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Production of a Potent Vaccine from the Attenuated M-44 Strain of *Coxiella burneti*

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The attenuated M-44 strain of *Coxiella burneti* was heated to free it of adventitious agents that may have been present. Parallel titrations of the strain before and after heating indicated that the virulence for guinea pigs was markedly decreased. Guinea pigs immunized with the strain derived after heat treatment were protected from the effects of challenge with *C. burneti* in either phase I or II.

The occurrence of Q fever in civilian populations and the potential importance of Q fever epidemics in troops to the outcome of military operations (1) have led several investigators to develop inactivated vaccines containing predominantly phase II (3, 16) and later phase I (14) organisms. *Coxiella burneti*, when freshly isolated or maintained by mammalian passage, occurs as phase I but on continued egg passage converts to phase II. Phase I organisms are more virulent for guinea pigs and react in the complement fixation (CF) test only with late convalescent sera. Phase II organisms react in the CF test with both early and late convalescent sera. Infection with organisms in phase I will produce both phase I and phase II CF antibody, whereas infection with phase II produces only phase II CF antibody (13). The vaccines containing phase II organisms were capable of serologically converting approximately 50% of those vaccinated (19). However, even vaccines without detectable CF antibody have been shown to be protected from aerosol challenge (2). The phase I vaccine is more effective on a per milligram of antigen basis than the phase II (14). Additionally, 10 of 14 (72%) vaccinees given a single 1.0-μg dose and 8 of 11 (73%) vaccinees given a single 3.0-μg dose were protected against aerosol challenge (R. B. Hornick et al., unpublished data).

Although both phase I and II products are effective, the occurrence of severe local reactions after vaccination with phase II material has been a problem (2, 19). To reduce the incidence of these reactions, investigators have proposed that persons with preexisting complement fixing antibodies not be vaccinated (2), or that persons reacting to a skin test dose of *C. burneti* antigen not be vaccinated (19).

Berman et al. (4) pointed out the importance of an available stockpile of vaccine. However, the published production methods for purified, inactivated products are involved and would not lend themselves to rapid production of large numbers of doses. The availability of a RIF-free attenuated strain that had been tested in animals would provide an additional, easily produced, and standardized vaccine for Q fever.

Our studies were undertaken to document thoroughly the properties of the M-44 strain of *C. burneti* as an attenuated vaccine. This strain has been employed in Russia since 1960 with reported conversion rates in humans of $\geq 80\%$ and few local or systemic reactions (5, 6, 7, 18, 20).

MATERIALS AND METHODS

Strains. The M-44 strain was received as vaccine intended for human administration. Additional passages performed at the Department of Biologics Research, Walter Reed Army Institute of Research (WRAIR) and in this laboratory to produce seed stocks were conducted in specific pathogen-free chicken eggs (SPAFAS, Inc., Norwich, Conn.). The strain was freed of adventitious agents that may have been present by heating a sample in a water bath at 56 C for 30 min. Subsequent to heating, this strain was tested and found free of avian leucosis viruses by the Bureau of Biologics, National Institutes of Health.

The Henzerling strain in the 3rd egg passage (EP-3) was obtained from the Merrel-National Drug Company, Swiftwater, Pa. This strain was furnished as an avian leucosis virus-free yolk sac pool. The Nine Mile strain in the 88th egg passage (EP-88) was available from our laboratory stocks. All seed and challenge materials were stored at −70 C in rubber-stoppered, aluminum-sealed vials.

Preparation of vaccines. Crude rickettsial pools were produced in embryonated eggs from avian leucosis virus-free hens (SPAFAS, Inc., Norwich, Conn.).

Seven-day embryonated eggs were inoculated with 0.2 ml of the seed stock containing $10^{7.9}$ median infectious doses for eggs (IDE_{50}) and incubated at 35 C. At 6 to 7 days postinoculation, when 20% of the embryos had died, the yolk sacs from the eggs with live embryos were harvested, drained, diluted to a 50% suspension with distilled water, and blended for 2 min in an Aseptic Dispersal (Waring Corp., Winstead, Conn.). The suspension was then stored at −70 C until further processed.

