blood samples were obtained and proved to contain no leptospiral antibodies. Antisera were obtained from rabbits during their initial peak titers. Antiserum adsorptions were carried out at room or refrigerator temperatures as multiple adsorptions, with more than sufficient antigen necessary to remove homologous antibodies. Complement-fixation tests were performed according to usual Kolmer procedure(5), using ½ volumes of all reagents, 4 units of hemolysin, 2 full units of complement, and 1% sheep erythrocytes. Precipitin tests were performed as described by Rothstein and Hiatt(6). HL and microscopic agglutination (MA) titrations were performed as previously described (1,3).

Results. Preliminary studies were made on the chemical nature of HL antigen. Negative ninhydrin and positive Biuret and Molisch tests were obtained on freeze-dried antigen. No significant alteration in titer was obtained by twice deproteinization by the Sevag method, nor by extraction of dried antigen with ether, acetone or chloroform for 2 hours at room temperature. In 24 hours at 37°C and pH 7.4, a final concentration of Bacto-trypsin of 0.1 mg/ml of a solution of antigen containing approximately 2 mg/ml did not alter the titer. Higher concentrations of trypsin, even after inactivation of the enzyme by heat, gave inconclusive results presumably because of its adsorption to erythrocytes. In 4 hours at 37°C 2.5 M urea reduced the titer to ½, and 5 M urea reduced it to ¼ its original activity.

TABLE I. Homologous Antibody Response in Rabbits.

Antisera *vs*	Titers (reciprocal) MA	HL
L. biflexa	10,000	10,000
HL antigen	100	1,000
HL antigen sens. rabbit RBC	10	1,000
L. biflexa ext. with 50% ethanol	10,000	—
75% "	10,000	10
50% ether	10,000	1,000

Extraction with N/5 HCl in boiling water bath for 10 minutes reduced the titer to 1/16, and similar treatment with N/5 NaOH reduced the titer to 1/64 of its original activity. Additional studies supported Chang's earlier reports that his ESS antigen was stable to temperatures of 100°C for 15 minutes, was not precipitated by trichloracetic acid, was soluble in 50-66% ethanol, relatively soluble in ethanol concentration of 80%, and insoluble in 90% ethanol. Chang(2) suggested that his antigen was "nonprotein," which is supported by above data; however, the positive Biuret and low order of inactivation by urea leads one to conclude that the polysaccharide is probably complexed with some low molecular weight nitrogenous material.

Homologous antibody responses in rabbits to various antigenic preparations from *L. biflexa* are shown in Table I. As anticipated, whole cells of *L. biflexa* stimulated formation of both MA and HL antibodies. HL antigen was also antigenic when injected by itself or adsorbed onto rabbit erythrocytes. Furthermore, it stimulated formation of homologous HL antibodies, and in addition some agglutinating antibodies. Further evidence was obtained for solubility of HL antigen in 50% ethanol, because 3 extractions of *L. biflexa* with 50% ethanol(1,3) rendered cells incapable of stimulating formation of HL antibodies. Similar extraction of *L. biflexa* with 75% ethanol did not completely remove the HL antigen, nor did extraction with 50% ether.

Tables II and III depict results of adsorption of antisera against *L. biflexa* and rabbit erythrocytes sensitized with HL antigen, with homologous and heterologous antigens. In

TABLE II. Adsorption Studies on *L. biflexa* Antiserum.

Adsorbed with antigen	Titers (reciprocals) MA		HL
	L. biflexa	*L. canicola*	
—	10,000	—	10,000
L. biflexa	—	—	—
L. canicola	10,000	—	—
L. biflexa ext. with 50% ethanol	—	—	10,000
L. biflexa ext. with 75% ethanol	—	—	—
HL sens. sheep RBC	10,000	—	—

