Demonstration of *Rickettsia rickettsii* in the Rhesus Monkey by Immune Fluorescence Microscopy

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Indirect immune fluorescence was used to detect rickettsiae in the tissues of a primate inoculated subcutaneously with *Rickettsia rickettsii*. Rickettsiae were identified by indirect immune fluorescence predominantly in skin, skeletal muscle, scrotum, testicles, nares, heart, kidney, liver, brain, spleen, pancreas, and larynx. Cell culture assay confirmed the presence of infectious organisms in those specimens.

The immunofluorescence (IF) technique is highly specific, useful as a diagnostic procedure, and has frequently been used for the detection of rickettsial antibodies (2, 3, 5, 8). Immune fluorescence microscopy has also been used to detect rickettsiae or rickettsial antigens in cell cultures infected with *Rickettsia prowazekii* (12, 14) and *Coxiella burnetii* (11, 13). Other authors using this technique have detected rickettsiae in the blood of mice infected with *R. prowazekii* (11) and *C. burnetii* (1); and in the organs of mice experimentally infected with *C. burnetii* (9, 16) and *R. tsutsugamushi* (15). Using IF, *R. rickettsii*, the etiological agent of Rocky Mountain spotted fever (RMSF), has been demonstrated in ticks (4, 19) and primates infected by inhalation (22). To further define the *R. rickettsii*-host model system that is being used in this laboratory, we report here the results of studies in which indirect immunofluorescence (IIF) was used to detect and localize *R. rickettsii* in a rhesus monkey experimentally infected by subcutaneous inoculation of the rickettsiae.

MATERIALS AND METHODS

Rickettsiae. Yolk-sac grown Sheila Smith strain *R. rickettsii* was used in this study. Seed stock was stored at -70°C as a 50% suspension and contained 1.5×10^7 plaque-forming units (PFU)/ml. Plaquing was performed on chicken embryo cells by the method of Weinberg et al. (21). Rickettsiae were stained with acridine orange by the method of Silberman and Fiset (20) to demonstrate the approximate dimensions of known preparations of organisms.

Primate model. One fully conditioned, normal, adult rhesus monkey (Prime Laboratory, Inc., Farmingdale, N.J.), weighing 6 kg and free from ectoparasites, was inoculated subcutaneously in the posterior thigh with 1 ml of suspension containing 10^5 PFU of rickettsiae per ml. The monkey was moni-

tored until its rectal temperature decreased substantially on day 6 denoting the terminal phase of the disease. Blood was then drawn from the femoral vein into a heparin-coated syringe and the monkey was killed by the intravenous injection of concentrated KCl solution.

Tissue isolation. Using sterile technique, sections of various tissues and organs were collected, frozen on solidified carbon dioxide (CO_2), and held at -70°C pending preparation for culture of rickettsiae. For assay the tissues were weighed to the nearest thousandth gram and homogenized in brain heart infusion broth using a glass tissue homogenizer immersed in ice. The resulting suspensions were serially diluted in brain heart infusion broth to quantitate rickettsiae by plaque assay. Additional slices of specimens, 4 to 6 mm thick, were covered with a tissue-embedding medium (O.C.T. Compound, Ames Co., Elkhart, Ind.), frozen on solidified CO_2 , and held at -70°C until processed for IF.

Antisera. Rabbit anti-guinea pig serum was obtained from Microbiological Associates (Bethesda, Md.). Antiserum against *R. rickettsii* (fluorescent antiserum titer 1:2,560) was obtained from guinea pigs which had been infected with these rickettsiae.

