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Evaluation of Different Fixatives and Treatments for Immunohistochemical Demonstration of *Coxiella burnetii* in Paraffin-Embedded Tissues

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Various fixatives and treatments such as acetone, methanol, Bouin fixative, modified Bouin fixative, 10% Formalin, modified methacarn, periodate-lysine-paraformaldehyde, acetone-methyl benzoate-xylene, and EDTA were evaluated for their effect on the immunoreactivity of *Coxiella burnetii* in paraffin-embedded tissues by using the avidin-biotin-peroxidase complex and the peroxidase-antiperoxidase procedure. *C. burnetii* antigen was shown to be present in liver, spleen, and uterus tissues of experimentally infected mice by all methods of fixation and treatment. A positive immunoreaction was seen in cytoplasmic vacuoles of macrophages, as extracellular rod-shaped organisms, and as residual particulate extra- and intracellular debris. Immunoreactivity and cellular preservation, however, varied substantially with the individual fixatives. Optimal immunostaining of *C. burnetii* was achieved by EDTA treatment and Bouin and acetone fixation. The avidin-biotin-peroxidase technique proved to be slightly more sensitive than the peroxidase-antiperoxidase procedure when primary antibody dilution was used as the criterion for sensitivity.

Coxiella burnetii is an obligate intracellular rickettsial organism, which causes both acute and chronic Q fever in animals and humans (13, 16). Currently, visualization of *C. burnetii* in cell cultures or paraffin-embedded tissues involves the use of histochemical methods and direct or indirect immunofluorescence (3, 6, 7, 13). Histochemical stains such as Stamp, Giménez, or Machiavello stain are useful for viewing *C. burnetii* in tissue sections, smears, and cell monolayers, but they have the disadvantage of being nonspecific (3, 13). Immunofluorescence allows no specific anatomic localization of the antigen, frequently exhibits nonspecific autofluorescence, requires special UV light equipment, and provides no permanent preparation (8). Therefore the application and development of new techniques for the detection of *C. burnetii* in clinical specimens is recommended by the World Health Organization (World Health Organization Workshop on Q fever, /VPH/COS 86.68, p. 1 to 14, 2 to 5 September 1986, Giessen, Federal Republic of Germany).

In contrast, high specificity and sensitivity (17), permanence of the reaction product, and usefulness in paraffin-embedded tissue, together with simultaneous histopathologic examination, make immunoperoxidase methods the techniques of choice at present. Therefore, to study the spread and pathogenesis of *C. burnetii* in experimentally infected laboratory animals (e.g., mice and guinea pigs) and in naturally infected humans and animals, we improved a recently described indirect immunoperoxidase method (14) and compared the effects of various fixatives and two immunohistochemical methods on the immunoreactivity of *C. burnetii* antigen in paraffin-embedded tissue.

MATERIALS AND METHODS

Tissue preparation. After 10 passages in mice, *C. burnetii* phase I strain Nine Mile (*C. burnetii* NMI) was propagated in the yolk sacs of embryonated chicken eggs, harvested, and purified by Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradient centrifugation as previously described (18). Twelve adult male and female BALB/c mice were each inoculated intraperitoneally with 3.85×10^6 inclusion-forming units of *C. burnetii* NMI in 400 μl of phosphate-buffered saline (PBS). Briefly, the titers of 10-fold dilutions of *C. burnetii* were determined in BGM cell cultures in flat-bottom tubes on round cover slips (diameter, 12 mm) without application of an agar overlay. After 3 days, infected cells were fixed in methanol-H_2O_2 and stained by the indirect immunoperoxidase technique (14). Immunopositive inclusions were counted, and titers were calculated and expressed in inclusion-forming units per milliliter of the original suspension (W. Schneider et al., manuscript in preparation). Three mice mock-infected with PBS served as a negative control. Infected mice were observed daily for overt clinical signs. The animals were sacrificed 3, 6, and 9 days postinoculation (p.i.). The spleen, liver, and uterus were immediately removed from each mouse after the animals were killed. Thin slices of tissue were immersed in one of the following fixatives or fluids and subsequently processed as described: (i) acetone, 3 h at room temperature (11); (ii) 10% Formalin, 48 h at room temperature (11); (iii) Bouin fixative (60 ml of saturated aqueous picric acid, 20 ml of 10% Formalin, 4 ml of glacial acetic acid), 24 h at room temperature (11); (iv) methanol, 6 h at room temperature (8); (v) modified Bouin fixative, 6 h at room temperature (15); (vi) modified methacarn, 24 h at room temperature (10); (vii) acetone-methyl benzoate-xylene (AMeX), 12 h at −20°C in acetone (12); (viii) periodate-lysine-paraformaldehyde (PLP), 20 h at 4°C (9); and (ix) EDTA with 7.5% (wt/vol) polyvinylpyrrolidone, 10 to 14 days at 4°C (5). After dehydration and/or clearing, the tissues were embedded in paraffin at 53°C.