Table II *L. biflexa* removed both type-specific (agglutinating) and genus-specific (HL) antibodies, whereas *L. canicola* removed only the HL antibodies. *L. biflexa* cells extracted with 50% ethanol, which previously was shown to extract the HL antigen (Table I), removed only type-specific antibodies; whereas *L. biflexa* extracted with 75% ethanol retained sufficient HL antigen to remove both types of antibodies. It is interesting to note that sheep cells adsorbed with HL antigen removed only the genus-specific homologous antibodies, which would support previous findings (Table I) that very minimal amounts of type-specific antigen adsorbed onto sheep or rabbit erythrocytes. Similar results were obtained by analogous adsorptions of *L. canicola* antiserum, which was included in these studies because of the heterologous relationships between *L. biflexa* and *L. canicola*. In Tables I and III it may be seen that the HL antigen not only contained some type-specific antigen, but that sufficient amounts of it remained adsorbed to rabbit erythrocytes after 3 washings to induce minimal, type-specific, agglutinating antibody response. Adsorption with *L. biflexa* removed both types of antibodies, whereas adsorption with *L. canicola* removed only the HL antibodies. Adsorption with sheep erythrocytes sensitized with homologous HL antigen removed both antibodies (Table III). Adsorption of HL antiserum with *L. biflexa* extracted with 50% ethanol removed the type-specific antibodies as expected, but also reduced the HL titer, which was not expected since *L. biflexa* extracted with 50% ethanol had previously been shown (Table I) incapable of stimulating formation of HL antibodies. These 2 experiments were carried out with separate batches of extracted leptospiral cells, and it seems probable in view of later duplicate experiments that HL antigen represented in this Table had not been completely extracted from the latter batch of cells. Later studies indicated that a fourth extraction in 50% ethanol carried out in a 56°C waterbath for 2 hours may increase the efficiency of extracting HL antigen.

TABLE III. Adsorption Studies on Antiserum against Rabbit RBC Sensitized with HL Antigen.

Adsorbed with antigen	Titers (reciprocals) MA		HL
	L. biflexa	*L. canicola*	
—	10	—	1000
L. biflexa	—	—	—
L. canicola	10	—	—
HL sens. sheep RBC	—	—	—
L. biflexa ext. with 50% ethanol	—	—	100
L. biflexa ext. with 75% ethanol	—	—	—

It was apparent that, by suitable adsorptive procedures, antisera could be prepared which contained only MA or HL antibodies. This permitted studies on precipitating and complement-fixing properties of each of the 2 types of antibodies. Results of precipitin tests using HL antigen and various antisera, adsorbed and unadsorbed, are shown in Table IV. HL antigen gave precipitation in low dilutions only with antisera containing MA antibodies, and this was apparently possible because of MA antigen impurities previously shown to exist in HL antigen. These findings suggest that precipitation was not due to the HL antigen-antibody reaction, which was substantiated by the very low titer obtained with antiserum against HL antigen and the finding that precipitating activity was adsorbed from such serum with 50% ethanol extracted *L. biflexa*.

L. biflexa cells, 50% ethanol extracted *L. biflexa*, and HL antigen were used as antigens in complement-fixation tests on homologous and heterologous rabbit antisera (Table V). All 3 antigens fixed complement only with antisera containing type-specific, MA anti-

TABLE IV. Precipitin Tests.

Rabbit antisera		Titer (reciprocals)		HL antigen dilutions				
vs	Adsorbed with	HL	MA	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
L. biflexa cells		1,000	10,000	+*	+	±	—	—
L. biflexa ext. with 50% ethanol	Rabbit RBC ads. with HL antigen	<10	"	+	+	—	—	—
HL antigen		1,000	100	+	—	—	—	—
Rabbit RBC ads. with HL antigen	*L. biflexa* ext. with 50% ethanol	10,000	<10	—†	—	—	—	—
Non-immunized		<10	"	—	—	—	—	—

* Positive results indicated were obtained only with antiserum dilutions of 1:4 or lower.
† Negative results obtained with undiluted antiserum, and double dilutions through 1:1024.

bodies. That complement-fixation with HL antigen was due to MA antigen impurities was shown by finding that antiserum with a high titer of only HL antibodies showed no complement-fixation with any of the 3 antigens. The possibility was considered that complete lysis (no fixation) might not be due to lack of complement-fixation, but rather to the HL reaction itself. Accordingly, appropriate controls lacking only hemolysin were included in this series of complement-fixation tests. Absence of hemolysis in these controls indicated that lack of complement-fixation in the indicated tests was actually due to absence of complement-fixation between HL antibodies and their homologous antigen in the impure HL antigen preparation.