Conjugate preparation. Antibodies were separated from rabbit anti-guinea pig serum by precipitation with 42.75% (vol/vol) methanol (7). Protein content, as determined by the Biuret test (10), was adjusted to 10 mg/ml in phosphate-buffered saline (PBS), pH 7.3 (10% final volume was carbonate-bicarbonate buffer, pH 9.0). Antibodies were conjugated at room temperature for 1 h with fluorescein isothiocyanate (FITC, BBL, Cockeysville, Md.) using 200 μg of dye:1.0 mg of protein. Unattached dye was removed by passing the conjugate through a column of G-25 coarse grade Sephadex (Pharmacia Fine Chemicals, Inc., Upsalla, Sweden). To reduce nonspecific fluorescence, the conjugate was diluted to 3.3 mg of protein per ml in PBS, absorbed twice with acetone-dried mouse liver powder (10 mg of powder:1 mg of protein), and centrifuged for 30 min at $35,000 \times g$ in an L2-65B ultracentrifuge (Beckman Instrument Co., Fullerton, Calif.). After ab-

sorption and centrifugation, the final product was passed through a 0.45- μ m filter and stored at -20°C in convenient volumes.

Standardization of conjugate. Working dilutions of both immune serum and FITC-conjugated globulin were established on WI-38-infected cells. WI-38 cells were grown in Leighton tubes on cover slips (9 by 22 mm). Each tube was seeded with 8×10^5 cells in 1 ml of Eagle basal medium containing Earl salts with L-glutamine and 10% fetal calf serum. After 72 h of incubation at 37°C in 5% CO_2 atmosphere, the cell monolayers were confluent (ca. 10^6 cells/Leighton tube) and were used for study. *R. rickettsii* stored as 50% infected yolk sac in sucrose phosphate glutamate buffer, pH 7.4, was diluted in brain heart infusion broth to approximately 3×10^6 PFU/ml for use. After removing growth medium each tube was washed with 1 ml of Puck saline A (18), pH 7.6, and then inoculated with 0.2 ml of the diluted rickettsial suspension. After stationary absorption for 1 h at 35°C the residual inoculum was removed, the cells were washed with 1 ml of Puck saline A, and 1 ml of Eagle basal medium containing Earl salts containing 2% fetal calf serum was added to each tube. After incubation for 72 h at 34 to 35°C maintenance medium was removed and each tube was washed twice with 1 ml of Puck saline A. Cells were then fixed in cold acetone and stored at -70°C until stained. IIF staining was consistently positive (4+) with 1:50 dilution of guinea pig anti-*R. rickettsii* serum and 1:38 dilution of rabbit anti-guinea pig conjugate.

Section preparation and staining. Frozen tissue sections (4 μ m) were cut in a Cryostat II microtome (Ames Co., Elkhart, Ind.), mounted on thin, pre-cleaned microscope slides, and fixed in cold (-70°C) acetone. Slides were stored at -70°C until examined. For IIF staining, sections were soaked for 10 min in PBS, dried with bibulous paper and placed in a moist staining chamber. Sections were then flooded with guinea pig anti-*R. rickettsii* serum (diluted 1:50 in PBS, pH 7.3) for 45 min, washed for 30 min in PBS with gentle agitation, and blotted dry. In a moist staining chamber slides were then flooded with FITC-conjugated rabbit anti-guinea pig conjugate (diluted 1:38 in PBS) for 45 min and washed with gentle agitation for 30 min in PBS. Cover slips were mounted using a phosphate-buffered glycerol (9 parts of glycerol:1 part of PBS) medium.

Histopathology. Tissues were fixed in neutral phosphate-buffered 10% formalin, processed through paraffin and sectioned. Representative sections were stained with phosphotungstic acid hematoxylin for fibrin, or Pinkerton's rickettsial stain (17).

IIF microscopy. A Carl Zeiss Standard microscope, equipped with an L-1 fluorescent illuminating system, KG-1 neutral heat shield, BG-38 and BG-3 exciter filters, an OG-1 barrier filter, oil immersion ultra dark field condenser (1.2/1.4), $\times 45$ oil immersion planapochromat objective with an iris diaphragm and $\times 10$ wide angle oculars, was used throughout these studies. Photographs were taken at 400 and 600 diameters with a Zeiss 35-mm camera using a $\times 10$ Zeiss or $\times 15$ Unitron ocular and Kodak high speed Ektachrome film.