Antibody. Antibodies were obtained by two intraperitoneal immunizations of New Zealand White rabbits with 3.85×10^5 inclusion-forming units of viable *C. burnetii* NMI; there was a 2-month interval between immunizations. The

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TABLE 1. Effects of different fixatives and treatments for immunohistochemical demonstration of *C. burnetii* in liver and spleen tissue[a]

Fixative or technique	Immunoreactivity[b] at following primary antibody dilution:				Tissue preservation
	1:3,000		1:6,000		
	PAP	ABC	PAP	ABC	
Acetone	++++	++++	++	++	Poor
Methanol	+	+	+	+	Poor to moderate
Bouin fixative	++++	++++	++++	++++	Good
Modified Bouin fixative	+	+	+	+	Moderate
10% Formalin	++	+++	+	++	Good
Modified methacarn	++	+++	++	++	Poor to moderate
PLP	++	+++	+	+	Poor to moderate
AMeX	++	+++	++	+++	Poor to moderate
EDTA	++++	++++	+	++	Poor

[a] Sections obtained from *C. burnetii*-infected mice 3, 6, and 9 days p.i.; mock-infected controls featured no immunoreactivity.
[b] Immunoreactivity scoring: ++++, intense; +++, good; ++, moderate; +, weak.

rabbits were bled weekly, and the development of phase II and phase I antibodies was monitored by using a complement fixation test with whole-cell phase II antigen and trichloroacetic acid extract of *C. burnetii* NMI, respectively. Serum specificity was determined as previously described (14). Serum samples with high antibody titers against phase I and II antigens were aliquoted and stored at −20°C.

Immunocytochemistry. Sections of paraffin-embedded tissue (thickness, 4 μm) were mounted on gelatin-covered slides, dried for 2 h at 58°C, deparaffinized in xylene (twice for 6 min each), rehydrated through graded alcohols, and washed in Tris buffer (0.05 M Tris base, 0.8% NaCl [pH 7.6]). Endogenous peroxidase was blocked by 0.5% H_2O_2 in methanol for 30 min at room temperature. The sections were then washed in PBS (pH 7.4) for 15 min and incubated for 10 min at 37°C with undiluted normal swine serum and then overnight at 4°C with rabbit anti-*C. burnetii* antibody at a dilution of 1:3,000 or 1:6,000. The sections were then washed in PBS and incubated sequentially with biotinylated goat anti-rabbit immunoglobulin G and avidin-biotin-peroxidase complex (ABC) (rabbit IgG Vectastain-Kit; Vector Laboratories, Inc., Burlingame, Calif.) as recommended by the manufacturer or swine anti-rabbit immunoglobulin G (dilution 1:40; Dako, Copenhagen, Denmark) and rabbit peroxidase-antiperoxidase (PAP; dilution 1:100; Dako) for 40 min at 37°C as described elsewhere (2). To visualize the peroxidase reaction, sections were washed and incubated with 0.01% (vol/vol) H_2O_2 in distilled water–0.1% (wt/vol) 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris buffer (pH 7.2) for 10 min, washed in tap water, and counterstained with hematoxylin. Control slides were prepared with tissues obtained from *C. burnetii*-infected and uninfected mice. To establish that staining was specific for *C. burnetii*, primary and bridge antibodies were substituted by normal rabbit serum and PBS, respectively. Effective blocking of endogenous peroxidase and absence of reactive tissue biotin were demonstrated by incubation with DAB and ABC. Adjacent sections were stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

