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Preparation of Purified Suspensions of *Coxiella burneti* by Genetron Extraction Followed by Continuous-Flow Ultracentrifugation[1]

DORIA R. DUBOIS, ERNEST C. CUTCHINS, SANFORD BERMAN, JOSEPH P. LOWENTHAL, AND ROBERT L. TIMCHAK

Department of Biologics Research, Walter Reed Army Institute of Research, Washington, D.C. 20012, and Department of Biology, Catholic University, Washington, D.C. 20017

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A method for the preparation of purified suspensions of *Coxiella burneti* by Genetron extraction followed by continuous-flow density gradient ultracentrifugation is described. Both phases of the Henzerling strain of *C. burneti* were found in a zone between 53 and 65% (w/w) sucrose. Based on chemical assays, the Genetron zonal rickettsial suspensions were found to be as pure as the rickettsial suspensions which were prepared by the ether extraction method currently in use for producing Q fever vaccines for human use.

In the preparation of purified Q fever vaccines or rickettsial antigens, ether generally is used to remove the major portion of the contaminating lipoprotein and fat from infected yolk sac membranes of embryonated eggs (3, 6, 12, 15). Because this solvent is highly inflammable there is a danger involved in its use, and disposal of large volumes of residual ether is a problem.

In recent years techniques employing continuous-flow zonal centrifuges have been developed for the preparation of concentrated suspensions of a number of viruses relatively free of extraneous materials (5, 7-9, 14). This paper describes the application of continuous-flow zonal centrifugation to the purification and concentration of *Coxiella burneti* organisms derived from infected yolk sac membranes and compares the products with those obtained by the ether extraction method used in this laboratory for preparing Q fever vaccines for human use (3).

MATERIALS AND METHODS

Rickettsial strains. The second egg passage of the Henzerling strain of *C. burneti* (predominantly in phase 1) and the 23rd egg passage of the same strain (predominantly in phase 2) were obtained from stock material prepared at Walter Reed Army Institute of Research for vaccine production. The seed material was comprised of 20% yolk sac membrane suspensions stored at -70 C in rubber-stoppered, aluminum-sealed bottles.

Preparation of rickettsial stock suspensions. Both the phase 1 and phase 2 rickettsial stock suspensions were produced by the method of Berman et al. (3). Seven-day-old embryonated eggs from pathogen-free White Leghorn hens (SPAFAS, Norwich, Conn.) were inoculated with seed suspensions of *C. burneti* diluted to give a 20% mortality rate occurring between postinoculation days 7 and 8. On the 8th day, the infected yolk sac membranes of the surviving eggs were harvested and homogenized with sufficient distilled water to make a 60% (w/v) tissue suspension. These suspensions were first subjected to one cycle of freezing (-70 C) and thawing and then diluted to 30% (v/v) with 0.85% saline containing sufficient Formalin and phenol to bring the final concentration to 0.3% Formalin and 0.75% phenol. Inactivation was carried out at room temperature for 4 days with shaking at least twice daily. The inactivated phase 1 and phase 2 30% yolk sac membrane suspensions were considered the stock materials from which the respective purified concentrates were made.

Preparation of Genetron zonal concentrates. The zonal centrifuge and the prototype of the Spinco B-XVI rotor used in these studies are described elsewhere (2, 5).

Initially, the infected 30% yolk sac membrane suspension was diluted following inactivation to a 10% (v/v) suspension with 0.85% saline and partially clarified by centrifugation at low speed. The supernatant fluid was collected and introduced into the B-XVI rotor of a model L4 Spinco ultracentrifuge. However, particulate matter remaining in the suspension blocked the small channels of the rotor and its seals, and fatty material collected on the inner core causing an abnormal rise in pressure within the rotor. Therefore, it was necessary to pretreat the

[1] A portion of these results are taken from a dissertation submitted to the Catholic University of America by D.R.D. in partial fulfillment of the requirements for an M.S. degree.

yolk sac membrane suspensions prior to zonal centrifugation.