RESULTS AND DISCUSSION

This report describes a comprehensive search for rickettsiae in multiple tissue specimens from a RMSF-infected primate using IIF, rickettsial culture techniques, and histologic examination. The animal followed a characteristic pattern of illness observed in the rhesus monkey model for RMSF used in this laboratory (L. S. Sammons, manuscript in preparation). The infected monkey developed significant fever by the afternoon of day 2 (rectal temperature exceeded 104°F). However, by day 6 the temperature had fallen to 97°F . During clinical illness the monkey became lethargic and anorectic and developed a prominent rash on the scrotum, ears, and nose.

The IIF technique was successfully utilized for the detection of *R. rickettsii* using heterologous antibody coupled with FITC. The observed fluorescence was specific for the presence of rickettsiae on the basis of control tissues from normal, uninfected monkeys. The presence of rickettsiae in sections which clearly demonstrated fluorescence by microscopic examination was confirmed by titration of tissues in cell culture. In this study those sections which showed the most conclusive evidence of *R. rickettsii* included: skin at the inoculation site, skeletal muscle of the thigh, skin of the scrotum, testicles, anterior nares, heart, kidney, liver, brain, spleen, pancreas, and larynx. Control sections from a normal monkey were negative. Shown in Fig. 1 are photomicrographs of rickettsiae. Figure 1A demonstrates, for comparative purposes, the size and morphology of known organisms stained with acridine orange (in this technique, the larger bacilli are standardized *Shigella dysenteriae*). An example of *R. rickettsii* propagated in WI-38 cells and examined by IIF is shown in Fig. 1B, again for comparative purposes. Figure 1C is a section clearly demonstrating vasculitis of a venule in skeletal muscle. There are neutrophils and macrophages within the wall and adventitia and thrombus within the lumen (arrow). A normal venule is seen at the left. Photomicrographs shown in Fig. 1D through 1H demonstrate the presence of *R. rickettsii* in tissues from the infected monkey when examined by IIF. Figure 1D shows a section of skeletal muscle from the thigh area (opposite that inoculated). The rickettsiae in this section are seen in what is most probably a capillary. The organisms found in the kidney sections (1E) appear to be located in the interstitia or in tubules, whereas rickettsiae found in sections of the testicle (1F) and anterior nares (1G) are located in the endothelium. One section taken from the heart of

FIG. 1. (A) Acridine orange stained *R. rickettsii* (smaller, coccobacillary forms) and *S. dysenteriae* (larger, bacillary forms) $\times 945$. (B) IIF method used to demonstrate *R. rickettsii* in WI-38 cells $\times 600$. (C) Hematoxylin and eosin stained section of skeletal muscle $\times 400$. (D) IIF method used to demonstrate *R. rickettsii* in skeletal muscle $\times 400$; (E) in kidney $\times 400$; (F) in testicle $\times 600$; (G) in anterior nares $\times 400$; (H) in heart $\times 400$.

the infected animal (1H) shows rickettsiae within a monocyte in a vessel.

High intensity nonspecific staining precluded absolute determination of the presence or absence of *R. rickettsii* in the bladder, sternum, regional and mesenteric lymph nodes, adrenal and salivary glands. Similar sections from the uninfected control monkey also stained nonspecifically. Attempts to block nonrickettsial fluorescence by counterstaining were marginally successful; however, this was too variable to be considered conclusive. Rickettsiae or rickettsial antigens were not detected by IIF in sections of the lung, stomach, small intestine, or auricular cartilage of the ear.

Results of rickettsial titrations of the various tissues are presented in Table 1. These data show conclusively that rickettsiae were disseminated throughout the animal. Sections which clearly demonstrated rickettsiae by IIF correlated very well with the presence of infectious organisms.

