COMPARISON OF Q FEVER CELLULAR AND CHLOROFORM-METHANOL RESIDUE VACCINES AS SKIN TEST ANTIGENS IN THE SENSITIZED GUINEA PIG

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Summary. – *Coxiella burnetii* phase I whole cell vaccine (WCV) is associated with risk of severe local delayed-type hypersensitivity (DTH) reactions in previously immunized individuals or those sensitized by natural exposure. We compared this vaccine to another investigational vaccine derived by chloroform-methanol extraction of phase I whole cells (chloroform-methanol residue vaccine, CMRV). Hairless guinea pigs, sensitized with either WCV or CMRV, were given 60, 600 and 6,000 ng of WCV or CMRV in an intradermal (i.d.) skin test. The i.d. administration of WCV consistently caused more host reactions than comparable doses of CMRV in guinea pigs sensitized with either WCW or CMRV, suggesting that CMRV may be a safer vaccine. However, the CMRV was not innocuous and caused significant indurated lesions and micro-abscesses at the 600 ng and 6,000 ng skin test sites.

Key words: Q fever; Coxiella burnetii; chloroform-methanol residue vaccine; whole cell vaccine; skin test

Introduction

Q fever, caused by the rickettsial organism *Coxiella burnetii*, is usually characterized by an acute febrile influenza-like syndrome which may present as pneumonitis or hepatitis and rarely evolves into endocarditis (Fries *et al.*, 1993; Raoult *et al.*, 1990; Richmond and McKinney, 1993). Non-immune agricultural workers or laboratory personnel with occupational exposure are at significant risk of contracting the disease (Timoney *et al.*, 1988). In addition, *C. burnetii* represents the rickettsial agent with the greatest risk of laboratory infection because it has a low infective dose (1 – 10 organisms) and is highly infectious by aerosol. Q fever has been reported to be the second most common laboratory-acquired infection (Richmond and McKinney, 1993).

Vaccination of individuals previously sensitized by either natural infection or repeated immunization has been associated with severe local reactions such as the formation of indurated masses, sterile abscesses, or granulomas at the vaccination site (Benenson, 1959). These reactions are due to DTH (Bell *et al.*, 1964; Luoto *et al.*, 1963; Ormsbee and Marion, 1990). Because of these potential reactions, vaccinees are screened with an i.d. skin test of 20 ng of *C. burnetii* phase I WCV before immunization to determine the sensitivity to the Q fever antigen. A positive test is defined as an area of induration greater than 5 mm in diameter on day 7 after administration. Those with a positive skin test are considered to be immune and are not vaccinated (Lackman *et al.*, 1962).

Current research is focused on developing a vaccine with diminished capacity to cause adverse tisuue reactions in individuals previously sensitized to *C. burnetii*. If successful,

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Abbreviations: CFA = complete Freund's adjuvant; CMRV = chloroform-methanol residue vaccine; DTH = delayed-type sensitivity; ELISA = enzyme-linked immunosorbent assay; i.d. = intradermal(ly); i.m. = intramuscular (ly); s.c. = subcutaneous(ly); WCV = whole cell vaccine

such a vaccine could eliminate the necessity for skin testing before vaccination. A new investigational vaccine, the residue obtained after chloroform-methanol extraction of WCV (CMRV), caused fewer adverse clinical and pathological reactions than the WCV in laboratory animals (Ascher *et al.*, 1983a). This could be due to removal of the lipophilic components of the organism during the chloroform-methanol extraction process. In a recently completed study, the CMRV was shown to have equivalent efficacy to a licensed cellular vaccine (Q-Vax) in rodents challenged with a lethal aerosol dose of *C. burnetii* (Waag *et al.*, 1994).

In this report, we compared the i.d. DTH responses of the two Q fever vaccines to determine their efficacy as skin test antigens.

