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

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Received for publication 23 April 1975

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 prowazeki (12, 14) and Coxiella burnetii (11, 13). Other authors using this technique have detected rickettsiae in the blood of mice infected with R. prowazeki (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 x 10^7 plaque-forming units (PFU)/ml. Plaques were 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 10^6 PFU of rickettsiae per ml. The monkey was monitored 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 (CO2), 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 serial 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 CO2, 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., Uppsala, 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 x g in an L2-65B ultracentrifuge (Beckman Instrument Co., Fullerton, Calif.). After ab-
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. dysenterii* (larger, bacillary forms) ×945. (B) IIF method used to demonstrate *R. rickettsii* in WI-38 cells ×600. (C) Hemotoxylin and eosin stained section of skeletal muscle ×400. (D) IIF method used to demonstrate *R. rickettsii* in skeletal muscle ×400; (E) in kidney ×400; (F) in testicle ×600; (G) in anterior nares ×400; (H) in heart ×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 uninoculated control monkey were 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 adrenitis, myocarditis, splenitis, lymphadenitis, interstitial pneumonia, and multifocal hepatic necrosis. Phosphotungstic acid hematoxylin staining confirmed the presence of rickettsiae 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-

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### Table 1. Titors of rickettsiae in organs and tissues of a monkey infected with RMSF

<table>
<thead>
<tr>
<th>Organ or tissue*</th>
<th>PFU/g of tissue</th>
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<tbody>
<tr>
<td>Spleen</td>
<td>10⁶</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>Testicle</td>
<td>7.7 x 10⁴</td>
</tr>
<tr>
<td>Bronchial lymph node</td>
<td>6.2 x 10⁴</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.8 x 10⁴</td>
</tr>
<tr>
<td>Liver</td>
<td>2.4 x 10⁴</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>1.4 x 10⁴</td>
</tr>
<tr>
<td>Brain</td>
<td>1.1 x 10⁴</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td>Heart</td>
<td>3.5 x 10³</td>
</tr>
<tr>
<td>Regional lymph node</td>
<td>3.2 x 10³</td>
</tr>
<tr>
<td>Sternum*</td>
<td>2.0 x 10³</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>5.5 x 10²</td>
</tr>
<tr>
<td>Skin at injection site</td>
<td>7.1 x 10¹</td>
</tr>
<tr>
<td>Bladder</td>
<td>Contaminated</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
</tr>
<tr>
<td>Nares*</td>
<td>0</td>
</tr>
<tr>
<td>Ear*</td>
<td>0</td>
</tr>
<tr>
<td>Blood</td>
<td>1.4 x 10⁹/ml</td>
</tr>
</tbody>
</table>

* Specimens were obtained on day 6 postinoculation with 10⁴ PFU of *R. rickettsii*.

* 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.

LITERATURE CITED