From 4 days after challenge until 9 days p.i., infected mice exhibited nonprogressive lethargy and ruffling of fur. Macroscopically, progressive splenomegaly was first noticed by increased splenic weights 6 days p.i.

Table 1 summarizes the effects of different fixatives, treatments, and both immunohistochemical methods on *C. burnetii* immunoreactivity in paraffin-embedded tissues. *C. burnetii* showed positive immunostaining with all fixatives used and with both the ABC and PAP methods.

C. burnetii antigen was present as brown inclusions in macrophagelike cells in the liver, spleen, and uterus during the observation period (Fig. 1). By 3 days p.i., few foci of stained antigen were seen in the splenic red pulp, randomly distributed in the liver, endometrium, and in the loose connective tissue between the inner circular layer and the outer longitudinal layer of the myometrium. The associated mild cellular infiltration consisted of macrophages and neutrophils as revealed by hematoxylin and eosin staining. At 6 and 9 days p.i., there was a marked increase in both the amount of *C. burnetii* antigen and the inflammation. The antigen was present within cytoplasmic vacuoles, presumably representing phagolysosomes of macrophages, as a dense, homogeneous DAB precipitation product (Fig. 2A). Some cytoplasmic inclusions were filled with spherical and rod-shaped particles, suggesting the presence of individual organisms. In addition, rod-shaped extracellular organisms and particulate extra- and intracellular antigenic debris were

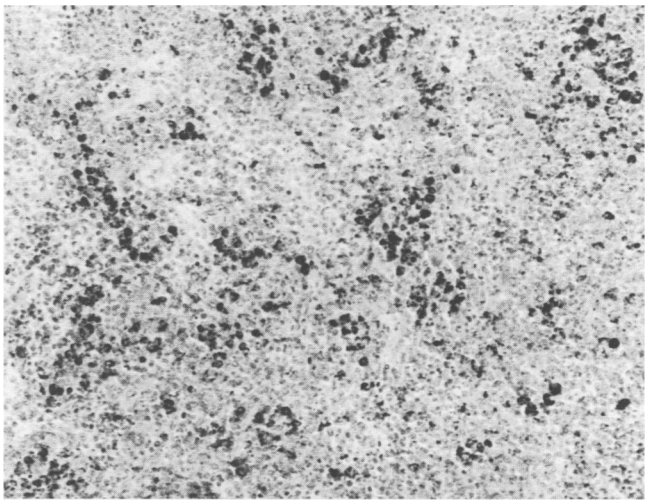

FIG. 1. ABC technique. Immunoperoxidase staining of *C. burnetii* in Bouin-fixed, paraffin-embedded mouse spleen 6 days p.i. Positive staining is seen in numerous macrophages in the red pulp. Rabbit anti-*C. burnetii* antibody was diluted 1:3,000. (Magnification, ×120.)