Because of the satisfactory experience in this laboratory with use of the fluorocarbon Genetron-113 (Allied Chemical, Morristown, N.J.) for purifying cholera organisms (1), this solvent was used at this step of the purification procedure. [Since these studies with Genetron-113 were initiated, Spicer et al. (16) reported on the use of Genetron in the preparation of *C. burneti* antigens from yolk sac membrane suspensions.] Preliminary trials indicated that maximum yields of rickettsiae in the aqueous layer were obtained when one part of the fluorocarbon was homogenized in a Waring Blendor at high speed for 1 min with four parts of the 10% yolk sac membrane suspension. The resulting mixture then was centrifuged at 800 × *g* for 30 min at 4 C. The upper aqueous layer was collected and could be processed through the Spinco B-XVI rotor at the recommended pressure without blockage or accumulation of fatty material on the rotor core.

Accordingly, 1 liter of a 10%-formalinized yolk sac membrane suspension of each phase was treated with Genetron-113, and the resultant aqueous layer (875 to 900 ml) was collected. Each of these aqueous layers was processed in the Spinco B-XVI rotor containing a sucrose gradient, and the sucrose concentration, density, and CF titers of the collected fractions were determined.

The rotor, having a capacity of 750 ml, was filled with distilled water and was spun at 3,000 rev/min. The gradient was made by introducing, with a peristaltic pump (Harvard Apparatus Co., Millis, Mass.), 550 ml of 65% (w/w) sucrose to the edge of the rotor. Approximately 100 ml of distilled water was circulated through the center of the core at 1.5 to 3 ml/min and the rotor was accelerated to 40,000 rev/min. At the end of 30 min the gradient had formed by diffusion of the sucrose toward the center of the rotor and washing of the sucrose "face." At this time the rickettsial suspension (875 to 900 ml) was pumped over the gradient at a flow rate of 1,440 ml/hr. After the introduction of the sample, the lines were flushed with distilled water. Thereafter, the rotor was allowed to spin at 40,000 rev/min for 2 hr to insure isopycnic sedimentation of the rickettsiae and then was decelerated to 3,500 rev/min. At this speed 65% (w/w) sucrose was pumped into the edge of the rotor, forcing the gradient containing the rickettsiae out through the core. A series of 50-ml fractions was collected and the density of each fraction was determined immediately by means of an Abbe-type refractometer. To each fraction 35 ml of 0.85% saline was added, and the fractions were centrifuged at 22,000 × *g* for 1 hr. The resulting pellets each were suspended in 5 ml of phosphate-buffered saline (PBS) containing 0.1% Formalin to concentrate each fraction 10-fold. The amount of rickettsial antigen in each fraction was determined by complement fixation (CF).

Preparation of ether-extracted suspensions (phase 1 and phase 2). The ether-extracted suspensions were prepared by the method of Berman et al. (3). One liter of 10%-infected yolk sac membranes was made by diluting the stock suspension with 0.85% saline and adjusting the *p*H to 5.7 with 1 N HCl. This suspension was mixed with 1.5 liters of sulfuric ether, and the mixture was held at room temperature in an explosion-proof fume hood for 24 hr. The aqueous bottom phase was collected and was placed under vacuum for 24 hr at room temperature to remove the ether. The product then was centrifuged at 22,000 × *g* for 1 hr at 4 C. The supernatant fluid was discarded, and the sediment was suspended in PBS containing 0.1% Formalin to a volume of 50 ml.

Chemical assays. Nitrogen content of the concentrated suspensions of rickettsiae was determined by the micro-Kjeldahl technique followed by nesslerization. Protein determinations were made by the method of Lowry et al. (11) with bovine serum albumin used as a standard.

