0019-9567/78/0019-0194$02.00/0
INFECTION AND IMMUNITY, Jan. 1978, p. 194-198

Vol. 19, No. 1
Printed in U.S.A.

Cell-Mediated Immune Responses of Guinea Pigs to an Inactivated Phase I *Coxiella burnetii* Vaccine

R. A. KISHIMOTO,* J. W. JOHNSON, R. H. KENYON, M. S. ASCHER, E. W. LARSON, AND C. E. PEDERSEN, JR.

U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Received for publication 10 August 1977

The ability of a killed phase I *Coxiella burnetii* vaccine to induce cell-mediated immune responses in guinea pigs was studied. Cell-mediated immune responses were assessed by the inhibition of macrophage migration and lymphocyte transformation assays. The macrophage migration response occurred rapidly and was detected at high levels, but was relatively short-lived. In contrast, the lymphocyte transformation response developed more slowly, and persisted for a longer period. The vaccine, given in a single dose or in two doses 1 week apart, protected guinea pigs from a subsequent virulent challenge.

Vaccines made from killed *Coxiella burnetii* have been shown to be effective in eliciting humoral antibodies in humans and guinea pigs (21). The protective efficacy of the vaccine has been demonstrated in humans and guinea pigs (2, 15, 23). Early Q fever vaccines were prepared from strains of *C. burnetii* predominantly in phase II; however, studies by Ormsbee et al. (18) showed that formalin-killed phase I rickettsiae possessed protective potencies 100 to 300 times greater than phase II organisms. Recent studies indicate that cell-mediated immunity also may play a role in the defense against *C. burnetii* infection. Peritoneal macrophages from guinea pigs vaccinated with Formalin-killed phase I rickettsiae are capable of killing ingested phase I rickettsiae in vitro in the absence of immune serum (12, 13). This activity is demonstrable at a time when macrophage inhibition factor is present in the peritoneal cavity (12). In guinea pigs infected with phase I *C. burnetii*, inhibition of macrophage migration (IMM) was noted as early as 3 days postchallenge and reached maximum levels between 14 and 21 days, at a time when there is no detectable circulating antibody to phase I antigen (11). Recently, Jerrells et al. (10) demonstrated marked lymphocyte transformation (LT) responsiveness in humans exposed to *C. burnetii* as long as 8 years previously.

In an effort to gain further insight into the cellular immune responses of animals vaccinated with a killed vaccine, we studied the IMM and LT responses in guinea pigs after vaccination. Our objective was to determine whether either response would prove to be a predictive correlative of vaccine-induced immunity to Q fever infection.

MATERIALS AND METHODS

Rickettsiae. The third egg passage (EP-3) of the phase I Henzerling strain of *C. burnetii* and the 88th egg passage (EP-88) of a phase II Nine Mile strain were grown in yolk sacs of embryonated eggs as previously described (19).

Guinea pigs. Outbred male Hartley strain guinea pigs, weighing approximately 350 to 450 g, were obtained from Buckberg Lab Animals, Tompkins Cove, N.Y. All animals were provided water and commercial guinea pig chow ad libitum.

Vaccination. The vaccine consisted of purified, Formalin-inactivated, particulate, Henzerling strain phase I *C. burnetii* (NDBR 105, lot 4) produced by the Merrell National Laboratories, Swiftwater, Pa. (22). Guinea pigs were vaccinated with 30 μg of antigen, a dose reported by Fiset (6) to evoke humoral antibody production and to protect humans against an aerosol challenge with phase I rickettsiae 10 months later. Guinea pigs, allocated into three groups, were given 30 μg of the antigen subcutaneously by one of the following regimens: (i) one group was vaccinated once with 1.0 ml of vaccine; (ii) another group, with 0.5 ml on day 0 and again 7 days later; and (iii) the third group, with 1.0 ml of the vaccine mixed with an equal volume of incomplete Freund adjuvant (IFA; Difco Laboratories, Detroit, Mich.).

Serological assays. Blood was collected from animals at selected times, and serum antibody activity against phase I and II *C. burnetii* was determined by the indirect immunofluorescent antibody technique of Bozeman and Elisberg (3).

