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The Pathogenesis of the Vascular Lesions in Experimental Rickettsial Disease of the Guinea Pig (Rocky Mountain Spotted Fever Group)

A Light, Immunofluorescent and Electron Microscopic Study

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Summary. Experimental rickettsiosis (Rocky Mountain Spotted fever group) was produced in guinea pigs and the vascular lesions studied by light, electron and immunofluorescent microscopy in an attempt to study the pathological aspects of this infection.

The vascular lesion falls roughly into two forms. In the early stage of the disease the non specific endothelial damage observed is probably due to a toxin activity, since no gamma globulin, complement or fibrinogen was detected in the lesion. At the later stage immunological factors superimpose on the initial action of Rickettsia at the vessel walls, leading to extensive deposits of gamma globulin and complement. The extensive damage in larger vessels is morphologically similar to an immediate-type hypersensitivity arteritis. This is probably due to lysosomal enzymes liberated from attracted neutrophils and the toxic activity of both Rickettsia antibody-complement complexes and the microorganisms themselves. Endothelial damage leads to platelet and extensive fibrin deposits.

Introduction

The fundamental lesion in Rocky Mountain spotted fever is a widespread vasculitis involving capillaries and small arteries (Wolbach, 1919; Manion, 1963; Lillie, 1941; Le Count, 1911). As previously reported (Allen and Spitz, 1945; Brito *et al.*, 1968) the glomerulus, a modified blood vessel, is similarly affected to a varying degree during the disease.

This paper is an attempt to elucidate some of the pathogenic aspects of the vascular lesion in experimental Rocky Mountain spotted fever of the guinea pig by use of light, electron and immunofluorescent microscopy.

Material and Methods

17 male guinea-pigs (200–250 g) were inoculated intraperitoneally with a strain of *Rickettsia rickettsii* isolated from a human case. The guinea pigs developed the disease and became moribund on the 9th–10th day after the inoculation. In order to study the early phase of the disease four of them were killed at the first or second day of fever, which corresponded to the 5th–6th day after the inoculation. The others were killed at the terminal phase of the disease. Three uninfected guinea-pigs served as controls.

The testes were removed and small fragments from the epididimis, tunica albuginea and underlying parenchyma of each were cut into small pieces of 0.2–0.3 mm, fixed for 2–4 hours in 2% glutaraldehyde (pH 7.4), post-fixed in osmium tetroxide, dehydrated in graded con-

centrations of ethanol, and embedded in Araldite. Sectioning was done on an ultramicrotome and medium-thin sections were selected after toluidine blue staining.

Immunofluorescent techniques were carried out on fresh tissue blocks obtained from the remainder of the testes of normal and infected guinea-pigs. These were quickly frozen in a solidified carbon dioxide-acetone bath and stored at −65° C. Four micra thick sections were cut in a cryostat, mounted on microscope slides, dried at room temperature and stored at −20° C for about 4 weeks without any decrease in the antigenic activity. The slides were fixed in cold acetone for 10 minutes before beginning the immunofluorescent procedures.

A standard control serum was collected from a patient with Rickettsiosis. This serum had a titer of 1/64 in complement fixation test and a titer of 1/1600 in seroagglutination-test.

Negative standard sera were obtained from clinically healthy individuals.

Anti-human gamma-globulin, anti-guinea-pig gamma-globulin, anti-guinea-pig C_3 and anti-fibrinogen sera were obtained by immunization of rabbits and chickens. The antisera were labelled as described by Silva *et al.* (1970).

Direct and indirect tests were carried out for the demonstration of gamma globulin, complement, fibrinogen and antigen on tissue sections.

Several controls were used:

Indirect tests—a positive serum was incubated with normal testes sections and a negative serum was incubated with Rickettsia-infected testes sections.

Direct tests—blocking of immunofluorescent staining by:

a) previous treatment of the conjugate with either gamma-globulin, zimosan-treated C_3 or fresh plasma.

b) previous treatment of the testes sections by an unlabelled rabbit-anti-guinea-pig sera before addition of the conjugates.

c) by heterologous conjugates. The sections were treated with rabbit anti-human gamma globulin and chicken anti-rabbit gamma-globulin.

