Identification of *Rickettsia rickettsii* in Formalin-Fixed, Paraffin-Embedded Tissues by Immunofluorescence

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With slight modification of a trypsin digestion technique, *Rickettsia rickettsii* were demonstrated specifically by immunofluorescence staining in Formalin-fixed, paraffin-embedded tissue sections from a human, rhesus monkey, and guinea pig with Rocky Mountain spotted fever and in infected membranes from a chicken embryo. Tissues were cut at 4 μ m and, using gelatin as a tissue adhesive, were hydrated in a routine manner. Sections were then digested in refrigerated 0.1% trypsin for 16 h, washed, and stained specifically for *R. rickettsii* by direct or indirect immunofluorescence. Rickettsial organisms were localized in affected vessels of the mammalian species and within the yolk sac epithelium of the chicken embryo. Specificity was confirmed by adsorbing antibody conjugates with *R. rickettsii* organisms. Trypsin digestion probably decreased tissue proteins which interfered with immunochemical attachment of antibody to the rickettsiae. The technique is valuable in that a diagnosis of Rocky Mountain spotted fever can be confirmed from Formalin-fixed tissues processed in a routine manner.

Rickettsia rickettsii is typically difficult to demonstrate histochemically in tissue sections because of (i) its small size, (ii) poor staining contrast between the organisms and tissues, and (iii) the affinity of the stains for normal tissue structures such as mast cell granules which, because of their size and morphology, can be mistaken for rickettsiae (12). These problems are encountered with a variety of special stains including Giemsa (3), Wright (11), Gimenez (3), and Pinkerton (11). Because of these difficulties and the lack of specific identification of *R. rickettsii* with the foregoing methods, immunofluorescence has been relied upon to demonstrate rickettsial organisms in pathogenetic studies of Rocky Mountain spotted fever (RMSF) (4-6, 9) and in human tissues submitted for diagnostic confirmation of the disease (12). This technique is preferred to routine histochemical procedures because of the ease of locating rickettsiae within tissue and the immunochemical specificity of the antibody conjugates. The primary drawback of the fluorescent-antibody technique to date, however, has been that it is necessary to examine sections from unfixed frozen tissues.

Repeated attempts in our laboratories to demonstrate rickettsial organisms by immunofluorescence in tissues fixed with 10% neutral Formalin were uniformly unsuccessful. With slight modification of a trypsin digestion procedure for fixed tissues, we were able to identify *R. rickettsii* by immunofluorescence in Formalin-fixed, paraffin-embedded tissue sections from an ex-

perimentally infected rhesus monkey, guinea pig, and chicken embryo yolk sac. In addition, *R. rickettsii* were demonstrated in tissues from a fatal human case of RMSF.

MATERIALS AND METHODS

Specimens. Paraffin blocks containing Formalin-fixed tissues from a guinea pig, rhesus monkey, and chicken embryo previously inoculated with *R. rickettsii* (Sheila Smith strain) were retrieved from storage in the Pathology Division. The guinea pig had been given 10^7 *R. rickettsii* intraperitoneally and was killed 3 days later. The monkey had been inoculated subcutaneously with 10^4 *R. rickettsii* and was killed 9 days later. The 5-day-old chicken embryo was inoculated into the yolk sac with 10^5 *R. rickettsii* and killed 3 days later. The inocula were prepared as previously described (4, 5). The paraffin blocks of tissue from each host had been in storage approximately 4, 3, and 1 years, respectively. Testis and epididymis from the monkey and guinea pig and yolk sac from the chicken embryo were examined. Frozen tissues and paraffin blocks of Formalin-fixed lung, heart, and testis from a human case of RMSF were referred for immunofluorescent confirmation. Diagnosis of RMSF was established by the contributors of this case by culture of *R. rickettsii* from fresh tissues, electron microscopic observation of rickettsial organisms within tissues, and characteristic clinical signs and lesions. Acetone-fixed smears of *R. rickettsii* grown in cell culture were also stained with the various antisera to determine effectiveness of the adsorption procedures.

Antisera. Antiserum against *R. rickettsii* was obtained from a previously infected monkey. A portion of this serum and goat anti-monkey immunoglobulin

were each conjugated with fluorescein as previously described (4, 6).

Tissue preparation and staining. The procedures of Huang et al. (1) were employed with the following modifications. Rather than using LePage Bond Fast resin glue (LePage's Ltd., Montreal, Canada), we found that gelatin, the tissue mordant routinely used in our laboratory, resisted trypsin digestion and did not autofluoresce. Approximately 0.2 g of gelatin (Difco Laboratories, Detroit, Mich.) was sprinkled over the surface of a 2-liter 37°C water bath, and tissue sections were floated from the water bath onto precleaned glass slides. Excess water was drained, and the slides were placed on a 60°C warmer for 1 h. The tissue sections were deparaffinized, hydrated, and immersed in a 0.1% solution of trypsin (1:250; Difco) and 0.1% CaCl_2 in distilled water, with the pH adjusted to 7.8 with 0.1 N NaOH. Tissues were incubated overnight (16 h) in the trypsin solution at 4°C, rinsed thoroughly in distilled water, and placed in phosphate-buffered saline for 30 min before staining. Direct and indirect staining was carried out as previously described (4, 6).