IMM. Peritoneal exudate cells were collected 4 days after intraperitoneal inoculation of guinea pigs with 25 ml of sterile mineral oil (Marcol no. 90; Humble Oil and Refining Co., Houston, Tex.). The peritoneal exudate cells were harvested and processed as previously described (13). The agarose technique for estimating direct IMM was performed as previously described (11). The area traversed by the migrating cells was measured, and the following formula was used to express the IMM.

$$\% \text{ Inhibition of macrophage migration} = 100 - \frac{\text{Mean area of migration with antigen}}{\text{Mean area of migration without antigen}} \times 100$$

LT assay. LT was measured using a whole-blood technique (10a). Weekly samples of cardiac blood from individual animals were collected in tubes containing 0.2 mg of heparin per ml of blood and diluted 1:10 in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with penicillin (200 U/ml) and streptomycin (200 μg/ml). Diluted blood (200 μl) was dispensed into flat-bottom microtiter plates (Cooke Laboratory Products, Alexandria, Va.). Six replicate wells were prepared, each containing 25 μl of one of the following components: (i) RPMI medium; (ii) 2×10^7 Formalin-killed phase I (EP-3 Henzerling strain) rickettsiae in RPMI medium; (iii) 4×10^6 phase II (EP-88 Nine Mile strain) Formalin-killed rickettsiae in RPMI medium; or (iv) phytohemagglutinin P (Difco) diluted 1:100 in RPMI medium. After wells had been incubated for 4 days at 37°C in a 5% CO_2 atmosphere, 0.02 μCi of [^{14}C]thymidine (50 mCi/ml, New England Nuclear Corp., Boston, Mass.) was added to each well. After 24 h, cells were harvested with a multiple automatic sampler harvester (MASH II; Microbiological Associates, Bethesda, Md.). The cultures were each aspirated with 30 ml of water through glass-fiber filter paper and 50 ml of absolute methanol. Dried filters were immersed in scintillation vials with 5 ml of toluene-Liquifluor (New England Nuclear) for scintillation counting. Stimulation indexes were calculated as counts per minute of stimulated culture/counts per minute of control culture. All counts per minute are given as the geometric mean of the six replicate cultures. Preliminary experiments indicated that the average standard deviation for each set of cultures was approximately 40% of the mean, and twofold differences were significant at the 5% level.

RESULTS

After vaccination of guinea pigs with the Formalin-killed, particulate, phase I Q fever vaccine, the temporal development of humoral antibody and cell-mediated immune responses was studied. The time of appearance and the persistence of humoral and cellular responses were dependent upon the vaccination regimen. The IMM responses of guinea pigs given vaccine in a single dose are shown in Fig. 1. Macrophage migration was inhibited significantly (40 to 50%) by either phase I or phase II *C. burnetii* antigens at 7 and 14 days postvaccination, but was unaffected by antigen on day 21. In comparable cultures with cells from nonvaccinated control guinea pigs, IMM did not exceed 5%. In contrast to the early cellular immune response, serum antibody to phase II antigen did not appear until day 14, and antibody to phase I antigen was not detected by day 21 (Fig. 1). Antibody to phase I antigen was not detected when assayed by two additional serological assays, the complement fixation (4) and microagglutination (7) tests (data not shown).

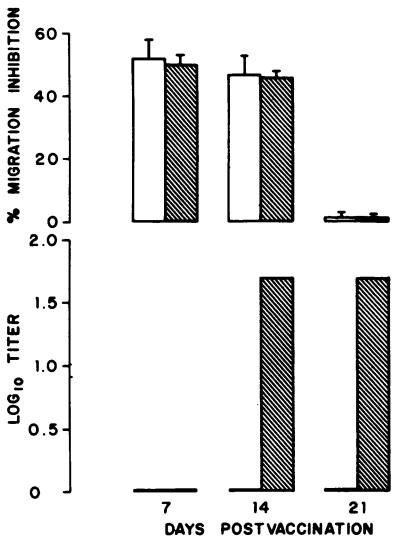

FIG. 1. *Percent migration inhibition and humoral antibody responses of guinea pigs after one dose of C. burnetii vaccine. Mean and standard error of the mean of four to six animals.*

