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Preparation of Phase 1 Q Fever Antigen Suitable for Vaccine Use

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Five lots of phase 1 Q fever antigen suitable for use as vaccine were prepared utilizing the Freon-brushite method of antigen purification. Antigen concentration levels were initially adjusted turbidimetrically and later confirmed by antibody response in guinea pigs. Data showed that all lots were essentially identical in purity and antigenicity.

Protection of man against Q fever infection by formalinized vaccines prepared from the Henzerling strain of *Coxiella burnetii* propagated in yolk sacs has been demonstrated (12). The discovery of the phase variation in natural host and egg-adapted strains of the rickettsia (14) led to studies that showed that phase 1 vaccines were more effective in protecting guinea pigs than a similar phase 2 vaccine (9). Phase 2 vaccines for use in man have been described, but none of these consisted of highly purified material, nor were they quantitated with regard to antigenic mass by other than biological means, i.e., complement fixation or animal potency tests.

Because of its greater immunogenicity and because strains of *C. burnetii* in nature appear to be in phase 1, it would seem more desirable to use phase 1 vaccines for human immunization.

A number of years ago, a controlled study of the efficacy of killed phase 1 vaccine was performed in man (4). Volunteers given a single subcutaneous dose of 30 μg of purified Henzerling strain phase 1 organisms developed humoral antibody. Ten months after vaccination all vaccinees resisted an aerosol challenge with phase 1 organisms.

This paper describes the preparation of five lots of a highly purified phase 1 Q fever antigen that were standardized by physical and biological means. The use of hazardous solvents and sophisticated equipment was avoided. Antigen preparations will be designated as vaccine in the remainder of this paper.

MATERIALS AND METHODS

Vaccine seed. The Henzerling (Italian) strain of *C. burnetii* was used for vaccine production. Seed was obtained from The United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md., and was certified by them to be free of avian leukosis and adventitious murine viruses. It had been isolated from a human case of Q fever and passaged six times in guinea pigs and then 22 times in eggs, followed by one guinea pig passage. After an additional four passages in leukosis-free chicken eggs and one passage in guinea pigs, it was passaged twice in leukosis-free chicken eggs and supplied to these laboratories as a 20% yolk sac suspension.

Experimental animals. Eggs were obtained from SPAFAS, Inc. (Storrs, Conn.). Guinea pigs were purchased from several commercial sources.

Vaccine preparation. Vaccine was prepared utilizing the procedures for growth and purification of *C. burnetii* previously described (13). Briefly, 10% infected yolk sac suspensions were inactivated with 0.5% (vol/vol) formalin. Inactivation was carried out for 18 h at 25°C followed by 70 h at 4°C. Stirring was maintained throughout the inactivation period; the fluid was transferred to a fesh sterile container after the 25°C period was completed. Inactivated rickettsiae were partially purified and concentrated by direct sedimentation; final purification was obtained by treatment of the resuspended rickettsiae with Freon followed by passage of the Freon-water phase through a brushite column. Sedimentation of the column effluent completed purification, and the pellet, which consisted of highly purified rickettsiae, was suspended in phosphate-buffered saline (pH 7.0) to a concentration of approximately 1 mg/ml. Merthiolate (Lilly) was added as a 1% (wt/vol) to give a concentration of 0.01% (wt/vol). This solution was designated as bulk vaccine.

After dilution to a rickettsial content of 120 μg/ml with 4% lactose (USP), the vaccine was dispensed in suitable aliquots and freeze-dried to facilitate handling and storage. Lactose was added as a bulking agent primarily to minimize entrainment of the antigen during drying.

One human dose of 30 μg in 0.5 ml was anticipated; therefore reconstitution with 13.0 ml of sterile physiological saline was required to obtain a final concentration of 60 μg/ml.

An outline of the steps and concomitant tests used for vaccine production are shown in Table 1.

