CONVERSION OF THE PHASE I ANTIGEN OF *COXIELLA BURNETII* TO HAPTEN BY PHENOL TREATMENT[1]

R. L. ANACKER, W. T. HASKINS, D. B. LACKMAN, E. RIBI, AND E. G. PICKENS

U.S. Public Health Service, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana

Received for publication 7 January 1963

ABSTRACT

ANACKER, R. L. (Rocky Mountain Laboratory, Hamilton, Mont.), W. T. HASKINS, D. B. LACKMAN, E. RIBI, AND E. G. PICKENS. Conversion of the phase I antigen of *Coxiella burnetii* to hapten by phenol treatment. J. Bacteriol. **85**:1165–1170. 1963.—Trichloroacetic acid extracts of *Coxiella burnetii* are converted to hapten by treatment with phenol. Such extracts react, like the original trichloroacetic acid extract, at high dilution in the complement-fixation test and produce zones of precipitate with specific antibody in gel diffusion tests; but, unlike the parent extract, injection of the phenol-treated extract neither induces resistance to challenge in guinea pigs nor antibody formation in guinea pigs, rabbits, or mice. This loss of antigenicity is correlated with removal of protein from the original product.

Effective whole-cell vaccines of *Coxiella burnetii* are available, but severe hypersensitivity reactions occasionally occur when the vaccine is given to individuals previously exposed to the organism (Smadel, Snyder, and Robbins, 1948; Meiklejohn and Lennette, 1950; Benenson, 1959; Lackman et al., 1962). We have attempted, therefore, to determine whether it is possible to obtain a rickettsial fraction which would immunize but would not induce the severe local reaction. Previously we reported (Anacker et al., 1962) that the phase I antigen of *C. burnetii* could be extracted with trichloroacetic acid and that this extract immunized guinea pigs and induced skin lesions in rabbits sensitized with ether-killed whole cells. However, the extract was approximately 10 times less potent than whole cells as an immunogen and 100 times less potent in inducing skin lesions.

Recently we prepared from the trichloroacetic acid extract, by treatment with aqueous phenol, a product which retained the serological properties of phase I antigen but which differed from the parent extract in that the phenol-treated product neither stimulated antibody production nor elicited a hypersensitivity reaction in sensitized rabbits. In this report the biological and chemical properties of the phenol-treated extract are compared with those of the trichloroacetic acid extract and the original whole cells.

MATERIALS AND METHODS

Purification of rickettsiae. Rickettsiae from yolk sacs of the fifth and sixth egg passages were purified by differential centrifugation in 1 M KCl, followed by several washes with decreasing concentrations of KCl solution, and finally washed twice with distilled water (Ribi and Hoyer, 1960).

Preparation of trichloroacetic acid extract. Trichloroacetic acid extracts were prepared as described previously (Anacker et al., 1962).

Phenol treatment of the trichloroacetic acid extract. Trichloroacetic acid extracts of *C. burnetii* were treated in a manner similar to that used by Westphal, Lüderitz, and Bister (1952) for extraction of endotoxin. The extract in distilled water was mixed with an equal volume of 90% phenol and then stirred for 30 min at 65 C. After chilling in an ice bath, the preparation was centrifuged at 1,250 × *g* for 30 min at 2 to 4 C to accelerate separation into an aqueous phase and a phenol phase. The aqueous phase was removed and stored. An equal volume of distilled water was added to the phenol layer, which included the interphase material, and the above procedure was repeated. The pooled aqueous layers and the phenol layer were dialyzed against running tap water for 24 hr and then against four 24-hr

[1] A preliminary report of this study was presented at the 62nd Annual Meeting of the American Society for Microbiology, Kansas City, Mo., May 6–10, 1962.

changes of distilled water at 4 to 6 C. Finally, the two fractions were lyophilized and designated as either *trichloroacetic acid-phenol extract (water phase)* or *trichloroacetic acid-phenol extract (phenol phase)*.

Chemical analyses. Nitrogen content was determined by the procedure of Johnson (1941). Total carbohydrate was estimated by the tryptophan method, using glucose as a standard (Dische, 1955). Phosphorus was determined colorimetrically after digestion with sulfuric acid and hydrogen peroxide (Dryer, Tammes, and Routh, 1957), and esterified fatty acids and amide-linked fatty acids, with palmitic acid as a standard, were determined by the method of Haskins (1961).