The crude pools were purified by a physical method in which the preliminary steps consisted of two cycles of alternating low-speed (900 × *g* for 30 min at 4 C) and high-speed (54,500 × *g* for 4 h at 4 C) centrifugations. The pellet from the final high-speed centrifugation was suspended in 10% sucrose and centrifuged onto a 70% sucrose "cushion" at 54,500 × *g* for 18 h at 4 C in a swinging-bucket rotor. The purified rickettsia were aspirated from the surface of the "cushion" and stored at −70 C.

Purified rickettsia were diluted with a volume of Snyder I buffer (9) calculated to give a final titer of $10^{9.5}$ IDE_{50} per ml and freeze-dried in a cabinet unit. The vaccine was standardized on the basis of IDE_{50} because this system was subject to less variation than direct counts or guinea pig titrations (unpublished data).

Infectivity titrations. *C. burneti* was quantified in 4-day-old embryonated eggs. Serial $\log_{10}$ dilutions in cold (4 C) buffered physiological saline (0.15 M NaCl, 0.006 Na_2HPO_4, pH 7.3) were inoculated in 0.2-ml volumes into the yolk sac. The surviving eggs were harvested after 10 days, and a yolk sac smear was examined with Gimenez stain (8). If rickettsia could be found the egg was considered positive. The titer, expressed as IDE_{50}, was calculated by the method of Reed and Muench (15).

Guinea pig assays. To determine the infectivity and efficacy of the vaccine and challenge strains in animals, 250- to 350-g Hartley strain guinea pigs (Animal Farm, Fort Detrick, Md.) were inoculated intraperitoneally (i.p.) or subcutaneously (s.c.) with serial 10-fold dilutions prepared in physiological buffered saline. Temperatures were taken daily for 14 days, and titers, expressed as median fever doses (FD_{50}), were calculated by the method of Reed and Muench (15). Fever, for the purpose of calculating the FD_{50}, was taken to be a temperature equal to or greater than 103.6 F (ca. 39.8 C) for 2 or more consecutive days (3). Blood was drawn by cardiac puncture 28 and/or 42 days postinoculation for determination of CF titers on individual sera.

To determine protection, the vaccine-experienced and virgin guinea pigs were challenged with selected FD_{50} of either phase I or phase II organisms. Temperatures were determined for 14 days or until the death of the animal, whichever occurred first. Protection was taken to be a reduction in duration of days of fever to 50% of that of the control animals (14). The titer of the vaccine, expressed as median protective doses for guinea pigs (PD_{50}), was calculated by the method of Reed and Muench (15).

Serological tests. CF tests were conducted by the method of the Communicable Disease Center (11). Commercially prepared phase II Nine Mile strain antigen (Lederle Laboratories) and phase I antigen, kindly supplied by Paul Fiset, University of Maryland, Baltimore, were used throughout these studies. A single pool of immune sera was titrated with each assay, and 4 units of each of the antigens were employed.

Nitrogen determinations. Nitrogen content of the samples was determined by digestion with the micro-Kjeldahl technique followed by nesslerization. The optical density was read at a wave length of 490 nm on a Spectronic 20 spectrophotometer (Bausch & Lomb Co., Rochester, N.Y.).

RESULTS

Pathogenicity before and after heating. In Fig. 1 are shown the responses of groups of eight guinea pigs each after i.p. administration of dilutions and the seed material (not heat treated) produced at WRAIR. The animals given $10^{10.4}$ IDE_{50} of rickettsia were all febrile by 2 days postinoculation, whereas those given $10^{9.4}$ IDE_{50} were all febrile by 3 days postinoculation.

All but one of the guinea pigs given $10^{8.4}$ IDE_{50} developed fevers on days 3 or 4 postinoculation, but only one of the animals given $10^{7.4}$ IDE_{50} developed a fever of 3 days duration beginning on day 2 postinoculation. No fevers were detected in animals given lower doses. The FD_{50} of the unheated seed material was $10^{8.8}$/ml. The mean temperature responses of guinea pigs given dilutions of the same preparation by the s.c. route are presented in Fig. 2. Generally, the febrile response of individual animals to s.c. inoculation of the rickettsia was more variable than to i.p. inoculation. Not all guinea pigs given $10^{9.3}$ IDE_{50} became febrile, but all of them did produce antibody detectable by CF. The mean duration of the febrile response to comparable doses was 4.7 days by the i.p. route and 2.3 days by the s.c. route, and the FD_{50} by the s.c.