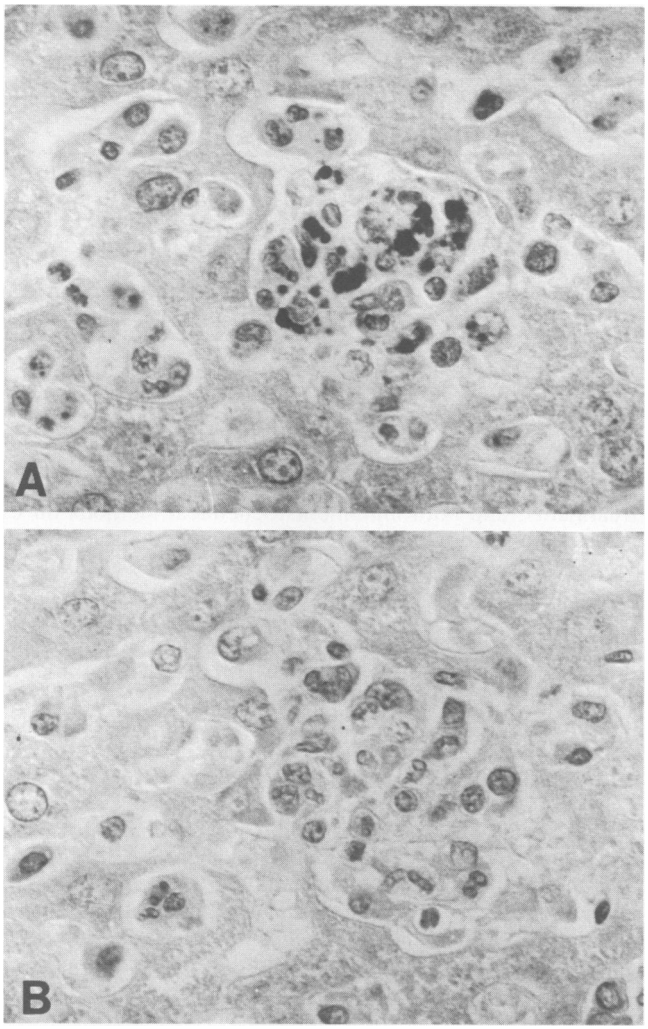

FIG. 2. ABC technique. (A) Immunoperoxidase staining of *C. burnetii* in Bouin-fixed, paraffin-embedded mouse liver 9 days p.i. The figure shows a hepatic granuloma with prominent cytoplasmic inclusions containing antigen in macrophages. Rabbit anti-*C. burnetii* antibody was diluted 1:3,000. (Magnification, ×480.) (B) Adjacent section to that in panel A. Note the lack of immunostaining. The section was incubated with normal rabbit serum (diluted 1:3,000) instead of hyperimmune serum. (Magnification, ×480.)

observed. Control sections from infected and uninfected mice were immunocytochemically negative, and nonspecific binding of ABC was not observed (Fig. 2B).

C. burnetii antigen staining varied substantially with different fixatives. EDTA, Bouin fixative, and acetone resulted in highly consistent and strong immunoreactivity; Formalin, AMeX, modified methacarn, and PLP displayed moderate immunostaining of *C. burnetii* antigen; and methanol and modified Bouin fixative were noticeably weaker.

The comparison of ABC and PAP methods revealed no substantial differences in their specific abilities to stain *C. burnetii*. However, the ABC method appeared to be slightly more sensitive than the PAP technique, and both unlabeled-antibody methods were more sensitive (with dilution of the primary antibody as the criterion for sensitivity) than the recently described indirect immunoperoxidase method (dilution of primary antibody, 1:200 versus 1:3,000 [14]). Furthermore, the staining intensity was greatly enhanced by the ABC and PAP methods (data not shown). These results are consistent with those of other studies (1, 4).

Bouin- and Formalin-fixed tissue had the best-preserved cellular morphology. Both immunohistochemical methods described in this paper have been applied successfully in our laboratory for the identification of *C. burnetii* antigen in paraffin-embedded tissues of experimentally infected mice and guinea pigs (W. Baumgärtner, unpublished results). To obtain both optimal immunostaining and cellular preservation, we routinely use Bouin fixation and the ABC method. This method allows a precise anatomic localization of *C. burnetii* and, simultaneously, correlation with the accompanying cellular immune response. Furthermore, immunohistochemical demonstration of *C. burnetii* in paraffin-embedded tissue also allows retrospective studies of suspected cases of human and animal Q fever infection. It also provides a permanent preparation which can be viewed by light microscopy and might be of particular interest for evaluation of biopsy material; it avoids the cumbersome, time-consuming, and hazardous isolation procedures of the pathogen in laboratory animals and embryonated eggs.

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