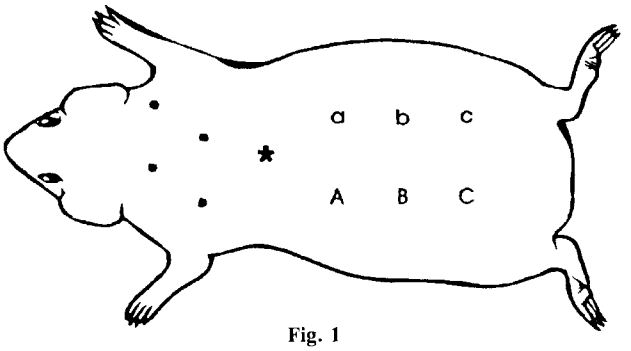

Fig. 1
Schematic of the dorsal view of a quinea pig showing sensitization and skin test sites
The sensitizing agents were administered s.c at multiple sites in the cervical region (•) at 0.1 ml per site. Sixty ng of WCV was given i.d. at site A, 600 ng at site B, and 6,000 ng at site C. Six thousand ng of CMRV was given at site a, 600 ng at site b, and 60 ng at site c. The saline skin test control site is depicted by the asterisk (*)

Materials and Methods

Animals. Twenty 300 – 400 g specific pathogen-free (Sendai, PVM, Reo-3, LCMV, *B. bronchiseptica* and *M. pulmonis*), female hairless Hartley guinea pigs (Crl:IAF(HA)-hrBR, Charles River Laboratories, Wilmington, MA, USA) were used in this study. The protocol was approved by the Laboratory Animal Care and Use Committee of the U.S. Army Medical Research Institute of Infectious Diseases. The guinea pigs were housed in standard stainless steel solid bottom cages with hardwood chip bedding, and were maintained in an AAALAC accredited facility under standard laboratory conditions at 20 – 21°C with a photoperiod of 12 hrs light/12 hrs dark. They were fed a commercial guinea pig diet (NIH34, Harlan Teklad, Madison, WI, USA) *ad libitum* and were provided filtered tap water through an automatic watering system. All animals tested negative for Q fever antibodies by an enzyme-linked immunosorbent assay (ELISA) before the start of the study (Uhaa *et al.*, 1994).

Anesthesia. All animals were sedated with a mixture of acepromazine maleate (10 mg/ml) (Promace, TechAmerica Group, Inc., Elwood, KS, USA) and ketamine hydrochloride (100 mg/ml) (Ketaset, Parke-Davis, Morris Plains, NJ, USA). One ml of acepromazine was mixed with 10 ml of ketamine, and 0.1 ml of this mixture was administered intramuscularly (i.m.) per animal. This prevented movement, facilitated proper placement of the immunogen, and allowed for accurate measurements of DTH response.

Sensitization. Two investigational vaccines were used as sensitizing and skin test agents: WCV (TSI-GSD 224, The Salk Institute, Swiftwater, PA, USA) and the investigational CMRV (TSI-GSD 217, The Salk Institute). The sensitizing agents were prepared by emulsifying 300 µg of vaccine in 1 ml of complete Freund's adjuvant (CFA) (Sigma). This gave a final concentration of 150 µg/ml (Ascher *et al*, 1983b; Ruble *et al*, 1994).

The 20 guinea pigs were sorted into four groups of five animals each. The groups were inoculated subcutaneously (s.c.) (0.1 ml/site) with either 0.4 ml (60 µg) of CMRV/CFA, 0.4 ml (60 µg) of WCV/CFA, 0.2 ml of CFA alone or 0.2 ml of saline (Fig. 1). As each animal was inoculated, it was consecutively numbered 1 through 5 on the back of the head with an indelible marker, which allowed individual animal identification within groups. This number was re-marked as needed.

The different sensitization groups (WCV/CFA and CMRV/CFA) were necessary to measure the influence of the sensitizing antigens on the skin test response. The CFA group was included to test non-specific reactions stimulated by the adjuvant itself. The saline group was used to test the inherent reactivity of the vaccines in an unsensitized animal.

Skin testing. Six weeks post sensitization, the animals were i.d. skin tested over the left thoracolumbar dorsum with three 0.1 ml doses of WCV (60 ng, 600 ng, or 6000 ng) in normal saline, over the right thoracolumbar dorsum with CMRV (60 ng, 600 ng, or 6000 ng), and at one saline control site (Fig. 1). Circles (approximately 2.5 cm in diameter) were drawn around each skin test site with an indelible marker to ensure the site could be located the next day. Skin test sites were monitored for induration daily through day 7, then on days 9, 11, and 14. The induration was measured in mm with a ruler laid across the site at the point of maximum induration. The same technicians performed consecutive measurements to decrease the variability attributable to different observers.