Serological tests. Sera of guinea pigs, rabbits, and mice were tested for specific antibody by the complement-fixation (CF) test, and the rickettsial preparations were titrated for antigenic capacity in the CF test as described previously (Anacker et al., 1962). Smears of the extracts were also examined for particulate material by the fluorescent-antibody technique (Burgdorfer and Lackman, 1960).

Biological tests. Skin tests of rabbits with rickettsial preparations and assays of immunogenicity of these preparations were also conducted in the same manner as before (Anacker et al., 1962).

TABLE 1. *Antigen titers* of preparations of Coxiella burnetii determined by the complement-fixation test*

Prepn	No. of samples	Recovery	Antiserum	
			Phase I and II	Phase II
		%		
Whole cells	5	100	16–32	0–4
Trichloroacetic acid extract	5	3.1	256–512	0–16
Trichloroacetic acid-phenol (water phase)	5	1.3	512–1024	0

* Initial concentration of antigen = 1 mg/ml.

TABLE 2. *Response of sensitized* rabbits to intradermal injection of preparations of Coxiella burnetii*

Prepn	SLD_{50} (μg)†	
	Expt 1	Expt 2
Whole cells	0.024	0.023
Trichloroacetic acid extract	0.44	0.88
Trichloroacetic acid-phenol (water phase)	>40	>40
Trichloroacetic acid-phenol (phenol phase)	≧5.0	1.4

* Ether-killed whole cells (50 μg) injected intradermally, 12.5 μg at each of four sites, 4 to 6 weeks before challenge.

† Based on lesions present 72 hr after challenge.

RESULTS

CF titers and yields of extracts. The yield and CF antigen titers of a number of preparations of *C. burnetii* are presented in Table 1. About 3% of the dry weight of the whole cells was recovered after extraction with trichloroacetic acid. Phase I CF antigen titers, based on an initial concentration of 1 mg/ml, varied between 256 and 512, and phase II CF titers were either 0 or very low, 4 to 16. Approximately half of the trichloroacetic acid extract, 1 to 2% of the original whole cell dry weight, was recovered in the aqueous phase after phenol treatment. Phase I CF titers of this product ranged from 512 to 1,024; phase II antigen was never detected. Smears of this product stained with fluorescent antibody and examined with an ultraviolet microscope were free of particulate material. This is the only serologically active material obtained from *C. burnetii* in which we have not been able to see at least a few fluorescent particles. Dilute solutions of this product are water-clear in contrast to the slight opalescence observed with other antigens derived from *C. burnetii*. The material recovered from the phenol phase accounted for less than 0.4% of the original dry weight and was not reactive in the CF test.

Skin test of rabbits. Serial twofold saline dilutions of each of the four kinds of preparation were injected intradermally into three or four sensitized rabbits to assess the ability of the fractions to elicit the hypersensitivity reaction. [Because of the insoluble nature of the trichloroacetic acid-phenol extract (phenol phase), it was necessary to pulverize this preparation with a Teflon grinder before dilution.] The results of two separate experiments are presented in Table 2. The dose required to produce skin lesions in 50% of the animals (SLD_{50}) was calculated from

the response at 72 hr. In both experiments the trichloroacetic acid extracts were considerably less potent than whole cells, and the trichloroacetic acid-phenol extracts (water phase) produced no lesions at the highest doses tested. The trichloroacetic acid-phenol extracts (phenol phase) had significant activity, even though only small lesions were produced (0.5 to 1 cm in diameter), whereas high doses of whole cells or trichloroacetic acid extracts can produce lesions 2 cm in diameter. It seems possible that the size of the lesion induced by the trichloroacetic acid-phenol extract (phenol phase) is related to the insoluble nature of the material.

