Inactivation of *Coxiella burnetii* by Gamma Irradiation

By G. H. SCOTT,1* T. F. McCaul† AND J. C. WILLIAMS2

1 United States Army Medical Research Institute of Infectious Diseases, Department of Intracellular Pathogens, Bacteriology Division, Fort Detrick, Frederick, Maryland 21701, USA
2 Office of the Director of Intramural Research Programs, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20204, USA

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The gamma radiation inactivation kinetics for *Coxiella burnetii* at −79 °C were exponential. The radiation dose needed to reduce the number of infective *C. burnetii* by 90% varied from 0.64 to 1.2 kGy depending on the phase of the micro-organism, purity of the culture and composition of suspending menstrum. The viability of preparations containing 10⁸ *C. burnetii* ml⁻¹ was completely abolished by 10 kGy without diminishing antigenicity or ability to elicit a protective immune response in vaccinated mice. Immunocytochemical examinations using monoclonal antibodies and electron microscopy demonstrated that radiation doses of 20 kGy did not alter cell-wall morphology or cell-surface antigenic epitopes.

INTRODUCTION

*Coxiella burnetii*, the causative agent of Q fever, is extremely resistant to adverse environmental conditions. The resistance of *C. burnetii* to various chemicals, and to heat and physical disruption (Babubieri, 1959) may be accounted for in part by the pleomorphism of the micro-organism, including the endogenous synthesis of small dense cells consistent with spore formation (McCaul & Williams, 1981). The micro-organism is easily transmitted aerogenically, both experimentally and in nature, producing Q fever in man with as few as 10 micro-organisms. Thus, work with materials contaminated with *C. burnetii* must be carried out under strict biological containment conditions. A variety of chemical disinfectants have been used to inactivate *C. burnetii*. However, recommended chemical methods occasionally fail to sterilize *C. burnetii* preparations (Bernard et al., 1982), and treatments sufficiently harsh to overcome the innate resistance of the micro-organism may destroy sensitive antigens. We became acutely aware of the resistance of *C. burnetii* to disinfectants during the production of a new-generation vaccine. Formalin treatment of purified *C. burnetii*, followed by chloroform/methanol extraction at 53 °C, failed to completely destroy infectivity. We sought a method that would ensure complete inactivation of *C. burnetii* while retaining structural and immunological activity. Such an inactivation procedure should also facilitate preparation of noninfectious diagnostic reagents for interchange among laboratories that do not have necessary containment for highly infectious agents. We therefore studied the effects of gamma radiation on *C. burnetii* cellular viability, morphology, antigenicity and immunogenicity.

METHODS

Organisms. *Coxiella burnetii* Nine mile strain in phase I (9MIC7) and phase II (9M1IC4) cloned in cell culture, were cultured in yolk-sacs (YS) of embryonated chicken eggs. Infected yolk-sac membranes were blended to a 50% (w/v) suspension in phosphate-buffered saline (PBS; 0.01 M-NaCl, 0.15 M-sodium phosphate, pH 7-0), frozen and

1† Present address: Electron Microscope Centre, University of Queensland, St Lucia, Queensland 4067, Australia.

Abbreviations: LCV, large-cell variants; SCV, small-cell variants; YS, yolk-sacs.

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stored at −70 °C. Organisms were extracted from infected YS and purified by Renografin density-gradient centrifugation as previously described (Williams et al., 1981).

Radiation treatment. Volumes (0·5 ml) of 50% infected YS (9MIC7-YS), purified 9MIC7 and 9MIIIC4 in PBS (1 mg ml⁻¹) were packed in dry-ice and exposed to 0, 1, 5 and 10 kGy in an AECL Gamma Cell 220, Cobalt-60 radiation unit.

Formalin treatment. Formalin was added to suspensions of 50% infected YS or 1 mg purified C. burnetii ml⁻¹ to a final concentration of 2.46% (w/v) formaldehyde; the suspension was stirred slowly at room temperature for 16 h, then dialysed against distilled water for 7 d, with water changes after 1, 2 and 6 d.