Histopathology. Three animals from each group were randomly selected for histopathological examination of the skin test sites on day 14. After animals were euthanized by CO_2 narcosis, an elliptical incision was made around each of the seven skin test sites, and the skin and s.c. tissues were undermined and removed. The tissue specimens were placed in labeled cassettes and immersion-fixed in 10% neutral buffered formalin. The formalin-fixed tissue specimens were processed and embedded in paraffin according to established procedures (Prophet *et al.*, 1992). Histological sections were cut at 5 – 6 µm on a rotary microtome, mounted on glass slides, and stained with Harris' hematoxylin-eosin. The slides were examined randomly and histopathological findings were determined by routine light microscopy. DTH responses were graded according to severity of the inflammatory response and abscess formation. Inflammation was graded on a scale from 0 to 5, based on the number of inflammatory cells and the overall extent

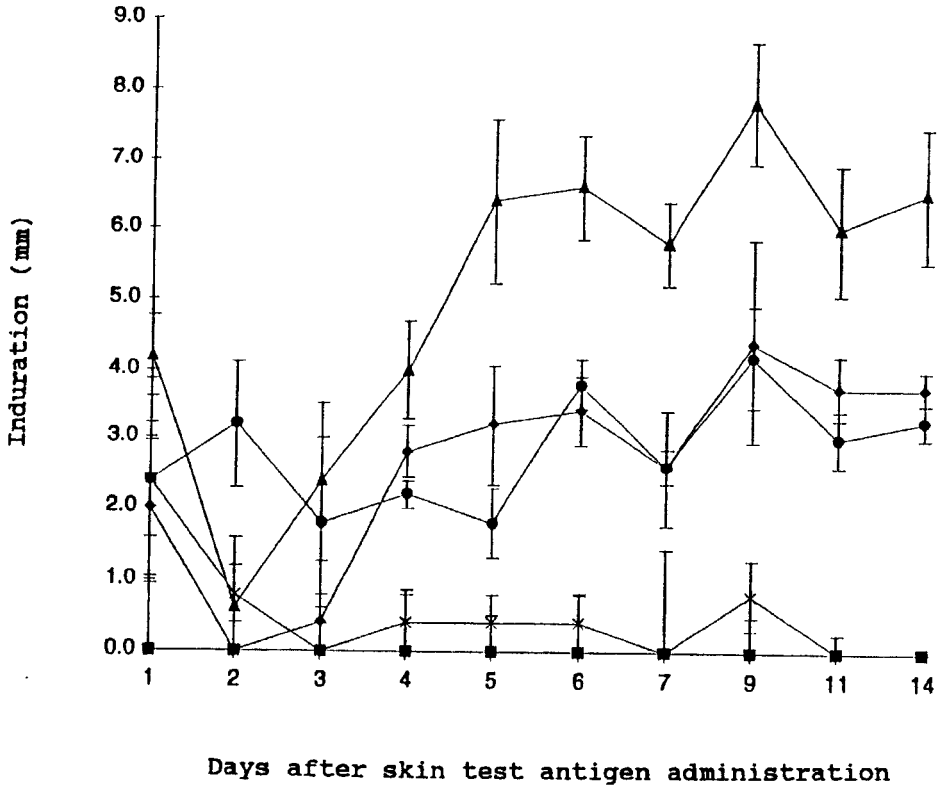

Fig. 2
Kinetics of induration at the skin test sites

Results are expressed as means ± SD. Six hundred mg of a skin test antigen was used. Skin test antigen, sensitization region: WCV, CMRV/CFA (◆); WCV, WCV/CFA (▲); WCV, saline (■) ; CMRV, CMRV/CFA (x); CMRV, WCV/CFA (●); CMRV, saline (■). There was no induration noted in animals „sensitized" with saline.

of their distribution. Abscesses were graded on a scale from 0 to 2, based on the presence and quantity of neutrophils.