All controls were consistently negative throughout the experiment.

After completing the cryostat sections the remaining testis tissue was fixed in buffered 10% formalin or Gendre's fluid and embedded in paraffin. Sections were stained with hematoxylin-eosin and Machiavello stain for the detection of Rickettsia. On a few sections the elastic stain was also done.

Results

A. Light Microscopy

The capillary injury was noted throughout the interstitium of the testes and in the larger vessels beneath the tunica albuginea and at the interstitium of the epididimis.

The vascular lesion in Rocky Mountain spotted fever is similar in man and the experimental animal and has been previously described in detail by Wolbach, Lillie, Le Count (1919) and by Piza *et al.* (1932). The present findings are essentially in agreement with these authors and need not to be elaborated. It is worth noting that the vascular lesion in the medium and small size arteries was of a necrotizing vasculitis resembling arteritis seen in hypersensitivity processes. The elastic membrane may be destroyed by the necrosis of the vessel wall. Neutrophiles were frequently found overlaying the endothelium and infiltrating the elements of the vessel wall (Fig. 1).

Rickettsia were seen at the late phase of the infection mainly in the muscular cells of the media and in the inflammatory infiltrate of the adventitia.

B. Electron Microscopy

The early capillary lesion was manifested by swollen endothelial cells with few free ribonuclein granules as compared with the controls and presenting

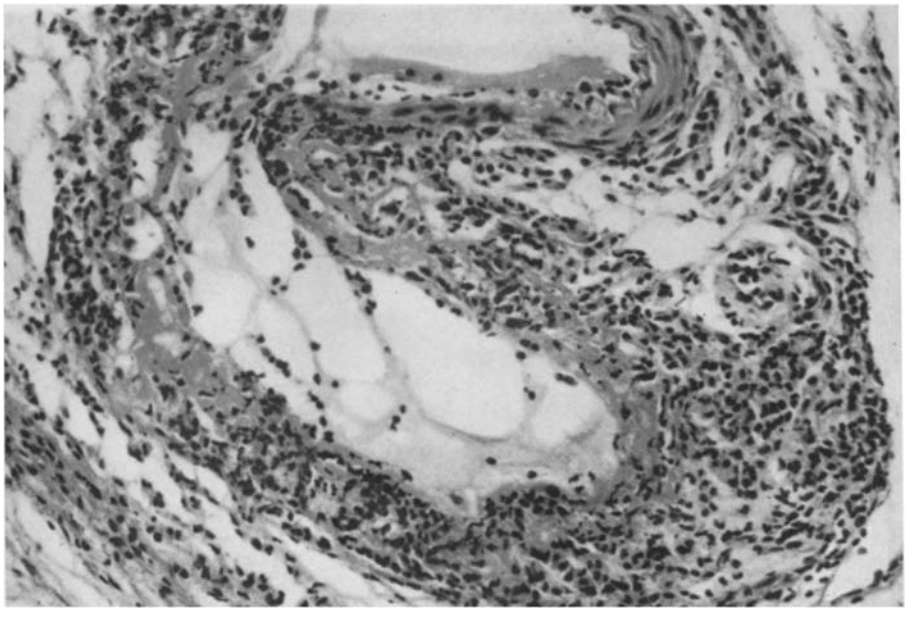

Fig. 1. Acute vasculitis with necrosis of the vessel wall and fibrin deposit. Marked inflammatory infiltrate. Neutrophils are observed among mononuclear cells and in the vessel lumina. HE ×400