The lipid determinations were performed by a modification of the methods described by Stoddard and Drury (17) and Youngburg and Youngburg (19). The test involved determination of the weight of lipids dissolved in an extraction solution which consisted of three volumes of 95% ethanol and one volume of ether. Samples of 1 ml in cellulose dialysis tubing were dialyzed against running tap water for 24 hr to remove salts. The dialyzed samples were transferred to Folin Wu tubes to which was added 1 ml of the extraction solution used to rinse the dialysis tubing. Eighteen milliliters of the extraction solution was added to the tubes which then were placed in a water bath and heated to boiling. The tubes were removed and allowed to stand at room temperature for 2 hr. The volumes were adjusted to the 35-ml mark with the extraction solution, and the contents were mixed and filtered through fat-free filter paper. Samples of 25 ml were pipetted into weighing flasks that previously had been dried and weighed. The contents of the flasks were evaporated to dryness under a vacuum hood; the flasks then were reweighed and the amount of extractable material was calculated.

Antigenicity. All CF testing was done by the method described by Kent and Fife (10). The reaction volume was 1.5 ml; thus, each of the five reagents of the CF test was in a volume of 0.3 ml. Five 50% units per 0.3 ml of complement were used, and the period of fixation was 18 hr at 4 C. The hemolytic system then was added, and the tubes were incubated in a water bath for 30 min at 37 C. The reaction tubes were centrifuged at 800 × *g* for 3 min and read by visual comparison with centrifuged standards simulating various degrees of hemolysis.

Antigenic activity of the different phase 1 and phase 2 preparations was obtained by determination of their capacity to fix complement with four units of 42- and 28-day postimmunization guinea pig antiserum, respectively. Serial twofold dilutions of the 10× concentrates of each fraction from the density gradients were prepared for testing. The titer of the final products was determined in the following manner. A set of dilutions comprised of five series of twofold serial dilutions was prepared using for each an initial dilution of 1:2, 1:3, 1:5, 1:7 and 1:9, respectively. This was done in duplicate, and the highest antigen titer was expressed as the reciprocal

of the average of the highest dilutions of the antigen in each set exhibiting less than 70% hemolysis.

Immunogenicity. Hartley-strain guinea pigs (250 to 350 g), in groups of six, each were given a single intraperitoneal injection of 1.0 ml of one of a series of twofold dilutions of experimental vaccines prepared from the concentrated preparations. At 21 and 42 days postinoculation, 5 ml of blood was removed from each animal by cardiac puncture and the blood was processed for the serum. Each serum was tested for specific Q fever antibodies by the CF test by using commercially prepared phase 2 Nine Mile strain antigen (Lederle Laboratories, Pearl River, N.Y.) and phase 1 Henzerling strain antigen prepared at this laboratory.

RESULTS

The gradients of sucrose concentrations and densities formed during zonal centrifugation and the relative distribution of organisms from the Genetron-extracted phase 1 and phase 2 rickettsial suspensions were the same in all experiments except for the minor differences described below. The results of a typical experiment with phase 2 rickettsiae presented in Table 1 are representative of those obtained throughout the study.

In the fractions subsequently found to contain the rickettsiae, a yellowish stringy material was present. This was separated easily by filtration through three layers of gauze. By using CF activity of the 10-fold concentrates of the 50-ml fractions as a measure of rickettsial content, it can be seen that the greatest concentration of organisms was in fraction 7 which contained 53% sucrose. Fractions 8, 9, and 10 also possessed an appreciable amount of rickettsial antigen.

When phase 1 suspensions were processed, the titers were slightly higher and fraction 11 also contained appreciable CF activity. The effluent (10× concentration) from the rotor in the phase 1 studies had a slight CF titer (2 to 4). The densities of the fractions ranged from 1.24 to 1.31 g/cm^3 for fractions 7 to 10, respectively. In a typical phase 1 experiment, fraction 7 had a CF titer of 512 and fraction 11 a CF titer of 64. The densities of these fractions were in the same range as those for phase 2 above.

The 10× concentrates of the fractions containing the bulk of the rickettsiae were pooled, and PBS containing 0.1% Formalin was added to give a final volume of 50 ml. These final products were designated G-Z phase 1 and G-Z phase 2.