Data analysis was performed by use of a commercial software package, v6.11 of SAS/STAT (SAS Institute, 1989, Cary, NC, USA). The peak value of induration at each skin test site was chosen for analysis without regard to the day on which the peak occurred. Thus each animal had seven responses, one from each site representing the maximum induration that occurred at that site over the experimental period. A one-way between groups analysis of variance was computed on each of the 7 sites. This permitted a comparison of each site between the 4 sensitization groups (e.g. 60-ng-CMRV on a CMRV/CFA-sensitized animal vs. 60-ng-CMRV on a WCV/CFA-sensitized animal vs. 60-ng-CMRV on a CFA-sensitized animal vs. 60-ng-CMRV on a saline-control animal).

A one-way repeated measures analysis of variance was computed for each of the 7 sites to compare vaccine antigen treatments within each group (e.g. 60-ng-CMRV vs. 60-ng-WCV skin test sites on the same animal). The alpha level for the F tests was set at 0.05. When the overall F test was significant ($P < 0.05$), pairwise comparisons of the group means were made with a least squares means test. The alpha level for these tests was also set at 0.05.

Results

Induration

The peak induration for each animal, for a given dose, was used to determine the group's average peak response. Peak induration at skin test sites occurred between days 6 and 9 after antigen inoculation, regardless of sensitization regimen or skin test antigen (Fig. 2).

The inherent reactivity of the antigens was demonstrated by the presence of indurated lesions in the unsensitized control groups (saline and CFA). The WCV 6,000-ng skin test dose caused an average 2.5 mm diameter of the indurated area in the saline control group (range 1 – 4 mm) and an average 1.8 mm diameter of the indurated area in the CFA group (range 0 – 3 mm) (Table 1). The CMRV 6,000-ng skin test dose caused an average diameter of induration of 0.5 mm in guinea pigs receiving saline, which included one animal with a zone of induration of 2 mm.

When comparing the different sensitization methods, the WCV/CFA group developed larger indurated lesions than

Table 1. Effect of skin test antigen on size of indurated skin test sites

Sensitization[a]	Dose[b]	Induration (mm)[c] WCV	CMRV	P[d]
WCV/CFA	60	4.4±2.8	0.8±1.0	0.03
CMRV/CFA	60	2.2±0.5	0.0±0.0	0.0004
Saline	60	0.0±0.0	0.0±0.0	–
CFA	60	0.0±0.0	0.0±0 0	–
WCV/CFA	600	8.0±2.0	5.2±0.8	0.07
CMRV/CFA	600	5.2±2.8	2.0±1.4	0.07
Saline	600	0.0±0.0	0.0±0.0	–
CFA	600	0.0±0.0	0.0±0.0	–
WCV/CFA	6000	11.2±0.8	10.2±1.3	0.14
CMRV/CFA	6000	9.8±1.1	7.8±0.8	0.02
Saline	6000	2.5±1.3	0.5±1.0	0.02
CFA	6000	1.8±1.6	0.0±0.0	0 07

[a]Guinea pigs were sensitized by s.c. route.
[b]Given by i.d. route.
[c]The mean diameter of zones of induration is expressed in mm ± SD.
[d]Probability, a measure of significance of the differences between the results of CMRV and WCV tests.

Table 2. Summary of histopathological results

Antigen-Dose[a]	Sensitization[b]	Inflammation[c]	Abscess[d]
Saline	WCV/CFA	0	0
	CMRV/CFA	0	0
	Saline	0	0
	CFA only	0	0
WCV 60 ng	WCV/CFA	2.5	0
	CMRV/CFA	1.8	0
	Saline	0.8	0
	CFA only	0.8	0
CMRV 60 ng	WCV/CFA	1.8	0
	CMRV/CFA	1.7	0
	Saline	0	0
	CFA only	0	0
WCV 600 ng	WCV/CFA	3.0	0.7
	CMRV/CFA	2.3	0
	Saline	1.3	0
	CFA only	1.7	0
CMRV 600 ng	WCV/CFA	2.7	0
	CMRV/CFA	1.7	0
	Saline	1.0	0
	CFA only	0.7	0
WCV 6000 ng	WCV/CFA	3.5	1.7
	CMRV/CFA	3.2	0.7
	Saline	1.5	0
	CFA only	1.8	0
CMRV 6000 ng	WCV/CFA	3.0	0.3
	CMRV/CFA	3.2	0.7
	Saline	1.2	0
	CFA only	1.5	0