Vaccine testing. Prior to inactivation with formalin, a suitable sample of the yolk sac suspension

TABLE 1. *Q fever vaccine production*

Step	Tests
Vaccine seed, EP_2	Adventitious agents Sterility Identity
↓	
Leukosis-free eggs	
↓	
40% Yolk-sac suspension	Sterility PPLO[a] Lowenstein-Jensen medium (TB)
↓	
Inactivation with formalin	
↓	
High-speed pellet	
↓	
Freon treatment	Water phase concentration
↓	
Pooled water phases	Concn
↓	
Brushite treatment	Effluent concentration
↓	
High-speed pellet	
↓	
Bulk vaccine	Concn Sterility Phase characterization Egg protein Microscopic Free formaldehyde Nondialyzable nitrogen Residual viable rickettsiae
↓	
Antigen dilution	Concn Sterility
↓	
Lyophilization	Sterility Moisture Safety Potency Concn Identity

[a] PPLO, Pleuropneumonia-like organisms.

was removed for use in testing for sterility. Tests were conducted in accordance with U.S. Government regulations (10) and included tests for *Mycobacterium* spp. and *Mycoplasma*.

A sample of the bulk vaccine prior to merthiolate addition was removed for sterility testing, residual free formaldehyde determination, phase characterization, egg protein content, nondialyzable nitrogen measurement, microscopic examination, and residual viable rickettsiae testing.

Residual free formaldehyde was determined using the method of Tannenbaum and Bricker (15). A 1-ml portion of the bulk vaccine prior to merthiolate addition was diluted with 3 ml of distilled water and centrifuged for 30 min at 20,000 rpm in a Spinco SW39 rotor. From the level found in the upper 2.5 ml of the centrifuged material, the formaldehyde content of the final diluted vaccine was estimated.

Rickettsial concentration was determined by optical density measurement in phosphate-buffered saline using a Spectronic 20 colorimeter (Bausch and Lomb). Measurements were made at 420 nm using a 1/2-inch (ca. 1.3-cm) tube and were calculated from the relationship: R (μg/ml) = $OD_{420}/(3.2 \times 10^{-3})$. This relationship was found to be valid, at least to an antigen concentration of 150 μg/ml. Instrument reproducibility was checked frequently with a $BaSO_4$ turbidity standard, and the same colorimeter was used for all determinations reported here.

Vaccine phase characterization of the bulk vaccine was determined using the microtiter version of the laboratory branch complement-fixation test (16). Guinea pig antisera obtained 17 days and 45 days after Q fever infection were used in the test.

Egg protein levels were estimated at the bulk antigen level by complement fixation using a guinea pig antiserum prepared by the method of Lackman et al. (8). This procedure had a sensitivity sufficient to detect 0.1 to 0.5 μg of egg albumin.

Nondialyzable nitrogen was determined by the Kjeldahl-Nesslerization method applied to a sample of bulk antigen that had been exhaustively dialyzed against physiological saline at 4°C.

The absence of residual viable rickettsiae was confirmed by inoculating twelve 6- to 7-day-old embryonated eggs via the yolk sac with 0.5-ml of the bulk vaccine. Yolk sacs were harvested at 12 days after incubation at 35 to 36°C; a 20% suspension was

prepared using Snyder I solution (sucrose, 74.6 g/liter; KH_2PO_4, 0.52 g/liter; Na_2HPO_4, 1.22 g/liter; L-glutamic acid, 0.72 g/liter [pH 7.2] [7]). Three additional passages in eggs were performed, and smears of yolk sacs from egg passages 3 and 4 were examined microscopically after staining (6). Six 350- to 450-g female guinea pigs were each inoculated intraperitoneally with 1 ml of a 20% yolk sac suspension of egg passage 4. Animals were prebled; after inoculation rectal temperatures were taken for 17 days, at which time their sera were examined for Q fever complement-fixing antibodies.

Vaccine was tested for sterility and rickettsial concentration prior to dispensing.

Final container testing. General sterility, safety testing, and moisture determination of the final dried product were performed as required by federal regulations (10).

Vaccine antigen concentration after reconstitution was confirmed by turbidity measurement (5).

For comparison of antigenicity, a guinea pig complement-fixing extinction dose was determined for each lot of dried vaccine. The extinction dose was defined as that total quantity of antigen, given subcutaneously as two equal doses 7 days apart, which produces a complement-fixation response in 50% of the inoculated animals 21 days after the initial inoculation. Dried vaccine was reconstituted with sterile physiological saline and diluted to obtain antigen concentrations of 40 μg/ml, 4 μg/ml, 0.4 μg/ml, and 0.04 μg/ml. Ten percent of the animals used were bled, and their individual sera were collected. These were used to confirm the absence of phase 2 antibodies prior to immunization. Each vaccine dilution was used to inoculate each of six female guinea pigs with 0.5 ml of a given vaccine dilution on day 0 and day 7. Reconstituted vaccine was stored at 4°C, and fresh dilutions were prepared for the day 7 inoculation. On day 21, all animals were bled and their sera were tested individually at a 1:8 dilution against a commercial phase 2 Q fever antigen (Lederle) in the microtiter version of the laboratory branch complement-fixation test (16). Calculations of the guinea pig complement-fixing extinction dose were performed by the Reed-Muench method (11).