Viability assays. The concentration of C. burnetii surviving irradiation was determined by a titration based on seroconversion of outbred, 7-week-old, female CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) injected with serial dilutions of the test samples. Irradiated test samples were also serially passaged three times in chick-embryo YS to amplify any viable micro-organisms. The irradiated samples were diluted 1:1000 in PBS and 0·3 ml vol injected into each of ten 5-d-old chick embryos. After incubation at 36 °C for 14 d, the YS were harvested, blended to a 50% (w/v) suspension in PBS and passed to another ten, 5-d-old embryos. Gimenez-stained, third passage YS were examined microscopically for C. burnetii. Similar serial passages of irradiated C. burnetii were done in mice. Mice injected with third passage spleens were tested for seroconversion.

Statistical analysis. Regression analysis of the concentration of surviving C. burnetii as a function of irradiation dose, the estimated sterilizing dose ($D_{100}$) and its confidence intervals were computed by using a statistical analysis system based on the work of Zerbe (1978).

Protection studies. The model for Q fever in inbred A/J mice was used for this study (Scott et al., 1987). Groups of 7-week-old, female A/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were injected intraperitoneally with 0·5 ml (30 μg micro-organism dry wt) of either an irradiated or formalin-treated preparation. Three weeks after vaccination, mice from each group were bled and the sera collected for antibody determination; the spleens were aseptically removed and weighed and organs examined for gross necrotic foci. In vitro proliferation responses of splenic lymphocytes to recall antigens and to T- and B-cell mitogens were measured by standard procedures (Damrow et al., 1985). All antibody measurements were by ELISA (Williams et al., 1986). Three weeks after the immunizing injections, mice from each group were challenged intraperitoneally with 10 LD₅₀ of the small cell variant of phase I C. burnetii. Mice were observed for morbidity and mortality for 3 weeks. Survivors were examined for cellular and humoral immune responses as described above.

Preparation of polyclonal and monoclonal antibodies for immunolabelling. Rabbit polyclonal antibodies were produced against C. burnetii phase I whole cells (Williams & Cantrell, 1982). Murine monoclonal antibody (#1135) was raised against lipopolysaccharide (LPS) by previously described procedures (Williams et al., 1984). Murine monoclonal antibody (#4D6) reactive against an immunogenic 29·5 kDa membrane protein was also selected for immunolabelling (McCaul et al., 1988). The normal control, hyperimmune and monoclonal antibodies were diluted in buffer containing 20 mM Tris-buffered saline (TBS) (pH 8·1) supplemented with bovine serum albumin (BSA) (1 mg ml⁻¹), Tween 20 (final concentration 0·05%, v/v) and sodium azide (20 mM).

Colloidal gold labels. Goat anti-mouse IgM and IgG, and goat anti-rabbit IgG colloidal gold conjugates were obtained from Janssen Life Sciences Products. Gold particles were either 5 or 15 nm in diameter. The conjugates were diluted 1/10 in TBS/BSA Tween 20 (pH 8·1).

Immunolabelling. Non-irradiated samples were fixed in 1·25% (v/v) glutaraldehyde in 66 mM-sodium cacodylate buffer (pH 6·8) at 4 °C for 4 h. Irradiated cells were fixed in 0·25% (v/v) glutaraldehyde and 1% formaldehyde in the same buffer at 4 °C for 15 min. Pre-embedding in 2% (w/v) Difco Noble Agar was followed by a 15 min rinse in the same buffer. The dehydration and embedding procedures were done with either LR White or LR Gold medium (method to be published). Thin sections (60–100 nm) were cut, transferred to nickel grids and stained first with 0·5% (w/v) uranyl acetate in 50% (v/v) ethanol and secondly with 0·2% (w/v) lead citrate.