Guinea pigs responded to the two-dose vaccine regimen with enhanced cellular and humoral responses, as compared with those given a single dose. On day 7 after administration of the second dose, IMM was approximately 70% in cultures with either antigen (Fig. 2). Inhibition decreased thereafter, but remained demonstrable for 28 days, compared with a 14-day duration after the single-dose schedule. Likewise, antibody production was also potentiated with the two-dose regimen. Phase II antibody was detected after 7 days, and phase I antibody was detected on day 21 (Fig. 2).

The humoral and cellular responses were also determined in guinea pigs vaccinated once with the vaccine emulsified in an equal volume of IFA. These animals demonstrated IMM and humoral antibody responses similar to those animals given two doses of the vaccine a week apart (data not shown).

LT. Antigen-specific responsiveness of peripheral leukocytes from vaccinated guinea pigs was evaluated with the LT test. These responses were measured on the same animal at weekly intervals. Animals inoculated with a single dose of vaccine developed a relatively low, delayed LT response; the stimulation index did not differ from base line on day 7 or 14, but increased

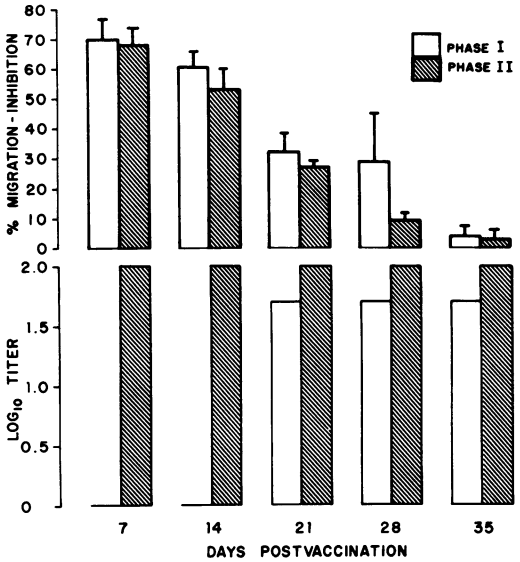

FIG. 2. *Percent migration inhibition and humoral responses of guinea pigs after two doses of C. burnetii vaccine. Mean and standard error of the mean of four to six animals.*

significantly on day 21 and persisted at an elevated level until day 35 (Fig. 3). In contrast, with the group vaccinated on the two-dose schedule, rickettsial antigens failed to stimulate a proliferative response at any time during the 42-day experimental period (data not shown).

Additional studies were performed with a group of animals immunized with vaccine in IFA. These guinea pigs demonstrated markedly enhanced leukocyte responsiveness. The response was minimal at 7 days (Fig. 3), but increased abruptly in the presence of either phase I or II antigen on day 14, and persisted at relatively high levels until day 42, when the experiment was terminated. In contrast, the stimulation indexes in cultures from animals given IFA without antigen remained at base line values throughout the experimental period. Stimulation indexes for phytohemagglutinin P control cultures ranged between 20 and 40 at each sampling time.

Responses of vaccinated animals to a virulent challenge. The efficacy of the vaccine given either in a single dose or in two doses a week apart was determined by in vivo protection studies. Evaluation of a protective effect was based upon inhibition of a febrile response (>40°C). Animals were challenged intraperitoneally 21 days after vaccination with approximately 10^5 50% egg infective dose of live phase I *C. burnetii*. Rectal temperatures of all guinea pigs vaccinated with either one or two doses, or one dose of the vaccine in IFA, remained within normal range after challenge, whereas all nonvaccinated animals became febrile by days 3 to 5 postchallenge (Table 1). Expanded studies on protection are being performed by one of us (J.W.J.). Humoral and cellular immune responses of vaccinated animals were also examined at selected times after challenge with virulent rickettsiae. Earlier results indicated that guinea pigs vaccinated with one dose of the vaccine had no demonstrable serum antibody to phase I antigen, and there was no IMM response with either phase I or phase II antigen at 21 days (Fig. 4). As early as day 1 after challenge, IMM became evident and reached significantly higher levels 3 days later; this response diminished by day 7 and was no longer present on day 14. In contrast, there was no phase I antibody present 1 day after challenge, but antibody titers for both phase I and II antigens were elevated on day 3, continuing to increase on day 7, and remained at high levels on day 14.