Discussion. Apparently the HL antigen-antibody system is not operative in leptospiral agglutination, precipitating, or complement-fixing reactions. However, the HL antigen is undoubtedly similar, if not identical, to the hemagglutinating antigen (ESS) described by Chang and McComb(2). Complement-fixing antigens described in the past include a deproteinized preparation from lysed leptospiral cells(7), supernatants of whole cultures(8), ethylene glycol extracts of leptospirae(9), sonically disrupted leptospirae(10) and suspensions of whole leptospiral cells(11). Relationships between active antigens in each of these preparations and their serotype specificity remains obscure. A more recent article by Rothstein and Hiatt described a clear precipitinogen obtained by electrodialysis of 70% ethanol extracts of leptospirae. They suggested that this precipitinogen probably contained a mixture of peripheral, type-specific antigen(P) and somatic, genus-specific, lipopolysaccharide antigen (S). They also suggested that the ESS antigen of Chang(2), which is probably very similar or identical to the HL antigen, probably consisted mainly of their S antigen. Although their precipitinogen and the HL antigen both contain polysaccharide, are heat stable and resistant to trypsin, there are several areas of divergence. The precipitinogen possessed complement-fixing

TABLE V. Complement-Fixation.

Rabbit antisera		Titer (reciprocals)		Reciprocal of CF titers *vs* antigens*		
vs	Adsorbed with	HL	MA	*L. biflexa* cells	*L. biflexa* ext. with 50% ethanol	HL antigen
L. biflexa cells		1,000	10,000	>128	>128	>128
L. biflexa ext. with 50% ethanol	Rabbit RBC ads. with HL antigen	<10	"	"	"	"
Rabbit RBC ads. with HL antigen	*L. biflexa* ext. with 50% ethanol	10,000	<10	—†	—	—

* All antigens used in dilutions of 1:8 to 1:32, based upon optimal activity determined by block titrations.
† No fixation with undiluted serum, nor with double serum dilutions through 1:128.

activity, and failed to sensitize human erythrocytes to hemagglutination. Furthermore, HL antibodies were easily removed by adsorption with leptospiral cells containing HL antigen, which was contrary to the findings of Rothstein and Hiatt with their precipitinogen. It is possible that variance in adsorptive technics between different laboratories may account for such differences. In this work leptospirae used in adsorptions were freshly harvested, non-formalinized cells. The most striking divergence is the difference in precipitating abilities. The relationship of HL antigen to the antigens of Rothstein and Hiatt remains unclear, although the HL antigen shows more resemblance to S than to P antigen. It is probable that the HL antigen and ESS, as suggested by Rothstein and Hiatt(6), contain their S antigen, and that differences in characteristics reflect differences in methods of preparation. Further clarification of relationships between leptospiral antigens and their chemical natures must await investigations using purified preparations, and purification will be difficult until leptospirae can be cultivated in chemically-defined, non-protein media.

Summary. Use of HL antigen adsorbed onto erythrocytes, and *L. biflexa* repeatedly extracted with 50% ethanol, permits preparation of antisera containing only type-specific (agglutinating) or genus-specific (HL) antibodies. HL antigen, as extracted from *L. biflexa* with 50% ethanol, contained small quantities of type-specific antigen, which was capable of adsorbing onto rabbit and sheep erythrocytes along with the HL reactive antigen. However, the type-specific antigen was apparently not operative in the hemolytic reaction. HL antibodies would not precipitate HL reactive antigen, agglutinate leptospiral cells, nor fix complement in the presence of HL reactive antigen or *L. biflexa* cells. Preliminary studies indicate that HL antigen contains polysaccharide which is probably complexed with some low molecular weight nitrogenous material.

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Inhibition of Arthus Reaction During Protracted Systemic Anaphylaxis in Mice and Guinea Pigs.* (24013)

SANFORD H. STONE (Introduced by J. Freund)

DHEW, PHS, NIH, NIAID, Bethesda, Md., and Public Health Research Inst., New York City

During studies on anaphylaxis in mice sensitized with antigens incorporated in paraffin oil adjuvants, it was found that protracted anaphylactic shock elicited by intralabial injections of antigen inhibited Arthus reactions at sites of injection. Furthermore, when mice sensitized to both hemocyanin and human serum albumin were injected into the lip with

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