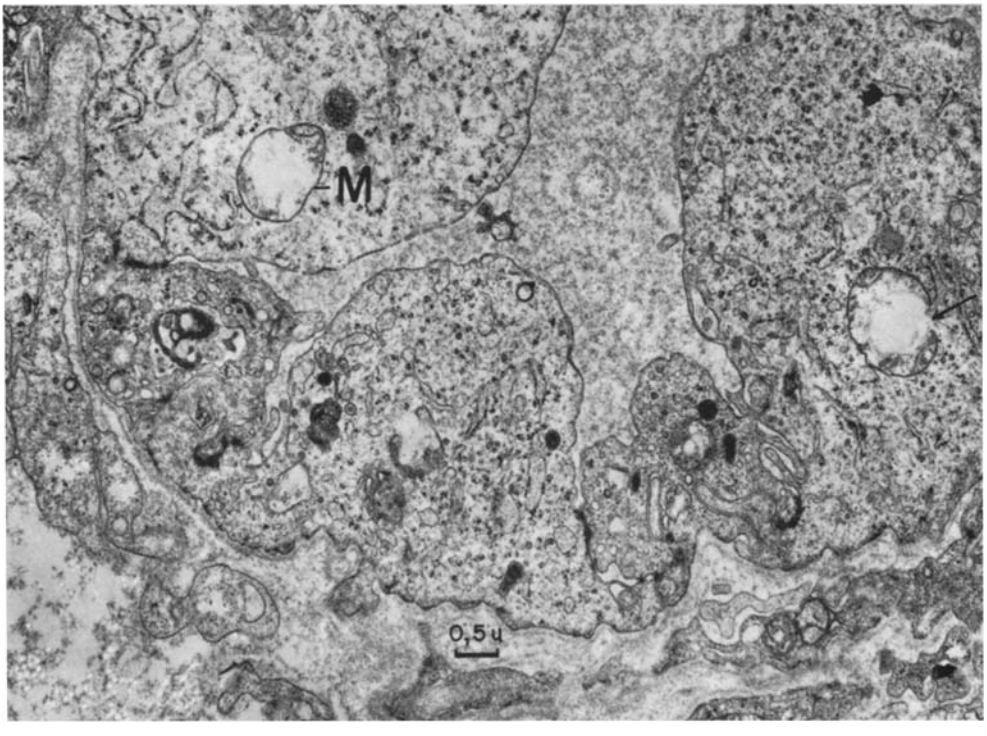

Fig. 2. Swollen endothelial cells with marked mitochondrial damage (*M*). Arrow point to a rupture of the mitochondrial membrane. Pinocytotic vesicles are seen in the cytoplasm nearby the cell surface. Electron dense bodies, some of them curled, are also seen in some of the endothelial cells