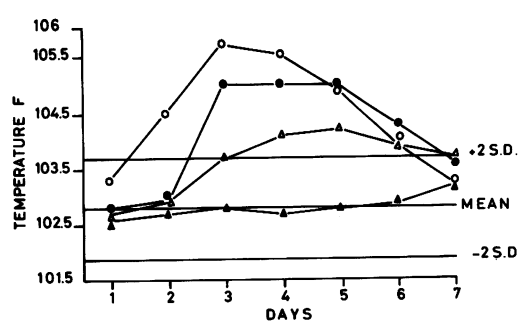

FIG. 1. *Mean temperature responses of groups of eight guinea pigs to the i.p. inoculation of selected doses of the M-44 strain. Symbols:* ○, $10^{10.4}$ IDE_{50}; ●, $10^{9.4}$ IDE_{50}; △, $10^{8.4}$ IDE_{50}; ▲, $10^{7.4}$ IDE_{50}.

route was $\leq 10^{2.8}$. Repetition of these titrations produced FD_{50} values of $10^{2.9}$ by the i.p. route and $10^{2.8}$ by the s.c. route. As in the first titrations, the duration of the fever produced by rickettsia given s.c. was approximately one-half of that produced by an equal dose of rickettsia given i.p. Because the normal route of administration of the vaccine was s.c. (7) or percutaneous (18), no further i.p. titrations were conducted.

After heating and subpassage of the strain, the intensity of the febrile response of the guinea pigs to comparable concentrations of organisms was diminished. The mean temperature responses elicited by s.c. inoculation of serial $\log_{10}$ dilutions of the heated strain are presented in Fig. 3. The FD_{50} was $10^{1.5}$, and a repeat titration produced no fevers on the inoculation of undiluted material. An s.c. titration of the unheated strain conducted concurrently with the repeat titration yielded an FD_{50} value of $10^{5.7}$. The mean duration of the febrile response was 2.5 days in both cases. Widely varying FD_{50} values were common when guinea pig titrations were repeated with animals procured at different times, but the relationships between the EP-3 and EP-88 were constant, with the EP-3 FD_{50} approximately 10,000 times greater in every case.

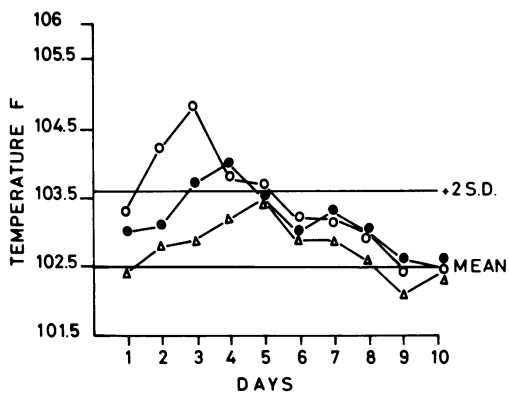

FIG. 2. *Mean temperature responses of groups of eight guinea pigs to the s.c. inoculation of selected doses of the M-44 strain. Symbols: O, $10^{10.3}$ IDE_{50}; ●, $10^{8.3}$ IDE_{50}; Δ, $10^{6.3}$ IDE_{50}.*

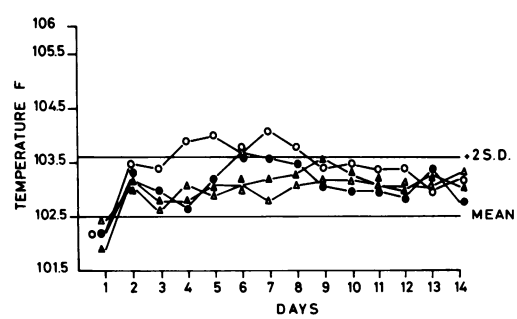

FIG. 3. *Mean temperature responses of groups of eight guinea pigs to the s.c. inoculation of selected doses of heated M-44 (R-M) strain. Symbols: O, $10^{11.3}$ IDE_{50}; ●, $10^{10.3}$ IDE_{50}; ▲, $10^{9.3}$ IDE_{50}; Δ, $10^{8.3}$ IDE_{50}.*

Because the 2nd and 3rd yolk sac passages after heating had been certified free of avian leucosis viruses, and the virulence for guinea pigs of pools with comparable titers in eggs was decreased after heating, the strain was redesignated R-M (RIF-free M).

