Ethylene-Glycol Extracts of Leptospirae in Complement-Fixation Tests. (22450)

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(Introduced by Gilbert Dalldorf.)
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Recognition of leptospirosis as worldwide public health problem has developed from recent studies(1-4). It has also been established that as many as 9 serotypes of pathogenic leptospirae occur in the United States (4-6). In search for diagnostic test that is easily performed and adapted to large-scale examinations, several workers have investigated complement-fixing antigens. The antigens considered have been crude whole-cell preparations grown in artificial media(7,8), formalinized infected chorioallantoic fluids of chick embryo(9), products of sonic vibration (10), weakly phenolized salt solution suspensions of microorganisms(11), and a soluble-specific antigen liberated into medium in which leptospirae are propagated(12).

Broad spectrum leptospirae antigens obtained by sonic vibration are reportedly sensitive and specific in acute leptospirosis and permit the use of fewer strains in diagnostic examinations(13). Since the wide scope of these antigens should offer an easier approach to detection of leptospiral antibodies of various serotypes, isolation of a similar fraction was attempted by means of ethylene-glycol extraction. Ethylene-glycol extracts derived as described below yielded complement-fixing antigens which were easily prepared and sensitive in the detection of leptospiral antibodies.

Material and methods. Antigens were prepared from type strains of leptospirae grown in modified Korthof medium at 30°C for 14 days. Serotypes included two strains each of *Leptospira icterohemorrhagiae, L. canicola,* and *L. pomona.** Leptospirae were killed by formalin (final 0.2% concentration) and collected by centrifugation at 10,000 rpm for 20 minutes at room temperature in Sorvall angle head centrifuge. The extraction method was similar to Morgan's(14). After washing twice with isotonic phosphate buffered sodium chloride solution (pH 7.2) cells were suspended, using a syringe and needle, in volume of ethylene glycol representing 2% of original culture fluid. The suspension was shaken with glass beads. Extracts recovered as supernates after centrifugation at 10.000 rpm for 1 hour were dialyzed first against running tap water and finally against phosphate buffered sodium chloride solution. The opalescent solution recovered was heated 15 minutes in boiling water bath. Cell residues were extracted a second time with one-half the volume first employed. After preliminary testing to determine potency, satisfactory extracts of single strains were combined. *Rabbit antiserum* was produced with each strain used in antigen production. Rabbits were immunized by intramuscular injection of killed culture in adjuvant emulsion(15). *Test procedure.* Complement-fixation test technic was that employed in this laboratory(16) using three 50% units of complement with fixation period of 4 hours at refrigerator temperature (3-6°C). Reactivity is expressed in Tables II and III as Titer equals $D(K_{SA}-1)$.

Results. Rabbit antiserum: Ethylene-glycol extracts of leptospirae fixed complement in the presence of rabbit antisera. The antigens were sufficiently potent to permit dilution and were neither lytic nor anticomplementary in the range of maximum reactivity with homologous serum. Inhibition of complement fixation was evident in zones of anti-

* *L. icterohemorrhagiae* 3228—Dr. P. K. Olitsky. Isolated from wild rat. 5309—Dr. Martha K. Ward. Wijnberg. *L. canicola* 39660—Dr. K. F. Meyer. Isolated from blood of icteric dog 1937 (*J. Am. Vet. Med. Assn.*, 1939, v95, 710). 5305—Dr. Martha K. Ward, Communicable Disease Center, Chamblee, Ga. *L. pomona* 5160—Col. R. H. Yager. Veterinary Division Army Medical Center, Washington, D. C. Australis C. 5310—M. K. Ward, Communicable Disease Center, Chamblee, Ga. L9 NIH.

TABLE I. Specificity of Ethylene-Glycol Extract Antigens of Leptospirae in Complement-Fixation Tests. Reactivity expressed as percentage of homologous antiserum.