Electron microscopy. Both irradiated and non-irradiated cells were fixed in 3% (v/v) glutaraldehyde in 66 mM-cacodylate buffer (pH 6·8) containing 2·5 mM-CaCl₂. Pre-embedding in 2% Difco Noble agar was followed by (1) a brief rinse with 66 mM-cacodylate buffer containing 0·37 M-sucrose, (2) post-fixation in 1% (v/v) osmium tetroxide buffered with 66 mM-cacodylate for 1 h at 4 °C, (3) a brief rinse in distilled water, and (4) dehydration through graded methanol. During dehydration steps, the blocks were stained for 1 h at room-temperature with 0·5% (w/v) uranyl acetate in 30% (v/v) methanol. The blocks were embedded in Spurr epoxy resin. Ultrathin sections were stained with potassium permanganate (McCaul & Williams, 1981) and examined in a Joel 100B transmission electron microscope operated at 80 kV.

RESULTS

Inactivation of C. burnetii

C. burnetii in YS suspensions were more resistant to radiation than purified organisms.

Purified phase II cells were more resistant than phase I cells. Radiation doses required to kill
Radiation inactivation of Coxiella

Fig. 1. Inactivation of C. burnetii with gamma radiation. O, observed value; P, predicted value. The sterilizing dose \( (D_{100}) \) is predicted to be 6-55 kGy, with confidence limits in kGy as follows: 95% (5-45, 8-45); 99% (4-99, 10-35); 99-9% (4-30, 21-09).

Table 1. Inactivation of C. burnetii by gamma radiation

<table>
<thead>
<tr>
<th>Gamma radiation (kGy)</th>
<th>log(<em>{10}(\text{ID}</em>{50}, \text{ml}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9MIC7, YS purified (1 mg ml(^{-1}))</td>
</tr>
<tr>
<td>0</td>
<td>10.8</td>
</tr>
<tr>
<td>1</td>
<td>10.0</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
</tr>
<tr>
<td>10</td>
<td>0.0</td>
</tr>
<tr>
<td>(D_{10})*</td>
<td>0.885</td>
</tr>
</tbody>
</table>

* Gamma radiation (kGy) required to reduce infectivity by 90%.

90% of the organisms varied from 0-64 to 1-21 kGy depending on phase and purity of the cell preparation (Table 1). C. burnetii in all preparations examined survived a dose of 5 kGy, but a dose of 10 kGy completely inactivated preparations that initially contained up to \(10^{11}\) \(\text{ID}_{50}\) ml\(^{-1}\) of infectious C. burnetii. To ensure that organisms were completely inactivated by 10 kGy, each sample was serially passaged three times in embryonated chicken eggs, as well as in mice. No C. burnetii were observed by microscopic examination of Gimenez-stained YS smears prepared 14 d after injection with third passage YS material. Mice injected with large numbers of irradiated organisms developed low titres of phase II antibody stimulated by the antigenic mass of dead cells, but mice injected with second- and third-passage spleen samples did not seroconvert. Regression of the concentration of surviving phase I 9MIC7 and Henzerling strain organisms, as a function of radiation dose, predicts complete inactivation with a dose of 6-55 kGy (Fig. 1).

Effect of radiation on antibody-binding capacity

Based on ELISA, there was little difference in the capacity of formalin-inactivated and irradiated cells to bind antibody (Fig. 2). Sterilizing gamma radiation did not diminish the antigen-binding capacity of polyclonal antibody directed against phase I or phase II whole cells in ELISA (Fig. 3).
Effect of radiation on the morphology and the distribution of antigenic determinants in *C. burnetii* cells

Electron micrographs of non-irradiated *C. burnetii* demonstrated a heterogeneous population of large (LCV)- and small-cell variants (SCV) (Fig. 4a). The multilayered cytoplasmic membranous intrusion, dense peptidoglycan and nucleoid regions distinguished the SCV from the LCV. The displacement of the ribonucleotide material to the periphery of the cell of the LCV, leaving a transparent zone between the nucleotide mass and the granular cytoplasm, also distinguished that cell type from the SCV (Fig. 4a). Electron micrographs of irradiated *C. burnetii* demonstrated similar morphological details (Fig. 4b). An effect of the irradiation was not apparent.