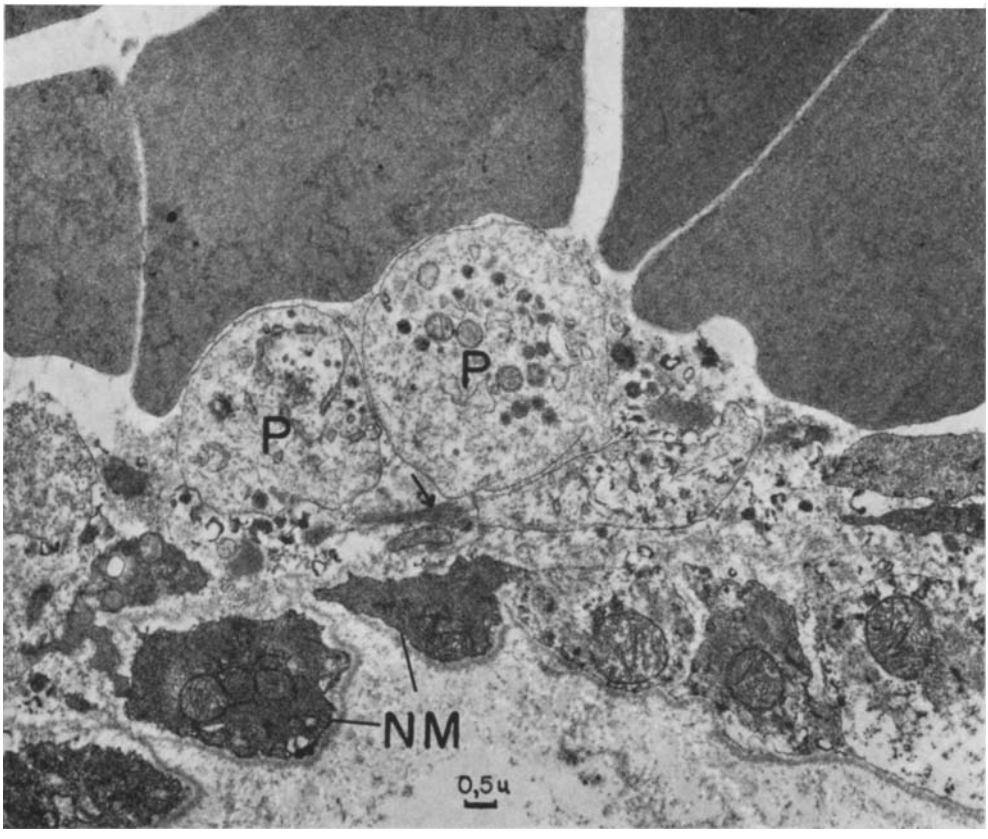

Fig. 3. Endothelial damage with platelet (*P*) deposition. Arrow point to fibrin strands partially obliterating the endothelial gap. *NM* designates necrotic muscle cells of the media

enlarged mitochondria with partial disappearence of cristae and rupture of the limiting membrane. Curled cristae resembling a myelin figure were sometimes formed. Sometimes electron dense elongated rods were observed inside the mitochondrial matrix. Such mitochondrial pathology was also seen in pericytes and in the endothelial and muscle cells of the small arteries and veins.

At a later phase the endothelial cells, besides exhibiting more marked mitochondrial alterations, showed many pinocytotic vesicles both at the luminal and basal membranes, mainly in the former (Fig. 2). In the swollen cytoplasm, with sparse free ribonuclein granules, small vacuoles were visible, particularly near the cytoplasmic limiting membranes. The few endoplasmic reticulum profiles appeared dilated.

The luminal projections of the cytoplasm were short and blunt as compared with the controls, and were made up of swollen cytoplasm. Rarely, short projections through the capillary basal membrane were seen. Both in capillaries and small arteries dense bodies were observed more frequently in the cytoplasm of