Immunogenicity of preparations of C. burnetii. The ability of two trichloroacetic acid-phenol extracts (water phase) to protect guinea pigs against challenge with the California AD strain was compared with that of whole cells and a trichloroacetic acid extract (Table 3). Despite the high activity of the trichloroacetic acid-phenol extracts (water phase) in the CF test (CF titers of 1,024), 25 μg of the extracts, the highest dose given, failed to prevent fever. However, whole cells (CF titer of 24) had an ED_{50} (dose which prevented fevers of 40 C or higher in 50% of the animals) value of 0.09 μg, a dose $\frac{1}{270}$th of the highest dose of the impotent trichloroacetic acid-phenol extract. The ED_{50} dose of the trichloroacetic acid extract, 0.4 μg, was approximately five times greater than that of whole cells.

Antibody titers of the sera collected from these guinea pigs 3 weeks after immunization, i.e., on the day of challenge, and of sera collected 13 days after challenge were determined by the CF test. Only low levels of phase II antibody were detected at the time of challenge in the sera of animals given either 5 μg and 1 μg of whole cells or 25 μg of trichloroacetic acid extract (see Table 3); antibody was not detected in sera from the

TABLE 3. *Protection of guinea pigs against challenge with Coxiella burnetii*

Prepn	Phase I CF antigen* titer	Dose injected	Geometric mean phase II CF serum titer (21 days)†	No. of animals with fever/total tested	ED_{50}
		μg			μg
Whole cells	24	5	3.6	0/8	
		1	1.3	0/8	
					0.09
		0.2	0	2/8	
		0.04	0	6/8	
Trichloroacetic acid extract	512	25	29.8	0/7	
		5	0	1/8	
					~0.4
		1	0	3/7	
		0.2	0	4/8	
Trichloroacetic acid-phenol (water phase) A	1,024	25	0	7/7	
		5	0	7/7	
					>25
		1	0	6/6	
		0.2	0	8/8	
Trichloroacetic acid-phenol (water phase) B	1,024	25	0	8/8	
		5	0	7/7	
					>25
		1	0	8/8	
		0.2	0	7/7	
Controls		0	0	7/8	—

* Initial concentration of antigen = 1 mg/ml.

† Sera collected on the day of challenge.

TABLE 4. *Geometric mean serum titers of guinea pigs bled 13 days postchallenge*

Dose injected	Animals injected with							
	Whole cells		Trichloroacetic acid extract		Trichloroacetic acid-phenol (water phase)			
					Expt A		Expt B	
	Ph. I*	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II
μg								
0	0	130						
0.04	0	190	—	—	—	—	—	—
0.2	6	854	2	184	0	33	0	65
1	28	2,078	10	1,131	0	217	0	73
5	80	587	9	1,192	0	141	0	464
25	—†	—	46	1,780	0	113	0	234

* Phase I CF antigen.
† Not tested.

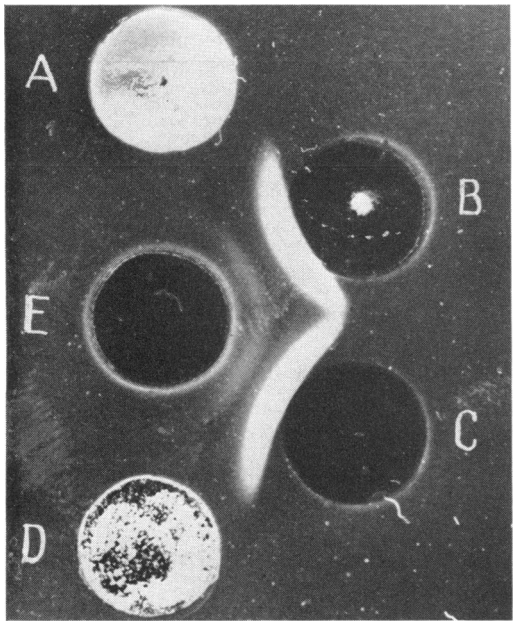

FIG. 1. *Zones of precipitate produced by preparations of Coxiella burnettii (1 mg/ml) and a hyperimmune rabbit serum. A, whole cells; B, trichloroacetic acid extract; C, trichloroacetic acid-phenol extract (water phase); D, trichloroacetic acid-phenol extract (phenol phase); E, antiserum.*

other animals at this time. The geometric mean phase I and phase II antibody titers of the sera from animals bled 13 days after challenge are presented in Table 4. Almost all of the animals injected with whole cells or trichloroacetic acid extract had both phase I and II antibody, indicating a significant antigenic stimulus, since it is well known that 10- 14-day postchallenge sera of guinea pigs given *C. burnetii* antigen several weeks before challenge have considerably higher CF titers than control animals which have been challenged only. However, the controls and the animals given the trichloroacetic acid-phenol extract (water phase) had no phase I antibody and relatively low levels of phase II antibody. The difference in geometric mean titers of the controls and the animals which received the trichloroacetic acid-phenol extracts is not statistically significant.