Both SCVs and LCVs were labelled by antibody against whole-cell vaccine (phase I) in irradiated and non-irradiated cells (Fig. 4c, d). The gold labels were mostly distributed on the outer membrane, periplasmic space and the region circumscribed by the LPS. In both non-irradiated (Fig. 5a) and irradiated (Fig. 5b) samples, the epitopes of phase I LPS (LPS-I), labelled with monoclonal antibody #1135, were present mainly on the outer membrane, cytoplasmic membrane and along the periphery of the nucleoid region of the SCV. Either few or no gold labels were found among the LCV.

Gamma radiation appeared to have no effect on the distribution of the epitopes of a 29.5 kDa major surface protein. In both non-irradiated (Fig. 5c) and irradiated (Fig. 5d) samples, the gold labels were detected exclusively on the outer membrane of the LCV. Little or no label was found on the SCV.

Protective immunity

Mice injected with 30 µg of either formalin- or irradiation-inactivated phase I *C. burnetii* cells developed similar antibody titres (1:81920) against phase I and phase II cells and survived challenge by a dose of virulent *C. burnetii* that killed 73% of non-vaccinated mice within an
Fig. 4. Electron micrographs of *C. burnetii* phaser I. (a, c) Non-irradiated samples; (b, d) irradiated materials. (c, d) Material embedded in blocks of LR White and labelled with rabbit antibody against *C. burnetii* whole cells, and anti-rabbit IgG conjugates with gold. S, small-cell variant; L, large-cell variant. Bars, 100 nm.
Fig. 5. Electron micrographs of *C. burnetii* phase I. (a, c) Non-irradiated samples; (b, d) irradiated materials. (a, b) Material embedded in blocks of LR White and labelled with mouse monoclonal antibody (#1135) against LPS-I, and anti-mouse IgM conjugated with gold. (c, d) Material embedded in blocks of LR gold and labelled with mouse monoclonal antibody (#4D6) against the 29.5 kDa protein, and anti-mouse IgG conjugates with gold. S, small-cell variant; L, large-cell variant. Bars, 100 nm.
Radiation inactivation of Coxiella

Table 2. Lymphocyte responses of vaccinated A/J mice before and after challenge with C. burnetii

<table>
<thead>
<tr>
<th>Vaccine*/Treatment</th>
<th>Challenge route†</th>
<th>Mean spleen wt (mg, n = 3)</th>
<th>Lymphocyte response to:‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS/None</td>
<td>None</td>
<td>48</td>
<td>49.0 3.0 5.2</td>
</tr>
<tr>
<td>9MIC7/Formalin</td>
<td></td>
<td>290</td>
<td>19.4 0.8 1.1</td>
</tr>
<tr>
<td>9MIC7/Irradiation</td>
<td></td>
<td>200</td>
<td>20.7 1.0 1.7</td>
</tr>
<tr>
<td>pPBS/None</td>
<td>Intra-peritoneal</td>
<td>733</td>
<td>0.7 0.0 0.1</td>
</tr>
<tr>
<td>9MIC7/Formalin</td>
<td></td>
<td>359</td>
<td>8.1 0.7 1.7</td>
</tr>
<tr>
<td>9MIC7/Irradiation</td>
<td></td>
<td>261</td>
<td>10.3 1.1 2.4</td>
</tr>
</tbody>
</table>

* Mice were injected intraperitoneally with 30 μg of Nine Mile phase I C. burnetii inactivated with formalin or by 10 kGy gamma irradiation.
† 10^8-2ID_{50} of C. burnetii, 21 d after vaccination.
‡ Stimulation index.

average of 7.2 d. Challenge infections stimulated an 8-fold increase in spleen weight and dramatically suppressed in vitro lymphocyte responses to mitogens and recall antigens in naive mice. Both splenomegaly and lymphocyte suppression were moderated equally by prior immunization with whole, phase I cells inactivated by either formalin or radiation (Table 2).