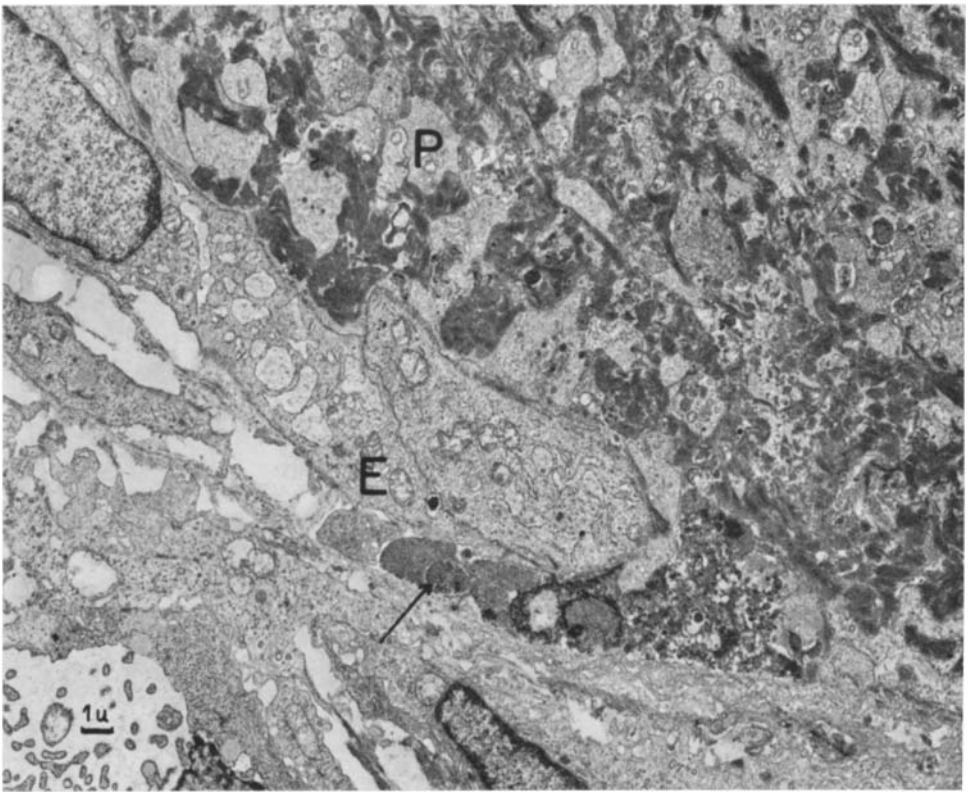

Fig. 4. Thrombotic deposit with platelets (*P*) and fibrin strands obliterating the lumen of a vessel. Endothelial cells (*E*) show mitochondrial damage. A gap of the endothelial lining is obliterated by the thrombotic deposit. Arrow point to a granulated electron dense material seen beneath the endothelial cell and in contact with the gap of the vessel wall

the endothelial cells as compared with the controls (Fig. 2). In the latter such bodies were also more frequently noted in the muscle cells of the media.

Inside the capillary lumen mononuclear cells, neutrophils and clumped red blood cells were seen. Neutrophils frequently exhibited marked regressive changes both in the cytoplasm and nuclei. Occasionally Rickettsia were seen in the cytoplasm of the endothelial cells.

Evidence of a more advanced capillary injury was characterized by occasional gaps between endothelial cells and later by disappearance of these cells. Platelets and fibrin were then observed to accumulate at the place, the former frequently degranulated (Figs. 3 and 4).

A deposit of an electron dense and non fibrillar material was occasionally observed over the endothelial lining in few blood vessels. More rarely it was seen beneath endothelial cells and inside vacuoles in the endothelial cell cytoplasm (Fig. 5). Such endothelial changes were more marked in small arteries and veins where they were followed by necrosis of isolated muscular cells (Fig. 3).