Antigenicity of the trichloroacetic acid-phenol extract (water phase). Since guinea pigs did not produce antibody after the injection of the extract, although the extract combined well with antibody in the CF test, it seemed possible that phenol treatment had converted the phase I antigen to hapten. To test this hypothesis, rabbits were given a total of 2 mg in two subcutaneous injections of the phenol-treated extract in incomplete Freund's adjuvant, and mice were injected intraperitoneally with doses ranging from 2 to 200 μg of the extract in incomplete Freund's adjuvant. Even though a single injection of a few μg of whole cells or trichloroacetic acid extract consistently stimulates antibody production, antibody was not detected in the sera from any of the animals injected with the phenol extract (water phase).

Gel diffusion study. A trichloroacetic acid extract and the two phenol-treated preparations were compared with whole cells by the agar gel diffusion test of Ouchterlony (1948), as illustrated in Fig. 1. Neither whole cells nor the trichloroacetic acid-phenol extract (phenol phase) exhibited a diffusible component capable of reacting with the hyperimmune rabbit serum, but both the parent extract and the phenol extract (water phase) regularly displayed the presence of two components, the predominant slow-diffusing component and a faster diffusing component present in low concentration. A third component occasionally was found between the other two. The fastest and the slowest diffusing components demonstrate reactions of identity in Fig. 1, but the concentrations of the intermediate components were too low to indicate whether or not they were immunochemically related.

Chemical analyses of rickettsial preparations. Chemical data, representing averages for at least two different rickettsial preparations for each analysis, are presented in Table 5. Trichloroacetic acid extracts from whole cells had approximately the same fatty acid content as whole cells but contained much less nitrogen and phosphorus and considerably more carbohydrate. Phenol treatment further reduced the nitrogen, phosphorus, and fatty acid content, and the carbohydrate content was correspondingly increased. There has not been enough of the trichloroacetic acid-phenol extract (phenol phase) for complete chemical analysis, but the nitrogen content of this fraction is, as one would expect, higher than that of the other extracts.

TABLE 5. *Chemical composition of preparations of Coxiella burnetii*

Prepn	Nitrogen	Total carbohydrate	Fatty acid*	Phosphorus
	%	%	%	%
Whole cells	11.1	7.5	27.2	2.52
Trichloroacetic acid extract	4.52	25.2	24.8	1.78
Trichloroacetic acid phenol (water phase)	2.83	32.4	11.8	0.83
Trichloroacetic acid phenol (phenol phase)	6.29	—†	—	—

* Total of ester and amidic bound fatty acids.
† Not tested.

Discussion

It is apparent that the trichloroacetic acid-phenol extract (water phase) behaves as a typical hapten, i.e., a substance which combines with antibody but which will not stimulate antibody production. This extract combined with antibody in the CF and gel diffusion tests but failed to induce antibody formation in guinea pigs, rabbits, and mice. After our original findings described above had been completed, Brezina, Schramek, and Urvölgyi (1962) reported in a brief communication that in preliminary experiments their extract of *C. burnetii* treated with phenol in their purification procedure failed to stimulate demonstrable antibody formation in rabbits and guinea pigs.

Even though the trichloroacetic acid-phenol extract (water phase) does not elicit the hypersensitivity reaction in the skin of rabbits sensitized with whole cells, we do not yet know whether or not the hypersensitivity state can be induced in rabbits by the injection of this extract. This problem is currently under investigation.