Histological lesions consisted of vasculitis and thrombosis in vessels of nares, larynx, tongue, testicle, epididymis, skeletal muscle, ear, and skin. Other microscopic lesions included adenitis, myocarditis, splenitis, lymphadenitis, interstitial pneumonia, and multifocal hepatic necrosis. Phosphotungstic acid hematoxylin staining confirmed the presence of

thrombi in vessels. In most tissues histological evidence of vasculitis correlated well with presence of rickettsiae by IIF. Although deBrito et al. (6) concluded that the vasculitis of RMSF was due to rickettsial invasion and multiplication in endothelial cells and we also found rickettsiae notably in the endothelial cells of blood vessels in many tissue specimens examined, the blood vessels of this animal had essentially normal morphological features in brain, kidney, and pancreas, even though these tissues contained rickettsiae. Inflammatory and necrotic lesions in other tissues and organs appeared to be referable to rickettsial growth or obstruction of blood vessels.

The distribution of lesions and disseminated rickettsiae implicated the involvement of several organ systems in RMSF. Other investigators have reported that the lung contained numerous organisms after inhalation (22) or inoculation by the intravenous or intraperitoneal routes (J. B. Moe, manuscript in preparation). As the lung is probably the initial capillary bed encountered upon aerosol, intravenous or intraperitoneal inoculation, organisms the size of rickettsiae would be readily entrapped. We are unable to explain the absence of rickettsiae by IIF and plaque assay from tissues in this study of a monkey inoculated by the subcutaneous route, other than by an unfortunate selection of negative specimens for study. Kundin et al. (15) found that mice inoculated subcutaneously with *R. tsutsugamushi* showed widespread distribution or rickettsial antigen. However, neither RMSF in the monkey nor scrub typhus in the mouse present with overwhelming numbers of rickettsiae in tissues. Since the organisms are indeed present in few cells, death is more likely due to cumulative effects of the rickettsiosis.

Other investigators (19) have reported that the fluorescent antibody technique is more sensitive than yolk-sac inoculation for the detection of small quantities of rickettsiae in ticks. We found that while intact rickettsiae could be readily detected by IIF and confirmed by cell culture assay in certain primate tissues, rickettsiae could not be identified with certainty in other tissues using only IIF (notably, node and gland sections) because of nonspecific fluorescence. Whereas there is a considerable body of information concerning the use of IF in studies involving rickettsiae, there is a paucity of micrographs conclusively demonstrating rickettsiae (especially *R. rickettsii*) in tissues.

Although only one monkey was used in this investigation, we feel that we have definitively examined the criteria required for positive iden-

TABLE 1. *Titers of rickettsiae in organs and tissues of a monkey infected with RMSF*

Organ or tissue ^a	PFU/g of tissue
Spleen	10 ⁵
Mesenteric lymph node ...	8.5 × 10 ⁴
Testicle	7.7 × 10 ⁴
Bronchial lymph node	6.2 × 10 ⁴
Kidney	3.8 × 10 ⁴
Liver	2.4 × 10 ⁴
Salivary gland	1.4 × 10 ⁴
Brain	1.1 × 10 ⁴
Adrenal gland	1.0 × 10 ⁴
Heart	3.5 × 10 ³
Regional lymph node	3.2 × 10 ³
Sternum ^b	2.0 × 10 ³
Skeletal muscle	5.5 × 10 ²
Skin at injection site	7.1 × 10 ¹
Bladder	Contaminated
Pancreas	0
Lung	0
Nares ^b	0
Ear ^b	0
Blood	1.4 × 10 ⁴ /ml

^a Specimens were obtained on day 6 postinoculation with 10⁵ PFU of *R. rickettsii*.

^b Tissues difficult to homogenize; therefore, titration may not be accurate.

tification of rickettsiae by IIF in laboratory animals. From the results of this study, it is obvious that the IIF technique is capable of demonstrating intact rickettsiae as well as rickettsial antigens in primate tissues. The IIF is a straightforward procedure and, using this technique, those tissue specimens which elicited clear positive IIF reactions and also showed high numbers of rickettsiae (for example: testicles, heart, kidney, liver, brain, and spleen) would be the material of choice for studies involving IIF screening of tissue samples. The IIF technique is more rapid and specific than conventional cell culture assays and, due to its relative simplicity, this method would be applicable to the examination of multiple specimens or biopsy tissues suspected of containing rickettsiae.

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