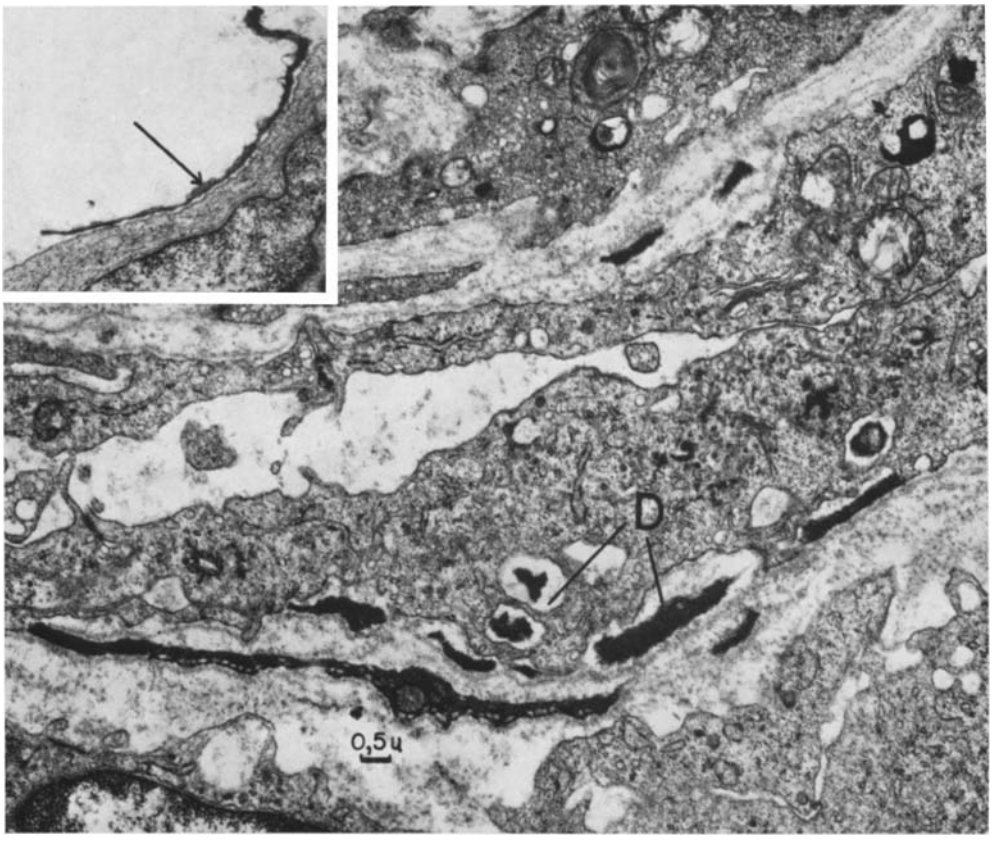

Fig. 5. Swollen endothelial cells with mitochondrial damage. Beneath the endothelial cells and inside cytoplasmatic vacuoles an electron dense material is seen (*D*), interpreted either as degraded fibrin or antigen antibody deposits. Insert show the same material over the endothelial cell surface

Around the injured vessels an inflammatory infiltrate made up of mononuclear cells was frequently seen. These cells sometimes exhibited mitochondrial changes and a dilated endoplasmic reticulum.

C. Immunofluorescent Microscopy

During the late phase of the disease antigens were demonstrated in large amounts as a granulated pattern in the endothelium and media of the larger vessels and capillaries (Fig. 6 A).

The large amount of antigens caused a clumped appearance in the vessel wall while smaller amounts were visible in the adventitia as a delicate granulated pattern.

At the early phase of the disease antigenic material was seldom demonstrated in the endothelium of the small vessels.

Gamma globulin, complement and fibrinogen were present only at the late phase of the disease.