Data are presented as the mean (N=3) of the histopathological reactions seen.
[a]Administered to sensitized guinea pigs by i.d. route.
[b]Guinea pigs sensitized by s.c. route.
[c]Severity of inflammation: none (normal tissue) = 0; minimal = 1; mild = 2; moderate = 3; marked = 4; severe = 5.
[d]Abscess formation: none = 0; microabscess not detectable at the subgross level = 1; neutrophilic abscess detectable at the subgross level = 2.

the CMRV/CFA group, regardless of skin test antigen used (Table 1). The relative ranking of lesion size did not depend on the sensitization method used. E.g., lesions at the CMRV 600-ng skin test site were smaller than those at the WCV 600-ng skin test site on both sensitization regimens. This implies that the method of sensitizing of the animals did not skew the results, regardless of the skin test antigen used.

In the WCV/CFA-sensitized animals, the WCV 60 ng test site developed an average induration of 4.4 mm (Table 1), which was significantly larger than the saline control site ($P = 0.02$) and the CMRV 60-ng site ($P = 0.03$). The CMRV 60-ng skin test site (0.8 mm induration) was not significantly larger than the saline control test site ($P > 0.05$), indicating that 60 ng of CMRV was below the threshold needed to detect previously sensitized animals. The induration at the 600- and 6000-ng WCV sites was consistently larger than that caused by CMRV, but these differences were not statistically significant ($P > 0.05$).

The extent of induration observed after skin testing on CMRV/CFA sensitized animals was smaller than that observed in WCV/CMRV-sensitized animals. Zones of induration at the WCV skin test sites were significantly larger than those noted for CMRV at the 60-ng ($P = 0.0004$) and 6,000-ng dose sites ($P = 0.02$).

Histopathology

Histopathology results are summarized in Table 2. There were mild histological changes at all skin test sites where WCV was injected and all sites receiving 600 or 6,000 ng of CMRV, regardless of the sensitization regimen. The inflammatory responses at the WCV and CMRV skin test sites were dose-dependent and consistent with the size of the indurated lesions observed grossly. Figs. 4–8 are photomicrographs of tissue samples from WCV/CFA-sensitized animals illustrating the "mild, moderate, and marked" grading scheme. Fig. 3 is a saline control skin test site representing essentially normal skin. Note the absence of inflammatory cells in the dermis. Figs. 4 and 5 show a "mild" infiltration of inflammatory cells, predominantly lymphocytes, macrophages, and occasional multinucleate giant cells, in the deep dermis and panniculus at a 60-ng WCV skin test site. Figs. 6 and 7 demonstrate the dose-dependent, increasing severity of the inflammatory reaction ("moderate" grade) at a 600-ng WCV skin test site. Note large numbers of inflammatory cells with an increased proportion of polymorphonuclear cells extending from the panniculus and deep dermis into the superficial dermis with thickening of the dermis. A "marked" reaction with abscess formation is seen in Fig. 8 (6,000-ng WCV skin test site).

Fig. 3
Photomicrograph of normal guinea pig skin
Tissue from a saline control skin test site. Magnification 30 x.