None of the several hundred animals that have been inoculated with undiluted and various dilutions of the M-44 or R-M strains have died as a result of Q fever infection regardless of the dose or route.

Serological responses prior to and subsequent to heating. To insure that the heating of the rickettsial suspension for purification purposes did not affect the infectivity, parallel titrations were conducted. The results of these titrations are presented in Table 1. The end points were comparable as were the geometric mean titers. None of the animals inoculated with the R-M strain developed fever in this assay or produced detectable phase I CF antibody. The FD_{50} of the guinea pigs inoculated with the M-44 strain was $10^{2.3}$.

Parameters during purification. Volumes and nitrogen levels obtained at each step during the purification process are listed in Table 2. The total N remaining in the purified rickettsia recovered from the sucrose gradient was 150 μg (11.5 μg/ml), which represented 0.008% of the N in the initial yolk sac suspension. In five other

TABLE 1. *Phase II CF responses of guinea pigs to the M-44 and R-M (heat-treated) strains of C. burnetti*

Dose inoculated (IDE_{50}[a])	M-44 strain (no. converted/no. inoculated)	GMT[a]	R-M strain (no. converted/no. inoculated)	GMT[a]
$10^{9.5\text{-}10}$	8/8	58	8/8	85
$10^{8.5\text{-}9.4}$	8/8	115	8/8	64
$10^{7.5\text{-}8.4}$	8/8	56	8/8	72
$10^{6.5\text{-}7.4}$	8/8	16	8/8	14
$10^{5.5\text{-}6.4}$	8/8	15	7/8	11
$10^{4.5\text{-}5.4}$	8/8	8	7/8	4
$10^{3.5\text{-}4.4}$	5/8	3	2/8	2
$10^{2.5\text{-}3.4}$	2/8	<1	1/8	<1
$10^{1.5\text{-}2.4}$	1/8	1	1/8	<1
$10^{0.5\text{-}1.4}$	0/8	0	0/8	0
CFD_{50}		$10^{7.8}$		$10^{7.0}$

[a] GMT, Geometric mean titer.

purified lots, N levels ranged from 7 to 43 μg/ml in the recovered sucrose band. These N levels represented from 0.002 to 0.04% of the initial yolk sac N. The final N levels were comparable with those reported by other workers using different techniques to purify inactivated organisms (4, 12, 17).

A comparison of the titers of the yolk sac pool and the freeze-dried vaccine prepared from this pool is presented in Table 3. Recovery rates (not shown) based on the IDE_{50} varied from 2 to 230% for different lots. In this case it was 2%. However, the median complement fixing doses (CFD_{50}) are equal, and no reproducible differences were seen in the titers after phase I or II challenge, whether the animals had prior experience to the crude yolk sac pool or to the purified vaccine. The initial concentration of rickettsia per microgram of N in the yolk sac pool was $10^{7.1}$ IDE_{50}, and the final concentration of rickettsia per microgram of N in the purified vaccine was $10^{11.1}$ IDE_{50}. No fevers were detected in guinea pigs inoculated with undiluted purified vaccine after either vaccination or challenge.

Challenge studies. Guinea pigs that had been inoculated with R-M strain from 28 to 42 days previously and had responded serologically were solidly protected against challenge with either the phase I or phase II strains. Furthermore, the immune status of the animals which had not responded serologically was directly related to the dose of R-M strain inoculated, i.e., those given an adequate dose of vaccine were protected in the absence of detectable antibody.

Mean temperature curves of groups of six immunized animals after a phase II challenge are presented in Fig. 4. The phase II challenge represented rickettsia in an undiluted yolk sac pool. A prompt, intense febrile response with an equally prompt remission was common in immune animals challenged with undiluted pool material. The PD_{50} was $10^{7.6}$ in this assay, and a repeat test yielded a value of $10^{8.1}$ (see Table 3). Serological responses to this level of challenge, which represented a preformed antigenic mass (unpublished data), were typical of anamnestic-type responses. Animals showing this response were protected against death from challenge regardless of their detectable antibody status after the R-M inoculation. Generally, animals without detectable antibody after vaccination had febrile reactions directly related to the titer of the vaccine inoculated, i.e., although 10^2 IDE_{50} of organisms protected the guinea pigs from the lethal effects of challenge, these animals had febrile responses indistinguishable from control animals.