One of the marked changes effected by phenol in the chemical composition of the trichloroacetic acid extract is the reduction in nitrogen content. It seems possible that carrier protein has been cleaved from a lipid-protein-polysaccharide phase I antigenic complex by phenol, and the predominantly polysaccharide haptenic component remains in the aqueous phase. The remaining nitrogen may be accounted for by the presence of peptidelike substances, as has been suggested for endotoxin extracted by the phenol method (Haskins et al., 1963). Recently, Ormsbee (1962) has shown that, compared to phase II whole-cell vaccines, phase I whole-cell vaccines stimulate much greater resistance in guinea pigs to challenge with phase I *C. burnetii* and equal resistance to challenge with phase II organisms. Obviously, the presence of phase I antigen in the whole-cell vaccine is very important, but, from the data reported here, it is probable that the phase I specificity depends upon a polysaccharide cell-wall component complexed to carrier protein. At present the ability of the haptenic phenol-treated phase I antigen to again become an antigenic determinant by coupling with carrier protein is under investigation.

As yet the component of the trichloroacetic acid extract responsible for the hypersensitivity skin reaction in sensitized rabbits is still unknown, but the protein portion of the extract is implicated for two reasons. First, reduction of the nitrogen content of the extract by phenol is correlated with the loss of the ability of the extract to elicit the skin reaction. Second, the material recovered from the phenol phase has a higher nitrogen content than the original extract and still retains the ability to induce skin lesions. The possibility exists, therefore, that the carbohydrate portion in the complex induces the formation of protective antibody in animals, and the protein moiety induces hypersensitivity.

Literature Cited

Anacker, R. L., D. B. Lackman, E. G. Pickens, and E. Ribi. 1962. Antigenic and skin-reactive properties of fractions of *Coxiella burnettii*. J. Immunol. **89**:145–153.

BENENSON, A. S. 1959. Q fever vaccine: efficacy and present status, p. 47–60. *In* J. E. Smadel [ed.], Medical Science Publication No. 6, Symposium on Q Fever. U.S. Government Printing Office, Washington, D.C.

BREZINA, R., S. SCHRAMEK, AND J. URVÖLGYI. 1962. Study on the antigenic structure of *Coxiella burnetii*. II. Purification of phase I antigenic component obtained by means of trichloroacetic acid. Acta Virol. **6**:278–279.

BURGDORFER, W., AND D. B. LACKMAN. 1960. Identification of *Rickettsia rickettsii* in the wood tick, *Dermacentor andersoni*, by means of fluorescent antibody. J. Infect. Diseases **107**:241–244.

DISCHE, Z. 1955. New color reactions for determinations of sugars in polysaccharides, p. 313–358. *In* D. Glick [ed.], Methods of biochemical analysis, vol. 2. Interscience Publishers, Inc., New York.

DRYER, R. L., A. R. TAMMES, AND J. I. ROUTH. 1957. The determination of phosphorus and phosphatase with N-phenyl-p-phenylenediamine. J. Biol. Chem. **225**:177–183.

HASKINS, W. T. 1961. Spectrophotometric determination of fatty acid amides in lipides. Anal. Chem. **33**:1445–1446.

HASKINS, W. T., E. RIBI, M. LANDY, R. L. ANACKER, AND K. C. MILNER. 1963. Preparation and properties of an aluminum citrate-endotoxin complex from *Salmonella enteritidis*. Proc. Soc. Exptl. Biol. Med. **112**:113–119.

JOHNSON, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. **137**:575–586.

LACKMAN, D. B., E. J. BELL, J. F. BELL, AND E. G. PICKENS. 1962. Intradermal sensitivity testing in man with a purified vaccine for Q fever. Am. J. Public Health **52**:87–93.

MEIKLEJOHN, G., AND E. H. LENNETTE. 1950. Q fever in California. I. Observations on vaccination of human beings. Am. J. Hyg. **52**:54–64.

ORMSBEE, R. A. 1962. The influence of phase on the protective potency of vaccines made from *Coxiella burnetii*. Federation Proc. **21**:34.

OUCHTERLONY, Ö. 1948. Antigen-antibody reactions in gels. Arkiv Kemi Mineral. Geol. **263**:1–9.

RIBI, E., AND B. H. HOYER. 1960. Purification of Q fever rickettsiae by density-gradient sedimentation. J. Immunol. **85**:314-318.

SMADEL, J. E., M. J. SNYDER, AND F. C. ROBBINS. 1948. Vaccination against Q fever. Am. J Hyg. **47**:71–81.

WESTPHAL, O., O. LÜDERITZ, AND F. BISTER. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **76**:148–155.