The LT responses were also determined for a comparable group of guinea pigs after challenge

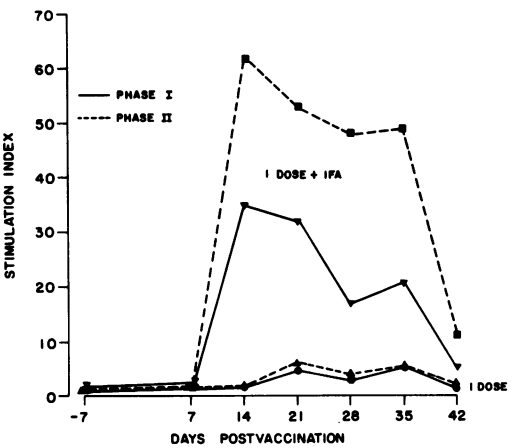

FIG. 3. *Stimulation indexes of guinea pigs after vaccination with one dose of C. burnetii vaccine with and without IFA. Mean of 4 to 12 animals.*

TABLE 1. *Protection of vaccinated guinea pigs against intraperitoneal challenge with phase I C. burnetii*

Treatment	Febrile response[a]
Single dose of vaccine	0/10
Two doses a week apart	0/10
Single dose of vaccine emulsified in IFA	0/10
Nonvaccinated	10/10

[a] Protection based on absence of a febrile response (>40°C).

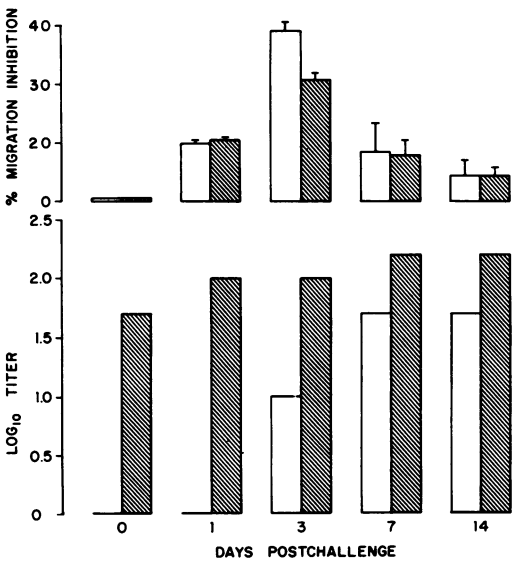

FIG. 4. *Percent migration inhibition and humoral responses of vaccinated guinea pigs (1 dose) after challenge with phase I C. burnetii. Mean and standard error of the mean of four to eight animals.*

at 21 days. At the time of challenge (21 days), the stimulation index value was approximately 5.0 (Fig. 3). Unlike the significant increase in the IMM responses, there was no significant elevation in the stimulation index up to 14 days after challenge (data not shown).

DISCUSSION

Guinea pigs vaccinated subcutaneously with Formalin-killed, particulate, phase I *C. burnetii* developed a cell-mediated immune response as measured by two different in vitro assays. The IMM activity was demonstrable at very high levels within 1 week after vaccination, but was relatively short-lived. This response to subcutaneous vaccination is similar to that observed in guinea pigs after infection with virulent phase I *C. burnetii* by the respiratory route (11).

The temporal course of the antigen-specific response as measured by the LT test did not correlate with IMM. After one dose of vaccine, LT response developed more slowly than IMM, but persisted at low levels for a longer period; sensitized populations of lymphocytes were present in peripheral circulation 35 days after vaccination. After two doses of vaccine, the blastogenic response failed to be detectable, although IMM reactivity was markedly enhanced. The hypothesis put forth by Oppenheim (17) explains this lack of response as caused by antigen-antibody complexes formed in antibody excess; Lee and Sigel (14) suggest that antigens complexed with immunoglobulin M antibodies can depress lymphocyte stimulation, with the same result. The observation that antibody to phase I antigen was present in guinea pigs that received two doses of vaccine, but not in those that received one dose, would seem to agree with this hypothesis. However, although antibody titers for guinea pigs that were inoculated with one dose of the vaccine suspended in IFA were similar to those for the two-dose group, the blastogenic response after vaccine in IFA was remarkably enhanced. This suggests that the lack of stimulation index response for the two-dose regimen was not due wholly to antibody suppression.