The temperature curves of immunized ani-

TABLE 2. *Purification of the R-M strain of C. burneti by physical methods*

Material tested	Vol (ml)	N (μg/ml)	N (total mg)	Remaining %[a]
50% yolk sac	300	6,200	1,860	
Low-speed supernatant	200	6,400	1,280	70
High-speed pellet	45	16,380	737	58
Low-speed supernatant	144	1,160	167	23
High-speed pellet	57	400	23	14
Sucrose gradient band	13	11.5	0.15	0.7
Final vaccine (1:75 dilution of band)	150	0.2[b]	NA[c]	

[a] N level of indicated fraction divided by N level of prior fraction times 100.
[b] Arithmetically determined.
[c] NA, Not applicable. Total will be the same as reported for 2 ml of the sucrose gradient band because that volume was diluted to 150 ml to prepare the final vaccine.

TABLE 3. *Parameters of the yolk sac pool and the purified, freeze-dried R-M (heat-treated) strain of C. burneti vaccine*

Material	N (mg/ml)	Titer	Serological response	Challenge response	
				Phase I	Phase II
Yolk sac pool	6,200	$10^{11.1}$[a]	$10^{4.8}$[b]	$10^{6.8}$[c]	$10^{7.3}$
Vaccine	0.2	$10^{10.4}$	$10^{5.1}$	$10^{7.1}$, $10^{7.7}$	$10^{7.6}$, $10^{8.1}$

[a] Median infectious titer in eggs.
[b] Median complement fixing antibody converting titer in guinea pigs.
[c] Median protective titer against a constant challenge of 10^{10} IDE_{50} of the indicated phase.

mals after phase I challenge are given in Fig. 5. The prompt febrile responses were not seen with phase I challenge doses that were diluted. After Formalin inactivation the phase I challenge material did not produce a CF antibody response in virgin guinea pigs. Uninfected yolk sacs also did not produce fevers when they were inoculated into immunized guinea pigs as 20% suspensions.

The PD_{50} of the R-M strain against a phase I challenge was $10^{8.2}$ when the challenge dose contained 10^6 IDE_{50}, and $10^{7.1}$ when the challenge dose contained 10^{10} IDE_{50}. However, as can be seen in Fig. 5, the animals given 10^4 IDE_{50} of the R-M strain had lower temperatures than control animals after challenge. Not all of these animals were considered protected in determining the PD_{50} where the criteria proposed by Ormsbee et al. were used (14).

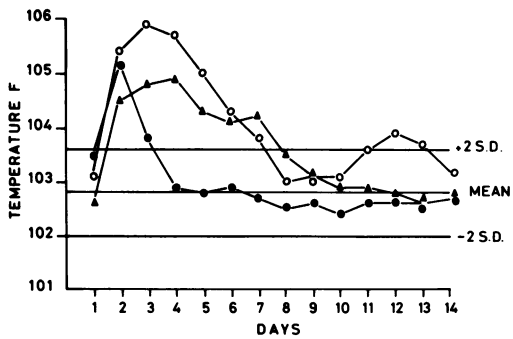

FIG. 4. *Mean temperature responses of groups of six immunized guinea pigs to phase II challenge. Symbols: O, control (no vaccine); ▲, 10^4 IDE_{50} inoculated s.c. 42 days prior to challenge; ●, 10^6 IDE_{50} inoculated s.c. 42 days prior to challenge Challenge dose was 10^{10} IDE_{50} of the EP-88 strain inoculated i.p.*

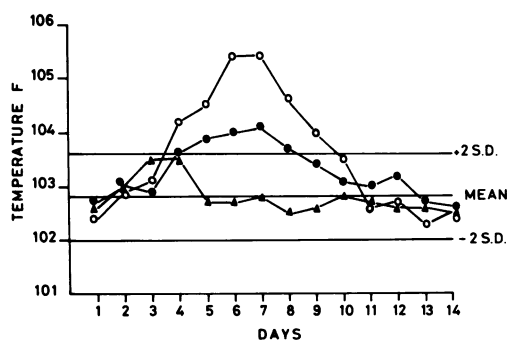

FIG. 5. *Mean temperature responses of groups of six immunized guinea pigs to phase I challenge. Symbols: O, control (no vaccine); ●, 10^4 IDE_{50} inoculated s.c. 42 days prior to challenge; ▲, 10^6 IDE_{50} inoculated s.c. 42 days prior to challenge. Challenge dose was 10^{10} IDE_{50} of the EP-3 strain inoculated i.p.*

From 30 to 50% of control animals died after inoculation of 10^{10} IDE_{50} of either phase I or II organisms.