		Rabbit antiserum produced with strain:					
		L. ictero-hemorrhagiae		*L. canicola*		*L. pomona*	
Extract antigen of strain:		3228	5309	39660	5305	5160	5310
L. icterohemorrhagiae	3228	*100*	10	33	33	33	33
	5309	10	*100*	33	66	33	66
L. canicola	39660	10	10	*100*	100	33	33–66
	5305	10	10	100	*100*	33	33–66
L. pomona	5160	10	10	66	33–66	*100*	100
	5310	10	10	33	33–66	100	*100*
"Sonic vibrated leptospiral antigen"*							
L. pomona		10		33		*100*	

* Antigen kindly furnished by Col. C. A. Gleiser, Army Medical Service Graduate School.

gen and serum excess.

As shown in Table I antigens had pronounced serogroup specificity in tests with rabbit antiserum. In addition, type specificity within a serogroup was demonstrated with strains *L. icterohemorrhagiae* (3228) and *L. icterohemorrhagiae* Wijnberg (5309). The degree of cross reactivity between serogroups was generally less at point of antigen concentration optimum for homologous antiserum. Results of comparative tests with sonic vibrated antigen are included in Table I. Similar relationships were demonstrated in tests refrigerated 16-22 hours.

Human serum. In tests with sera from human cases of leptospirosis diagnosed serologically, extracts appeared less serogroup specific than with immune rabbit sera, although not so broadly genus specific as to assure detection of all leptospiral serotypes with individual strain antigen (Table II).

Triple antigen. To obtain an antigen for screening tests, extracts of each of the 3 serotypes employed were combined to form a pool. Potency of the triple antigen thus prepared was equal to or better than that of the individual components. In some cases reactivity was enhanced, the antigen being more sensitive (Table II). Broad valency was shown by reactions obtained with specimens containing serum antibody of unrepresented types (*L. autumnalis* and *L. grippotyphosa* by agglutination).

Tests with specimens from 202 presumably normal human adults yielded a reaction rate of 9%; however, 7% of these were of weak reactivity (titers under 10), with some unconfirmed on retest.

TABLE II. Reactivity of Ethylene-Glycol Extract Antigens of Leptospirae in Complement-Fixation Tests with Serum from Human Cases of Leptospirosis.

Extract antigen of strain:	Complement-fixation test titers with serum from cases: 1	2	3	Killed culture antigen of strain:	Endpoint of agglutination in tests of serum from cases: 1	2	3
1. *L. icterohemorrhagiae*				*L. icterohemorrhagiae* AB	32	8192	4096
a 3228	10	40	90				
b 5309	10	340	110				
2. *L. canicola*				*L. canicola*	0	256	2048
a 39660	10	20	60				
b 5305	10	30	70				
3. *L. pomona*				*L. pomona*	512	32	0
a 5160	10	30	60				
b 5310	10	50	80				
Triple antigens:							
1. Pool of 1a, 2a, 3a	20	120	110				
2. Pool of 1b, 2b, 3b	10	380	140				

TABLE III. Serologic Examinations of Serial Specimens from Human Cases of Leptospirosis.

Case No.	Collection date	Complement-fixation with triple antigen (ethylene-glycol extracts)	Hemagglutination*	Agglutination with killed culture of strain:† *L. icterohemorrhagiae* AB	*L. canicola*	*L. pomona*
1	8/23/54	220	1280			
	9/10	170	1280			
	10/28	20	160	32	0	512
2	7/11/55	280	640	4096	0	0
	8/15	120	160	8192	256	32
	10/24	10–20	160	1024	0	0
3	9/22/55	110	320			
	30	110	160	4096	2048	0

Clinical data:

Case 1. Male, age 30. Hospital admission 8/12/54 with malaise, chills, and fever for 2 days. Temp. between 101° and 104.6°. Complained of heaviness of head, stiff neck, and severe low back pain. Employed in slaughter house eviscerating calves, sheep, and lambs. Denied contact with adult cattle or pigs.

Case 2. Male; butcher. Onset of illness 7/2/55. Admitted to hospital 7/8/55 with temp. of 103°, headache, nausea, vomiting, and muscle aches particularly in calves of legs. Jaundiced 3 days; several bouts of loose stools. Improved without antibiotics. 7/19/55 WBC 26,000; CSF: elevated protein and cells. Drank Genesee River water in mid May 1955. Cut finger on meat cutting job June 1955. Contact with pet dog ill in June.