The ether-extracted purified rickettsial suspensions prepared from yolk sac membranes infected with phase 1 and phase 2 rickettsiae, as previously described, were designated E-E phase 1 and E-E phase 2, respectively. The E-E preparations were comparable to the G-Z preparations because each contained in the same final volume the rickettsial organisms recovered by the respective procedures from the same mass of infected yolk sac membranes.

In Table 2 the nitrogen, protein, and lipid content, and CF activity of the final rickettsial products are compared.

The results of the chemical analyses of both phase 2 preparations were essentially the same. The G-Z phase 1 preparation, however, assayed lower in every category than the E-E

TABLE 1. *Sucrose concentration, density, and complement-fixing (CF) titer of fractions obtained following density gradient zonal centrifugation of a Genetron-extracted phase 2 rickettsial suspension*

Fraction	Sucrose (%)	Density	CF titer of 10× concentrate[a]
1	11.0	1.042	2
2	17.6	1.070	2
3	22.2	1.091	2
4	28.8	1.121	2
5	36.6	1.159	0
6	45.2	1.204	8
7	53.0	1.246	256
8	62.0	1.298	128
9	62.8	1.303	32
10	64.0	1.310	16
11	64.5	1.313	8
12	65.0	1.316	8
13	65.0	1.316	8
14	65.0	1.316	4
Affluent[b]	Not done	Not done	8
Effluent[c]	Not done	Not done	0

[a] Reciprocals of highest dilution with less than 70% hemolysis.

[b] Genetron-treated membrane suspension before introduction into the rotor.

[c] Genetron-treated membrane suspension after processing through the rotor (10× concentration).

TABLE 2. *Comparison of the nitrogen, protein, lipid, and complement-fixing (CF) content of the final rickettsial products*

Preparation	Nitrogen (mg/ml)	Protein (mg/ml)	Lipid (mg/ml)	CF titer[a]
G-Z phase 2	.158	.714	2.24	72
E-E phase 2	.156	.802	1.50	52
G-Z phase 1	.21	.616	1.68	160
E-E phase 1	.357	1.313	2.38	224

[a] Reciprocals of highest dilution with less than 70% hemolysis.

phase 1.

Also shown in Table 2 is a comparison of antigenic content of the rickettsial preparations as indicated by CF titer. The four preparations contained different amounts of antigen. Before comparative immunogenic studies were done, the antigenic content of the preparations was adjusted to correspond to one of two reference vaccines which have been used for human immunization. Because DP-7 (phase 2) and Lot 1 (phase 1) had CF antigen titers of 8 and 6, respectively, the G-Z phase 2 preparation was diluted 1:9 and E-E phase 1 was diluted 1:37 with PBS.

The chemical characteristics and CF antigen titer of each of the vaccines are compared in Table 3.

Table 4 shows the response of the guinea pigs to immunization with serial twofold dilutions of each of the vaccines. No significant difference in immunogenicity, as indicated by serological conversion of guinea pigs, was observed between the vaccines of the same phase.

TABLE 3. *A comparison of the nitrogen, protein and lipid content of the rickettsial preparations after dilution to comparable complement-fixing (CF) content*

Experimental vaccine	Nitrogen (mg/ml)	Protein (mg/ml)	Lipid (mg/ml)	CF titer[a]
G-Z phase 2	.018	.079	.249	8
E-E phase 2	.024	.123	.230	8
G-Z phase 1	.008	.023	.06	6
E-E phase 1	.0096	.035	.06	6

[a] Reciprocals of highest dilution with less than 70% hemolysis.