The temperature data for all the dilutions of vaccine were compiled as degrees of fever per animal during the experimental period according to the method of Kenyon et al. (10). The mean value derived from control animals was arbitrarily selected as the 100% level, and the values derived from animals' experience with serial $\log_{10}$ doses of the R-M strain were expressed as a percentage of this value. Any temperature above 103.6 F for any period of time was considered a fever. Temperatures below this value were not included in the determination. Figure 6 represents these temperature responses after a uniform challenge dose of phase I or II organisms. The points plotted represent degree days per group of six animals as a percentage of days in unvaccinated control animals. Appreciable protection against the febrile effects of challenge was present at vaccine doses equal to or greater than 10^4 IDE_{50} against a phase I challenge and 10^3 IDE_{50} against a phase II challenge. When the assay was repeated, values from 10^5 to 10^3 IDE_{50} were obtained and no conclusion could be drawn as to the difference in efficacy of the vaccine against the two challenge strains.

Figure 7 presents the CF antibody responses of vaccinated animals to a constant challenge dose of phase II organisms. The response of the animals to the original vaccine was determined by bleeding the animals at 28 days. The response to the challenge was determined on bloods drawn at 48 days (14 days postchallenge). High doses of vaccine inhibited the

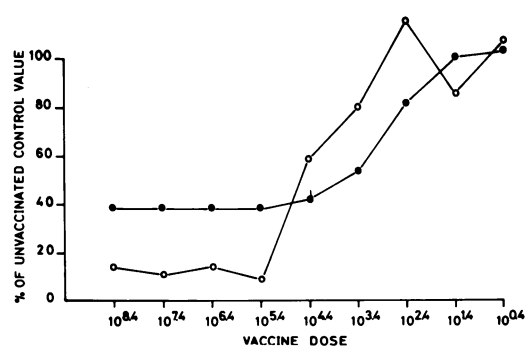

FIG. 6. *Mean febrile responses of vaccinated guinea pigs to phase I and II challenge expressed as a percent of control values. Symbols: ●, 10^{10} IDE_{50} of EP-88 inoculated i.p. at 42 day postvaccination; O, 10^{10} IDE_{50} of EP-3 inoculated i.p. at 42 days. Vaccine doses expressed as IDE_{50}.*

production of an anamnestic-type response. However, low doses of vaccine that did not protect the animals from challenge by the criteria of Ormsbee et al. (14) "primed" the guinea pigs to respond to the subsequent challenge with anamnestic-type responses. Only when 200 IDE_{50} or less were given did the response equal that of the surviving challenge controls.

The CF antibody response to a phase I challenge did not reflect an anamnestic-type response as was seen with the phase II challenge (Fig. 8). In fact, the CF response was inhibited by doses of vaccine that had enhanced the response to a phase II challenge. When these challenge doses were inactivated with Formalin and inoculated into guinea pigs, the phase II challenge produced a CF antibody response, whereas the phase I challenge did not.

DISCUSSION

The loss of virulence after purification was fortunate, especially because there was no concomitant loss in infectivity. This decrease of greater than 100-fold in the fever dose for guinea pigs was unexpected and remains unexplained. Duplicate titrations conducted in eggs immediately prior to and after heating yielded IDE_{50} titers of $10^{7.0}$ and $10^{7.1}$, respectively. This indicates that minimal inactivation of the rickettsia occurred during the heating. Therefore, there was minimal chance for any selection based on heat sensitivity.

The precision of replicate guinea pig titrations was poor, but concurrent titrations gave results within a $\log_{10}$ of each other. Because it was not possible to titrate all materials of interest at one time, all dosages, recovery rates, and titers were based on IDE_{50}. The variation in titer as measured by the IDE_{50} was about 10-fold. The published work on the M-44 strain is also based on egg titrations (5, 6, 7, 18, 20).