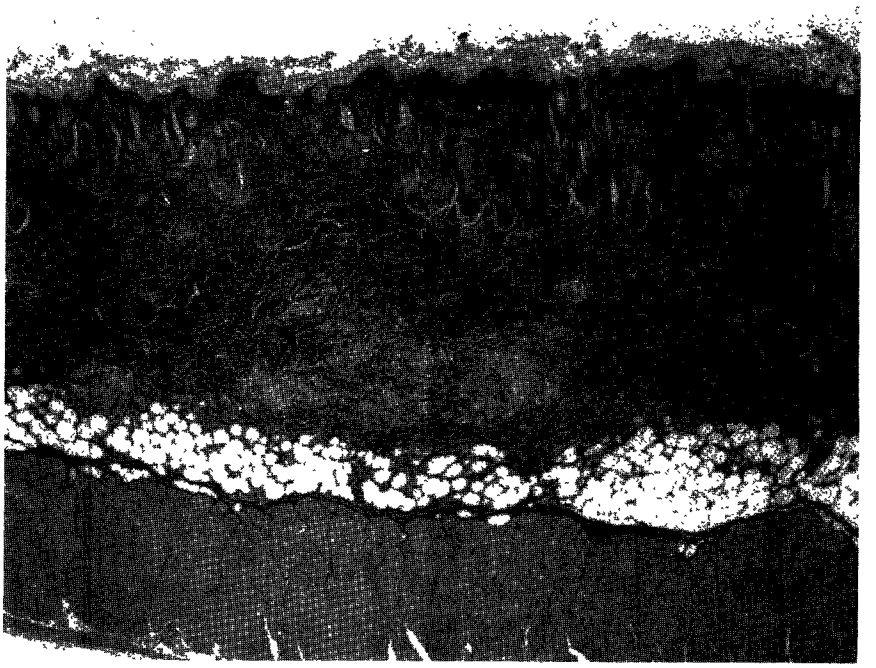

Fig. 4
Photomicrograph of a mild inflammatory reaction (severity grade 2)
Tissue from a WCV 60-ng skin test site Magnification 30 x.

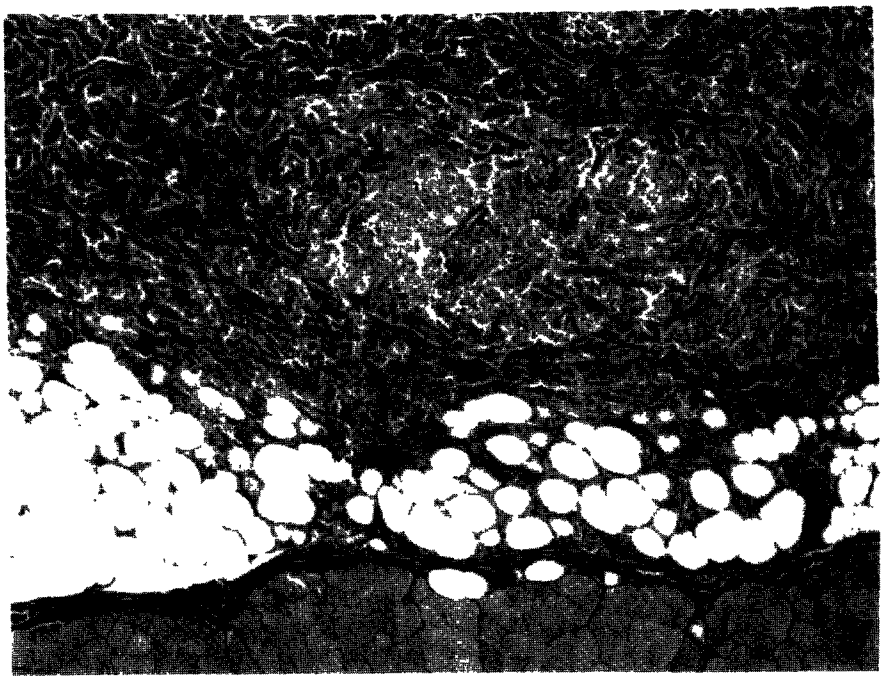

Fig. 5
Higher magnification (150 x) of Fig. 4
Note mild cellular infiltrates in dermis.