The ability of killed rickettsial vaccines to induce a cellular immune response has been noted by others. Coonrod and Shepard (5) reported that lymphocytes from humans vaccinated or infected with spotted fever or typhus group rickettsiae exhibited in vitro LT when cultured with specific antigen. Kenyon et al. (10a) found that Formalin-killed *Rickettsia rickettsii* produced negligible LT but high levels of IMM in guinea pigs.

The present studies indicate a possible role for cell-mediated immunity in protection. Guinea pigs vaccinated with one dose of the vaccine produced no detectable serum antibody (by microagglutination, complement fixation, and indirect immunofluorescence), but developed antigen-specific lymphocyte responsiveness. Suggestive evidence for a role for cell-mediated immunity in resistance to infection with *C. burnetii* was described in our previous studies (12, 13), in which macrophages from guinea pigs vaccinated with a phase I Formalin-killed antigen were capable of destroying phase I rickettsiae in the absence of immune serum. Likewise, Benenson (2) showed that, whereas only 50% of human volunteers vaccinated with killed phase I antigen developed detectable antibody, all were resistant to aerosol challenge with virulent rickettsiae.

The role of lymphokines, such as the macrophage inhibition and blastogenic factors, in the pathogensis of, and/or host defense against, Q fever infections is not known. Enhancement of antigen-specific blastogenic responses could result in an increased production of functional T and B lymphocytes and/or in stimulated production of lymphokines or antibodies capable of affecting host resistance in a variety of ways. It has been suggested by other investigators that macrophage inhibition factor not only localizes macrophages at the site of infection, but also participates in the activation of macrophage; phagocytic, bacteriostatic, and even bactericidal activities of macrophages are enhanced in the

presence of either sensitized lymphocytes or their soluble products (8, 16, 20). Studies by Hinrichs and Jerrells (9) provide evidence that normal guinea pig peritoneal macrophages cultured in vitro with immune lymphocytes or with macrophage inhibition factor-rich supernatant fluid inhibit the growth of ingested *C. burnetii*.

Our data suggesting a role for cell-mediated immunity do not eliminate a contributory role for humoral antibody in the overall defense of the host. Serum antibodies for phase II antigen were present at high levels in those guinea pigs given two doses of the vaccine, and antibodies against phase I antigen rapidly increased in vaccinated animals after challenge. In our previous studies, pretreatment of rickettsiae with immune serum not only enhanced ingestion of organisms within the phagocyte but also potentiated the destruction of ingested organisms (12, 13). Moreover, while immune serum is not known to have a direct rickettsicidal effect on *C. burnetii*, rickettsiae incubated with specific antiserum before inoculation do not cause infection in guinea pigs (1). The rapid mobilization of both humoral and cellular responses after challenge of vaccinated guinea pigs suggests that both facets of immune activity participate in defense against Q fever infection. Further work is needed to define the specific contribution of humoral and cellular immune responses in protection.

ACKNOWLEDGMENTS

We thank Robert W. Stockman and Chester L. Redmond for their technical assistance.