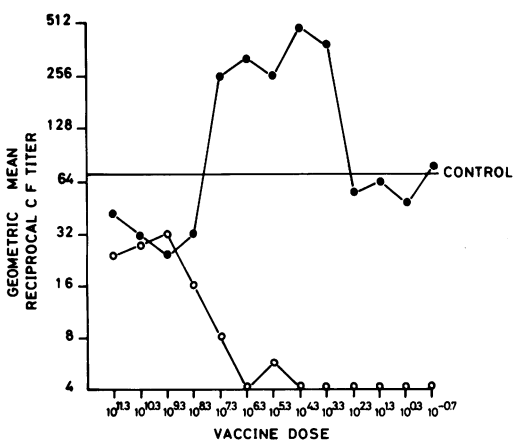

FIG. 7. *Antibody responses of vaccinated guinea pigs prior and subsequent to challenge with phase II organisms. Symbols: O, prior to challenge; ●, subsequent to challenge. Vaccine dose expressed as IDE_{50}. Challenge dose equaled 10^{10} IDE_{50} of the EP-88 strain.*

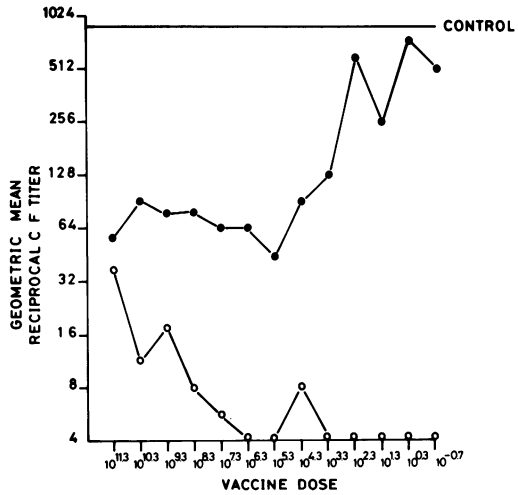

FIG. 8. *Antibody responses of vaccinated guinea pigs prior and subsequent to challenge with phase I organisms. Symbols: O, prior to challenge; ●, subsequent to challenge. Vaccine dose expressed as IDE_{50}. Challenge dose equaled 10^{10} IDE_{50} of the EP-3 strain.*

Serological conversions based on CF tests were disappointing. However, other workers have found satisfactory protection from challenge in animals using Rocky Mountain spotted fever (12) and in humans using Q fever (2) in the absence of detectable CF antibody. Furthermore, Ormsbee et al. (14), when testing inactivated phase I and II vaccines, found serological converting doses measured in micrograms of N to be equal, whereas fever suppression doses were 195-fold smaller with the phase I product.

Serological responses indicate that a phase II challenge containing a threshold antigenic dose produced an anamnestic-type response to phase II antigen in guinea pigs that had not responded to the original vaccination with antibody detectable in the CF tests. These animals had apparently been "primed" by an antigen similar to the phase II CF antigen. This occurred in some animals when a dose of $10^{3.3}$ IDE_{50} of R-M strain was given, but not when a dose of $10^{2.3}$ IDE_{50} was given. Guinea pigs that were challenged with a similar dose in IDE_{50} in phase I (one that did not constitute a preformed, antigenic threshold dose) responded in a different

manner. The CF titers of animals given $10^{3.3}$ IDE_{50} of vaccine were less than the control values in virgin animals. From these results a dose of 2,000 IDE_{50} (which is not a preformed antigenic mass) apparently replicates in vivo and elicits an immune response that is not detectable by CF. This response does not protect the animals against the febrile effects of challenge but does protect them from the lethal effects.

Many animals responded to challenge with an initial temperature rise rapidly followed by a period of temperatures below the mean value of the control animals. Because the onset of fever was more variable after challenge than after vaccination, the concurrent occurrence of fever and submean values biased the calculated mean values. The comparison of values of animals with fevers rather than temperature values for all animals eliminated this bias. The elevated febrile response to phase II challenge when high doses of vaccine were given was a reflection of the prompt febrile response mentioned previously. The uniformity of this response over such a wide range of vaccine doses (from 10^8 through 10^5 IDE_{50}) indicated that these animals were protected and had responded maximally to the immunizing dose. Also, the challenge elicited an anamnestic-type antibody response in these animals.

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