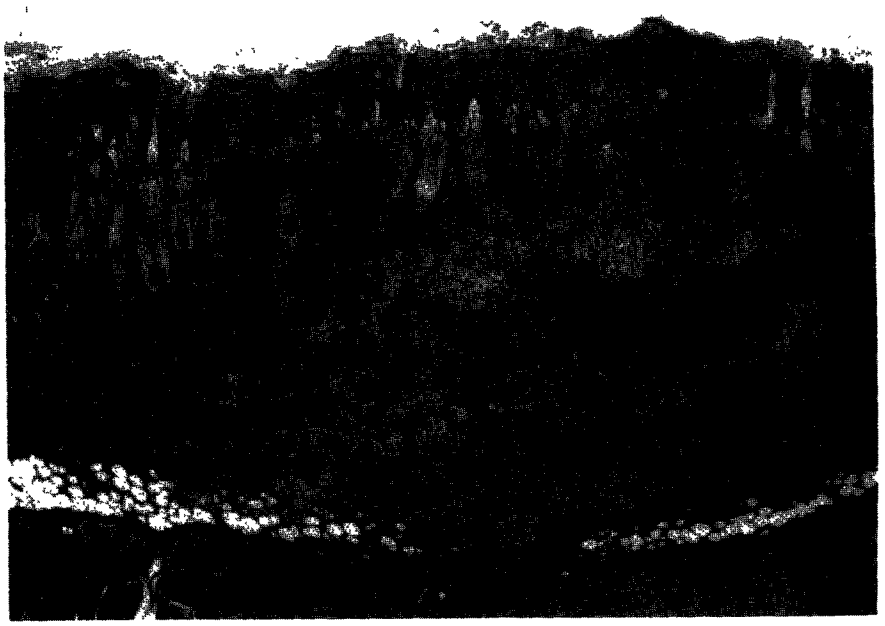

Fig. 6
Photomicrograph of a moderate inflammatory reaction (severity grade 3)
Tissue from a WCV 600-ng skin test site. Magnification 30 x.

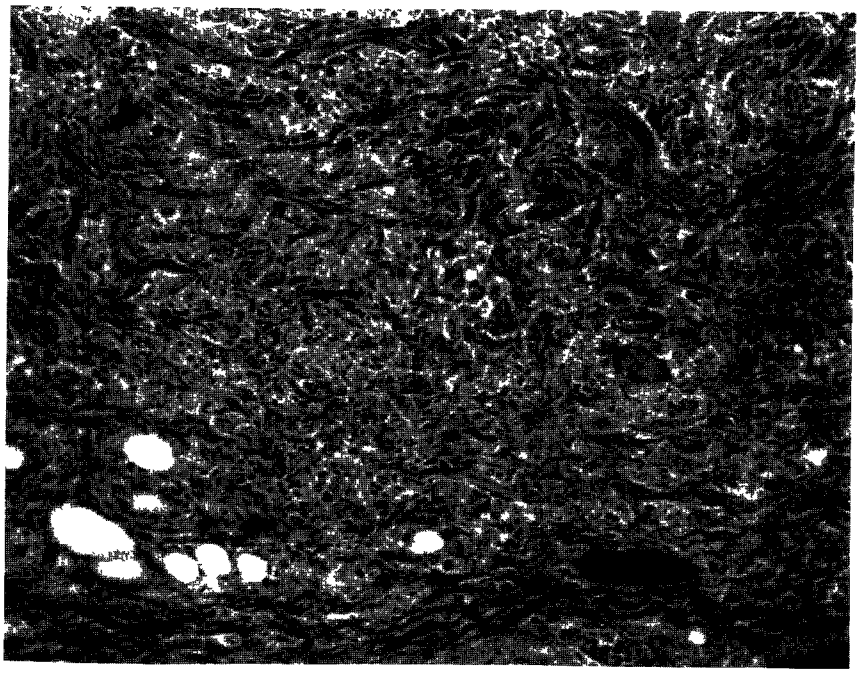

Fig. 7
Higher magnification (150 x) of Fig. 6

Fig. 8
Photomicrograph of a marked inflammatory reaction (severity grade 4) with a neutrophilic abscess (abscess grade 2)
Tissue from a WCV 6,000-ng skin test site. Magnification 30 x

The host inflammatory response at the WCV skin test sites was slightly greater at each dose than at an equivalent dose of CMRV in guinea pigs previously sensitized with either WCV or CMRV. Abscess formation (visible at the subgross level) in the WCV/CFA-sensitized animals was seen at the WCV 600-ng test site in one out of three guinea pigs, while none was observed at the 600-ng CMRV test sites. No abscesses occurred at the 600-ng WCV or 600-ng CMRV test sites in guinea pigs sensitized to CMRV/CFA. Abscesses or microabscesses (visible at magnification of 30 x) were present at four of the six 6,000-ng WCV test sites on WCV/CFA- and CMRV/CFA-sensitized animals (Fig. 8). Microabscesses were also seen at three of the six 6,000-ng CMRV test sites on the same guinea pigs. No abscesses were noted at any test site in the unsensitized control groups (CFA and saline) with either antigen. However, the WCV test sites consistently exhibited a slightly increased inflammatory response compared to the CMRV test sites in those unsensitized control guinea pigs.