DISCUSSION

Since the CF activity of suspensions of *C. burneti* is directly related to the concentration of the organisms and not to soluble components, the antigen titer can be used to estimate the efficiency of continuous-flow zonal ultracentrifugation in recovering and concentrating rickettsiae from crude suspensions. The CF titer of 10% yolk sac membrane suspension of phase 2 rickettsiae after extraction with Genetron was 8, and the titer of the phase 1 material was 16. Because 10-fold concentrated portions of the effluents from the centrifuge after processing the phase 2 and 1 suspensions titered 0 and 3, respectively, the procedure had removed 95 to 100% of the rickettsiae. More than 50% of the total CF antigen content of the crude suspensions could be accounted for in the fractions collected from the gradient. Approximately 90% of the organisms captured in the gradient were contained in four to five 50-ml fractions. However, the quantity of organisms in these fractions compared favorably with the yield obtained when the same amount of crude suspensions was extracted with ether and concentrated to $\frac{1}{20}$ of the original volume. The yield of phase 1 antigen with both methods was two to four times greater than the yield of phase 2 organisms.

The difference in yields of phase 1 and phase

TABLE 4. *Serological conversion of guinea pigs following inoculation with the rickettsial vaccines*

Vaccine dilution	Phase 2 vaccines[a]		Phase 1 vaccines[b]			
			E-E		G-Z	
	E-E phase 2[c]	G-Z phase 2[c]	Phase 2	Phase 1[d]	Phase 2	Phase 1
1/8	5/6[e]	5/6	6/6	6/6	4/6	4/6
1/16	6/6	6/6	6/6	5/6	4/6	4/6
1/32	6/6	6/6	6/6	5/6	6/6	6/6
1/64	3/6	4/6	5/6	3/6	6/6	5/6
1/128	2/6	1/6	6/6	2/6	6/6	2/6
1/256	1/6	1/6	6/6	0/6	5/6	1/6
1/512	Not done	Not done	6/6	0/6	5/6	0/6
ED_{50}[f]	1/100	1/87	>1/512	1/64	>1/512	1/78

[a] Sera obtained 21 days postinoculation.
[b] Sera obtained 42 days postinoculation.
[c] Lederle antigen.
[d] Antigen prepared at this laboratory from yolk sacs infected with the Henzerling phase 1 strain of *Coxiella burneti*.
[e] Number positive [complement-fixing titer of 4 or greater]/number tested.
[f] Calculated by the method of Reed and Muench (13).

2 organisms can be attributed to the relative concentrations of the respective organisms in the infected yolk sac membranes. A similar difference has been reported by Stoker (18) and by Bobb and Downs (4).

The techniques developed in the present study can be used for preparing vaccines for Q fever. There were relatively few differences among the nitrogen, protein, and lipid contents and potencies of the experimental vaccines prepared from crude suspensions of the same phase organisms by each method. However, the phase 1 vaccines contained about four times less lipid and two- to four fold lower concentrations of nitrogen and protein than the phase 2 vaccines. Based upon the phase 2 antibody response in guinea pigs, both of the phase 1 vaccines were at least five times as potent as the phase 2 vaccines.

The procedure developed by Spicer et al. (16) for preparing purified suspensions of *C. burneti* included the use of centrifugation, fluorocarbon, and brushite treatments. After fluorocarbon extraction, the water phase was collected and processed through brushite columns. Samples of 150 to 200 ml at a flow rate of 0.5 to 1.0 ml/min were found to be optimal and the column packing pressure could not exceed 1 psi or high losses were encountered.

The results contained in this report were obtained with initial 1-liter volumes of crude suspensions of rickettsiae. To determine the suitability of the procedure for handling larger volumes, 5 liters of a Genetron-extracted yolk sac membrane suspension containing phase 2 organisms were processed with the Spinco B-XVI rotor. The bulk of the antigen was found again in three 50-ml fractions corresponding to the 53 to 63% sucrose portion of the gradient. Chemical analysis of the 10× concentrates of these fractions indicated that approximately the same degree of purification was achieved as was observed with the 1-liter volumes. Indeed, the total particle capacity of the gradient in the rotor was not exploited with the 5-liter volumes and it is felt that even larger volumes could be conveniently and safely processed.

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