DISCUSSION

The influence of physical conditions on the killing effect of gamma radiation for microorganisms has been well-documented (Jordan & Kempe, 1956; Raghu & Kesavan, 1986). The radiation dose, reaction temperature, composition of the suspending medium, presence of oxygen, as well as the complexity and size of the genome of the organism, are important factors governing the inactivation rate. Our study examined high concentrations of C. burnetii under relatively protective conditions at -79 °C in crude suspensions of chick YS membranes, and under less protective conditions obtained by suspending purified organisms in buffered saline. Predictably, the radiation dose required to kill C. burnetii in YS suspension was higher than needed in cleaner, less complex preparations, and we detected a difference in killing rate between purified phase I and phase II cells. Purified phase II organisms retain more host-cell material throughout the purification process, and are generally more difficult to purify than are phase I preparations (Williams et al., 1981). The inactivation dose for C. burnetii was less than that required for most viruses (Sullivan et al., 1971; Thomas et al., 1981). This is consistent with variation in genome sizes since the smallest viruses have predictably been found to be the most radiation-resistant (Elliott et al., 1982). However, the radiation dose required to inactivate C. burnetii was severalfold higher than that required to abolish lethality for mice and plaque-forming ability of scrub typhus rickettsiae (Eisenburg & Osterman, 1977), even though the organisms have genomes of similar size.

McCaul & Williams (1981) demonstrated two cell types, a large-cell and a small-cell variant, in cultures of C. burnetii and described 'spore-like' forms that occur within the large-cell types. The small-cell variants are resistant to extreme adverse conditions, retaining their morphology and viability through osmotic shock and ultrasonication. Perhaps they also contribute to the radiation-resistance of C. burnetii.

Our data resemble that from most virus studies, indicating an exponential dose-survival relationship for C. burnetii. A plot of the logarithm of the number surviving against dose is a straight line, suggesting that a single ionizing hit is sufficient to destroy infectivity. Extrapolation of this line suggests that no C. burnetii would survive radiation doses in excess of 6.55 kGy. Amplification procedures which detect one infectious micro-organism failed to reveal any viable C. burnetii in samples given 10 kGy, the upper level of the 99% confidence interval for the estimated sterilizing dose.
Our results demonstrated that the antigenic and immunogenic properties of *C. burnetii* were not significantly altered by a radiation dose in excess of that needed to ensure complete inactivation. Mouse-protection studies indicated that radiation- and formalin-inactivated organisms provided vaccinated animals with similar levels of protection against lethal challenge with *C. burnetii*. No significant alterations in antigenicity were detected by using gamma-sterilized cells in ELISA, or in lymphocyte transformation tests. The small variance observed between the antibody-binding capacity of formalin-treated and irradiated cells may simply reflect leaching of LPS or protein from the surface of unfixed cells, or the release of irradiated cells from the surface of ELISA plates.

Electron microscopy indicated that sterilizing radiation did not destroy the morphology of the organisms. Although electron microscopy combined with immunolabelling techniques cannot be used to quantify the antigenic distribution between non-irradiated and irradiated materials, the sensitivity of the technique allowed us to relate antigenic determinants with structure, and to test the effect of radiation on the distribution of the antigens. Immunolabelling revealed that epitopes for the LPS-I, a 29.5 kDa major surface protein, and phase I antigenic determinants remained intact after irradiation. We have used monoclonal antibodies against the LPS-I and the 29.5 kDa protein as immuno-probes to distinguish the cell types of both phase I and phase II cells (unpublished). Similar distribution of the label in irradiated materials, using such monoclonal antibodies, implies that such immuno-probes could also effectively be used on irradiated samples.

REFERENCES