Discussion

Ascher *et al.* (1983a) previously reported that induration caused by an i.d. skin test with CMRV peaked at day 2. Our study showed an increase in induration on day 2 at the CMRV skin test sites, however, the peak induration occurred between days 6 and 9 for both the WCV and CMRV skin test antigens. This discrepancy could be due to experimental design differences. We sensitized the animals by injecting 30 μg of antigen (WCV) s.c. and skin-tested them at 6 weeks with 60 ng of WCV or CMRV. The abovementioned authors sensitized the animals with 12 mg of antigen (WCV) by footpad injections, and skin-tested them at 2 weeks with 50 ng of WCV or CMRV. However, they also noted that the severity of DTH reaction increased five-fold in WCV-sensitized animals, if the animals were held and skin-tested at 6 weeks post sensitization.

The smallest dose of skin test antigen, which will yield a positive test in previously sensitized animals/people, should be used. The skin test antigen currently used in assessing immunity to *C. burnetii* in humans is a 20 ng dose of WCV (Lackman *et al.*, 1962). A pilot study to investigate adverse reactions stimulated by *C. burnetii* vaccines using WCV skin test doses of 0.6, 6.0, and 60 ng in sensitized guinea pigs did not provide statistically significant results (data not shown). We therefore chose 60, 600, and 6,000 ng as our test doses. The induration at the WCV 60-ng skin test site was significantly larger than that at the saline control skin test site in both WCV/CFA- and CMRV/CFA-sensitized animals ($P = 0.02$ and 0.04, respectively). The 60-ng CMRV skin test sites, however, did not develop indurated lesions significantly larger than the saline control sites with either sensitization method ($P > 0.05$). In fact, induration at the 60-ng CMRV skin test site was below detectable levels in the CMRV/CFA-sensitized group. This indicates that 60 ng of CMRV was below the minimum amount of antigen necessary to evaluate prior sensitization in this model, and that WCV at 60 ng was a superior skin test antigen to CMRV.

These results also indicate that CMRV may stimulate fewer potentially adverse reactions if given as a vaccine to previously immune individuals. Although CMRV is antigenically similar to WCV (McCaul *et al.*, 1991), extraction with chloroform-methanol removes immunomodulatory components (Waag and Williams, 1988) and may account for the differences noted between the two antigens in this study.

Although, in most cases, the i.d. CMRV caused significantly smaller lesions than WCV ($P < 0.05$), CMRV was not innocuous. The lesions at the CMRV 600- and 6,000-ng sites were not significantly smaller than those with corresponding doses of WCV ($P = 0.07$ and 0.14, respectively) in the WCV/CFA-sensitized group.

We were also interested in comparing histologically adverse tissue reactions initiated by these vaccines. As Q fever vaccines generate a vigorous cell-mediated immune response (Kishimoto *et al.*, 1978), we expected an inflammatory reaction to the antigen. We saw this as a minimal to mild inflammatory reaction in the unsensitized control groups and as dose-dependent inflammatory responses to WCV and CMRV in all test groups. Although the histological differences were slight, the inflammatory response to WCV was more severe than that to CMRV. The abscess formation was consistently seen at the higher skin test doses (6,000 ng) in the sensitized animals.

As the proposed human immunizing dose for CMRV is between 30 and 100 μg (5–17 times greater than our highest skin test dose), CMRV may cause granulomas or abscess formation in previously sensitized vaccinees. However, because of the s.c. route of vaccination, the CMRV antigen may diffuse more readily and be better tolerated than when given i.d.. Studies are being conducted to evaluate the abscess potential of the CMRV when given to a sensitized guinea pig at the proposed s.c. immunization doses (Wilhelmsen and Waag, 1993).

Three conclusions can be drown from this study. First, WCV was more effective than CMRV in sensitizing guinea pigs to develop detectable skin test responses to these antigens. Second, WCV was more effective as a skin test antigen than an equivalent amount of CMRV. Third, CMRV was associated with fewer i.d. pathological changes than WCV, but those differences were primarily seen at doses below 6,000 ng.

Note. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals;* the facilities used were fully accredited by

the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International.

The views, opinions and/or findings contained herein are those of the authors and should be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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