Case 3. Male, age 21. Admitted to hospital 8/31/55 with 2-day history of abdominal pain, nausea, vomiting, chills, fever and headache. Temp. 105°, muddy sclera, and abdominal tenderness. Jaundice 5th hospital day. Patient swam in canal in Tonawanda 10 days before admission.

* Chang's technic(19). Reactivity is expressed as highest serum dilution exhibiting definite agglutination (2+ or greater).

† Tests kindly performed at Leptospira Research Laboratory, Communicable Disease Center, Chamblee, Ga., Mrs. Mildren Galton in charge. Agglutination reported with certain of other serotypes employed was less than maximum recorded here. All dilutions started in range 1:16-1:160.

Three human cases of leptospirosis were brought to our attention during this study. Some of the serial specimens were insufficient for complete serologic examination. With at least one specimen from each patient, however, confirmatory data were furnished by agglutination tests with strains of numerous serotypes of leptospirae.† Results of serologic tests, agglutination, complement-fixation (with triple antigen), and hemagglutination, are recorded in Table III.

Our ethylene-glycol extract antigens are now routinely employed in serologic examinations for evidence of leptospirosis and have yielded reproducible results. They have retained reactivity when stored at −20°C or at 3-6°C for at least 3 months.

Composition of ethylene-glycol extracts of leptospirae was not studied. Proteins are said to be relatively insoluble and bacterial polysaccharides readily soluble in this reagent. Morgan and Partridge(14,17) identified products obtained by similar extractions of *Shigella dysenteriae* with diethylene glycol as loosely bound complexes consisting of 3 principal components: a carbohydrate which behaved as a hapten and was responsible for serologic specificity; a phosphorous containing conjugated protein which was antigenic in itself; and a nonantigenic phospholipid. The antigen described yielded a positive Molisch test and a negative biuret test.

Discussion. In view of the incidence of reacting sera in the normal population, examination of serial specimens, before making a diagnosis, is the routine practice of many workers regardless of serologic tests routinely employed.

In agglutination tests of specimens from patients with leptospirosis, antibody concentration increases from the first (acute-phase)

† Kindly performed at Leptospira Research Laboratory of Communicable Disease Center in Chamblee, Ga., under direction of Mrs. Mildred Galton.

to the second (convalescent) serum specimen. On the other hand, in complement-fixation tests with ethylene-glycol extracts of leptospirae, titers reached a maximum early in the disease. Thus a decline in complement-fixing titer from the first to the second specimen would not necessarily obviate a diagnosis of leptospirosis.

Extracts of leptospirae made with desoxycholate solution in a manner similar to that reported for the preparation of *Treponema pallidum* complement-fixation test antigen by Portnoy and Magnuson(18) proved reactive with rabbit antiserum but poorly reactive with serum from human leptospirosis.

Summary. A colloidal aqueous solution obtained by ethylene-glycol extraction of leptospirae appears to offer a complement-fixing antigen of considerable promise. Extract pools have broad valency and high sensitivity in the detection of leptospiral antibodies.

We wish to acknowledge the technical assistance of June McVeigh and Catherine Moczulski.

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Received April 20, 1956. P.S.E.B.M., 1956, v92.

Mechanism of Oxygen Deficit.* (22451)

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After studying the origin of oxygen debt during human exercise, Hill, Long, and Lupton(6) concluded that it resulted entirely from accumulation of lactic acid. Margaria and his co-workers(10) divided the oxygen debt repayment curve into two components, a rapid one with no significant decreases in lactic acid, and a slower one during which accumulated lactic acid was removed. These workers observed no accumulation of lactic acid during exercise producing oxygen debts of less than 2.5 l in man. They postulated that the component of the debt which is rapidly repaid results from a depletion of phosphocreatine in the muscles. A number of observations seem to support this theory. Lundsgard(9) showed that in anaerobic conditions, lactic acid accumulates and organic phosphate dissipates in muscle in tetanus, and that phosphorylation follows a short tetanus. Tiegs(15) found much more creatine, a fission product of phosphocreatine, in fatigued than in rested muscle, a finding confirmed by Eggleton(2) who observed three times the

* This research was supported in part by U. S. Air Force under Contract No. AF 18(600)-1467, monitored by Alaskan Air Command, Arctic Aeromedical Laboratory, APO 731, c/o Postmaster, Seattle, Wash.