LITERATURE CITED

1. **Abinanti, F. R., and B. P. Marmion.** 1957. Protective or neutralizing antibody in Q fever. Am. J. Hyg. **66**:173–195.
2. **Benenson, A. S.** 1959. Q fever vaccine: efficacy and present status, p. 47–60. *In* Symposium on Q fever. Medical Science Publication no. 6. Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.
3. **Bozeman, F. M., and B. L. Elisberg.** 1963. Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc. Soc. Exp. Biol. Med. **112**:568–573.
4. **Casey, H. L.** 1965. Standardized diagnostic complement fixation method and adaptation to micro test. II. Adaptation of the LBCF method to microtechnique, p. 31–34. Public Health Monogr. no. 74. U.S. Government Printing Office, Washington, D.C.
5. **Coonrod, J. D., and C. C. Shepard.** 1971. Lymphocyte transformation in rickettsioses. J. Immunol. **106**: 209–215.
6. **Fiset, P.** 1966. Vaccination against Q fever, p. 528–531. *In* Vaccines against viral and rickettsial diseases of man. PAHO Science Publication no. 147. Pan American Health Organization, Washington, D.C.
7. **Fiset, P., R. A. Ormsbee, R. Silberman, M. Peacock, and S. H. Spielman.** 1969. A microagglutination technique for detection and measurement of rickettsial antibodies. Acta Virol. **13**:60–66.
8. **Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David.** 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. J. Exp. Med. **138**:952–964.
9. **Hinrichs, D. J., and T. J. Jerrells.** 1976. In vitro evaluation of immunity to Coxiella burnetii. J. Immunol. **117**:996–1003.
10. **Jerrells, T. R., L. P. Mallavia, and D. J. Hinrichs.** 1975. Detection of long-term cellular immunity to *Coxiella burneti* as assayed by lymphocyte transformation. Infect. Immun. **11**:280–286.
10a. **Kenyon, R. H., M. S. Ascher, R. A. Kishimoto, and C. E. Pedersen, Jr.** 1977. In vitro guinea pig leukocyte reactions to *Rickettsia rickettsii*. Infect. Immun. **18**:840–846.
11. **Kishimoto, R. A., and G. T. Burger.** 1977. Appearance of cellular and humoral immunity in guinea pigs after infection with *Coxiella burnetii* administered in small-particle aerosols. Infect. Immun. **16**:518–521.
12. **Kishimoto, R. A., B. J. Veltri, F. G. Shirey, P. G. Canonico, and J. S. Walker.** 1977. Fate of *Coxiella burnetii* in macrophages from immune guinea pigs. Infect. Immun. **15**:601–607.
13. **Kishimoto, R. A., and J. S. Walker.** 1976. Interaction between *Coxiella burnetii* and guinea pig peritoneal macrophages. Infect. Immun. **14**:416–421.
14. **Lee, J. C., and M. M. Sigel.** 1974. A differential effect of IgM and IgG antibodies on the blastogenic response of lymphocytes to rubella virus. Cell. Immunol. **13**:22–31.
15. **Meiklejohn, G., and E. H. Lennette.** 1950. Q fever in California. I. Observations on vaccination of human beings. Am. J. Hyg. **52**:54–64.
16. **Nathan, C. F., M. L. Karnovsky, and J. R. David.** 1971. Alterations of macrophage functions by mediators from lymphocytes. J. Exp. Med. **133**:1356–1376.
17. **Oppenheim, J. J.** 1972. Modulation of *in vitro* lymphocyte transformation by antibodies: enhancement by antigen-antibody complexes and inhibition by antibody excess. Cell. Immunol. **3**:341–360.
18. **Ormsbee, R. A., E. J. Bell, D. B. Lackman, and G. Tallent.** 1964. The influence of phase on the protective potency of Q fever vaccine. J. Immunol. **92**:404–412.
19. **Robinson, D. M., and S. E. Hasty.** 1974. Production of a potent vaccine from the attenuated M-44 strain of *Coxiella burneti*. Appl. Microbiol. **27**:777–783.
20. **Simon, H. B., and J. N. Sheagren.** 1972. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell. Immunol. **4**:163–174.
21. **Smadel, J. E., M. J. Snyder, and F. C. Robbins.** 1948. Vaccination against Q fever. Am. J. Hyg. **47**:71–81.
22. **Spicer, D. S., and A. N. DeSanctis.** 1976. Preparation of phase 1 Q fever antigen suitable for vaccine use. Appl. Environ. Microbiol. **32**:85–88.
23. **Tigertt, W. D., and A. S. Benenson.** 1956. Studies on Q fever in man. Trans. Assoc. Am. Physicians **69**:98–104.