The direct procedure was controlled by staining normal tissue with fluorescein-labeled monkey anti-RMSF globulin and infected tissue with either fluorescein-labeled rabbit anti-Q fever or the labeled monkey anti-RMSF globulin which had been adsorbed with *R. rickettsii*. Controls for the indirect technique consisted of (i) staining normal tissue with hyperimmune monkey anti-RMSF globulin and fluorescein-labeled goat anti-monkey globulin, (ii) direct staining of infected tissue with only fluorescein-labeled goat anti-monkey globulin, (iii) reacting the sections with RMSF-negative monkey serum and applying the fluorescein-labeled goat anti-monkey globulin, and (iv) reacting the section with monkey anti-RMSF (adsorbed with *R. rickettsii*) and fluorescein-labeled goat anti-monkey globulin.

Fluorescein-labeled and untagged anti-RMSF globulin were exhaustively adsorbed by reacting samples of each with *R. rickettsii* organisms. The antigen used for adsorption was grown in static chicken embryo cell culture and inactivated with 0.1% Formalin. Pellets of approximately 1.5×10^8 rickettsiae were added to 1-ml samples of immune serum and continually mixed in the dark for 1 h at 37°C. A portion of each serum without the addition of *R. rickettsii* was treated identically. Each sample was centrifuged twice at $5,000 \times g$; the supernatant fluid was removed and reabsorbed as described above. The supernatant fluid and paired unadsorbed sera were used to stain tissue sections.

RESULTS

Microscopic examination of testis and cremaster muscle from the guinea pig and monkey revealed segmental vascular necrosis and inflammation. These lesions were characterized by perivascular accumulation of mononuclear cells, fibrinoid degeneration of the media of larger vessels, degenerative and proliferative endothelial changes, and thrombosis of some of the more severely affected vessels. In the human, micro-

scopic alterations of vessels were minimal and consisted principally of congestion of pulmonary capillaries accompanied by exudation and moderate congestion of vessels in the testis. Tissues from the chicken embryo and embryonic membranes were essentially normal.

When trypsin digestion was used, *R. rickettsii* were demonstrated in the endothelium of affected vessels with both direct and indirect immunofluorescent procedures of Formalin-fixed, paraffin-embedded tissues of the three mammalian species. In addition, rickettsiae were found in the endothelium of unaltered vessels of the heart and testis of the man and in the yolk sac epithelium of the chicken embryo (Fig. 1). The organisms appeared as small pleomorphic rods or coccobacilli. Staining intensity was brighter in the indirect as compared with the direct method; staining intensity was slightly reduced in Formalin-fixed, paraffin-embedded tissues as compared with fresh human tissues and smears of *R. rickettsii* grown in cell culture. The morphology of Formalin-fixed, paraffin-embedded tissues used for this immunofluorescent procedure was markedly improved over that of frozen tissues so that identification of various cells and tissues harboring rickettsiae was readily apparent. Normal tissues without rickettsiae did not stain; conjugates not specific for *R. rickettsii* and adsorbed anti-RMSF globulin failed to demonstrate rickettsiae both on smears and within frozen and fixed tissues containing the organisms.

DISCUSSION

The usual method for examination of tissues by immunofluorescence is to section frozen tissue, fix in acetone or alcohol, and stain with fluorescein-labeled conjugates (7). Frozen sections have obvious advantages for use in diagnostic situations because they can be processed rapidly and can also be used for isolation of infectious organisms. However, in retrospective study of specimens from RMSF patients or animals, fresh tissues are often unobtainable, thus necessitating the use of alternative techniques for diagnosis.

Except for some antigens such as those in certain bacteria (10), mycoplasma (D. H. Lein, Ph.D. dissertation, University of Connecticut, Storrs, 1974), fungi (2), and immunoglobulins (1), tissue fixation in Formalin frequently precludes subsequent demonstration of the antigens by immunofluorescence. Trypsin treatment of the Formalin-fixed, paraffin-embedded tissues permitted specific immunochemical attachment of antibody to *R. rickettsii*. The exact mechanism is not known, but it may result from re-

FIG. 1. *R. rickettsii* in chicken embryo yolk sac epithelium. Direct immunofluorescence. $\times 400$.

removal of nonspecific protein which interferes with the staining of rickettsiae by labeled antibodies (2). With this treatment, staining intensity of rickettsiae was only slightly reduced in the Formalin-fixed, paraffin-embedded tissues, as compared with nontrypsinized frozen tissues from the human case of RMSF stained in an identical manner. However, as indicated previously, tissue morphology was better preserved in the Formalin-fixed tissue so that cellular detail and localization of the rickettsiae were readily apparent (1).

The procedure of trypsin digestion as described by Huang et al. (1) was modified by replacing the tissue adhesive with gelatin. Because this is a mordant commonly used by many pathology laboratories, the preliminary steps do not deviate from routine histotechnology procedures. Thus, routine unstained sections submitted to pathologists can be utilized for immunofluorescence. The advantages of the technique in a diagnostic situation, especially in retrospective tissue examination, are readily apparent in that (i) a diagnosis of RMSF can be made on autopsy or biopsy tissue specimens processed in a routine manner, (ii) specific identification of *R. rickettsii* can be made in infected tissues, and (iii) the risk of potential infection by sectioning frozen tissues is eliminated by fixation in Formalin. Preliminary results indicate that the technique is equally applicable to other rickettsiae (*Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia sibirica*) as well.

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ADDENDUM

Since submission of this manuscript, another paper was published which demonstrated *R. rickettsii* in paraffin-embedded tissues by immunofluorescence. Essentially, the technique as described by Walker and Cain differed from the foregoing in the tissue mordant used (8).

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