Identity of the vaccine was confirmed by the nature of the antibody evoked in the potency test. Q fever antibodies have not shown cross-reactions with any other microorganisms or viruses. A commercial Q fever antigen, phase 2 (Lederle), was used in the potency complement-fixation test.

RESULTS

Pertinent data from the preparation of five consecutive lots of phase 1 Q fever vaccine are summarized in Table 2. Uniformity of the purified vaccine from lot to lot was highly satisfactory. Host protein (as egg albumin) was undetectable in the bulk vaccine, as was phase 2 reactivity. The average antigen nitrogen content for all lots of bulk vaccine was 11.7%, a figure identical with that reported earlier for highly purified *C. burnetii* (13).

Additional evidence of vaccine uniformity is shown by the antigen concentration values obtained after reconstitution and by antigenicity responses in guinea pigs. The former ranged from 56 to 58 μg/ml and the antigen required to produce a 50% complement-fixing antibody response averaged from 0.42 μg to 0.98 μg.

DISCUSSION

Other methods devised for the purification of *C. burnetii* for vaccine purposes have been reported (1-3), and of these, two utilized zonal ultracentrifugation for the final step. One of these (2) is not comparable to the work reported here since it was applied to a phase 2 vaccine.

The other procedure employing zonal ultracentrifugation required that the phase 1 formalin-inactivated yolk sac suspension be

TABLE 2. *Q fever vaccine preparation: summary of test results for five consecutive lots of vaccine*

Vaccine lot no.	Bulk vaccine						Final product		
	Concn (μg/ml)	Nitrogen content (μg/mg)	Egg protein content[a] (μg/ml)	Residual formaldehyde (μg/ml)	Phase characterization		Residual moisture (%)	Reconstituted vaccine antigen concn (μg/ml)	GPCFED[c] (μg)
					Phase 1	Phase 2[b]			
1	920	131	<0.4	71.2	64	—[d]	0.51	56	0.42
2	960	115	<0.4	24.2	64-128	—[e]	0.49	58	0.67
3	835	104	<0.4	25.2	64	—[f]	0.83	58	0.56
4	1,020	125	<0.2	23.4	512	—[g]	0.93	56	0.98
5	1,020	110	<0.2	10.5	256-512	—[g]	0.49	58	0.67

[a] As egg albumin.
[b] Reciprocal complement-fixing titer.
[c] GPCFED, Guinea pig complement-fixing extinction dose. Average of two determinations. Based on a calculated 60 μg/ml reconstitution.
[d] Undetectable at 920 μg/ml.
[e] Undetectable at 960 μg/ml.
[f] Undetectable at 835 μg/ml.
[g] Undetectable at 1,020 μg/ml.

treated with Freon prior to zonal centrifugation to avoid rotor line blockage. This phase 1 vaccine was reported to be more highly purified than a phase 1 vaccine prepared using ether by the method of Berman et al. (1).

Comparison of the zonal method for preparation of Q fever vaccine with the Freon-brushite method reported here is not possible, since previous authors failed to indicate the purity of their final product on a weight basis and did not evaluate the host (egg) protein content of their preparation. Although suspensions of *C. burnetii* organisms have been shown to be effective vaccines in man (1, 4), an effective vaccination regimen without serious side reactions has not been established. The occasional production of deep sterile abscesses in those persons receiving several inoculations of vaccine has been a drawback and is of great concern.

For the vaccines described here, purity and antigenicity values are based on an antigen mass basis. Host protein (egg) was not detectable and was estimated to be less than 0.03 μg/ml in the final product when measured as egg albumin.

Considering the purity and uniformity of the phase 1 vaccines described here, they may show reduced reactogenicity, particularly to sterile abscesses, and provide an effective immunization regimen in man. Future planned field trials should determine this.

The data presented show that the Freon-brushite method of purifying *C. burnetii* provides a simple procedure for the preparation of phase 1 Q fever antigen suitable for vaccine use. No difficulties were encountered in processing 12-liter lots of yolk sac suspension by these techniques, which require neither hazardous solvents nor sophisticated equipment.

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