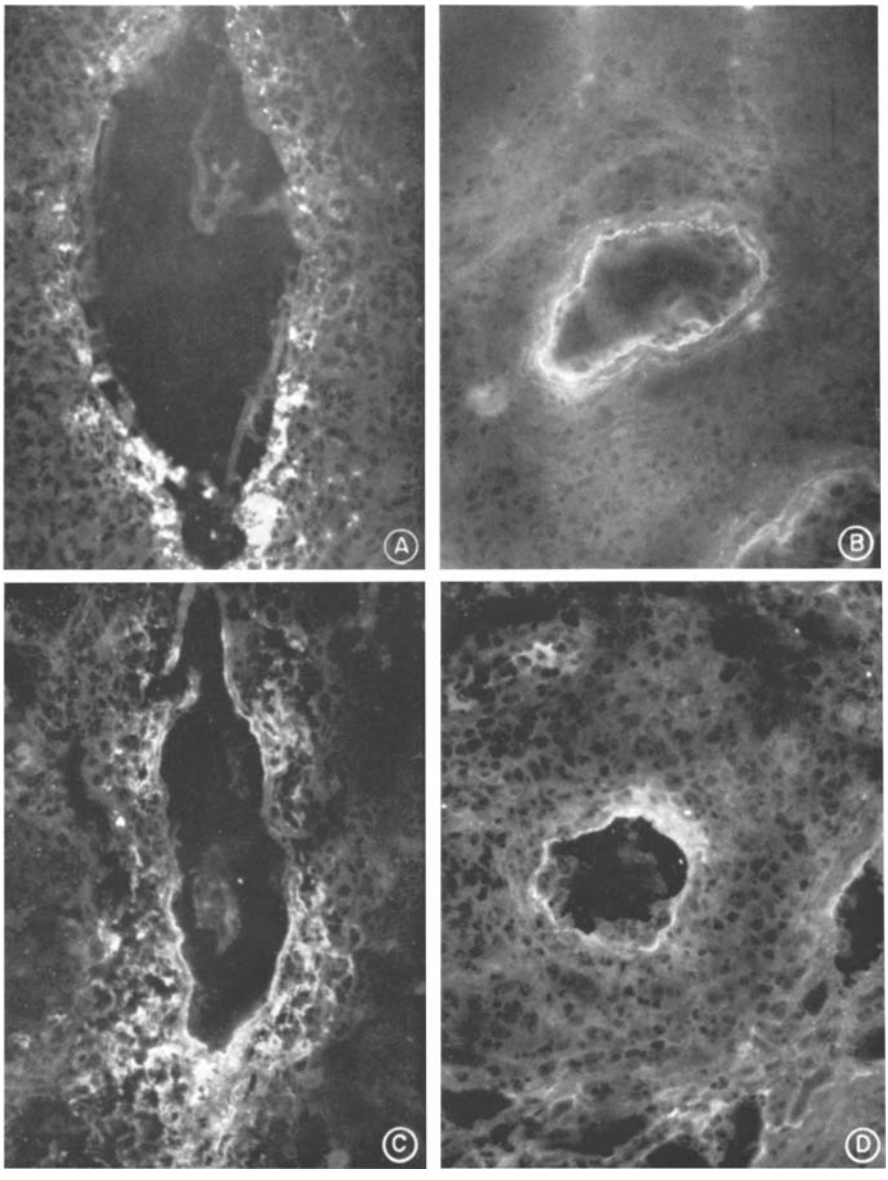

Fig. 6 A—D. Immunofluorescent pattern of the vascular lesion: A Granulated antigen deposits in the vessel wall in the inflammatory infiltrate of the adventitia; B complement deposit in a linear and granular pattern also delimiting the lumen of the vessel; C fibrinogen deposit in the vessel wall spreading throughout the adventitia; D linear deposit of gamma globulin seen delimiting the lumen of the vessel. ×300 (reduced to 5/6)

The gamma globulin deposit was linear in larger vessels and capillaries, occupying the place of the endothelium (Fig. 6 D). In larger vessels where necrosis of the wall occured deposits of gamma globulin were seen in the media. Smaller

amounts of gamma globulin with a granular pattern were seen among the cells of the periperal inflammatory infiltrate.

The pattern of the complement deposits roughly followed that of the gamma globulin. However, in a few vessels it was seen as linear and granular deposits over the endothelium (Fig. 6 B). It was rarely seen at the media and/or adventitia.

Fibrinogen was deposited in a linear fashion over the endothelium and was seen spreading through the adventitia as a reticular network (Fig. 6 C).

Discussion

The widespread vasculitis characteristic of Rocky Mountain spotted fever is due to Rickettsial invasion and multiplication initially in endothelial cells. In larger vessels the invasion progresses to the muscular cells of the media.

The main pathological finding at this stage was a marked mitochondrial injury and evidence of hydropic swelling of the cell. Other evidence of cellular injury was the reduction of ribonuclein granules and dilation of the endoplasmic reticulum.

Antigens represented by Rickettsia and their products were detected in small amounts at this stage of the disease but no gamma globulin, complement or fibrinogen were demonstrated by immunofluorescent techniques. The intensity and diffusibility of the cellular injury together with the small number of Rickettsia present seems to correlate well with the action of a toxin which is presumably produced by Rickettsia (Davis *et al.*, 1967).

At the late stage of the disease the above findings persisted in a more marked fashion. In capillaries and larger vessels an increase of pinocytotic vesicles was seen which sometimes involved all the endothelial cells and, in larger vessels, the muscular cells of the media. The cytoplasm, chiefly at the cell periphery, was crowded with small vesicles, which had become detached from the surface, suggesting a more active uptake of fluid from both surfaces (Bruns, 1968, I and II; Fawcett, 1963). Considering the marked mitochondrial injury present, this phenomenon probably required little energy. Alternatively the energy may have come from anaerobic sources. The fluid transport across the cytoplasm was at least partially impaired because the concentration of the vesicles was higher at the cell periphery. However, in certain instances, capillaries and isolated endothelial cells might show a complete absence of pinocytotic vesicles.

At this phase of the disease, large amounts of antigenic material in a granulated pattern was demonstrated at the walls of the small and large vessels.

Typical arteritis similar to that seen in an immediate type of hypersensitivity reaction appeared together with secondary thrombosis. The "fibrinoid" change of vessel walls was due to endothelial and muscular cell necrosis as well as fibrin deposition. Gamma globulin and complement were also demonstrated by immunofluorescence techniques. In a few instances fine granular and non-fibrilar electron dense materials were present, both above and beneath the endothelial cells. These could be interpreted as either degraded fibrin or antigen-antibody complexes as described in the Arthus phenomenon (Udaka, 1971). In one instance the material was seen inside a cytoplasmic vesicle.

Both gamma globulin and complement were demonstrated in a linear pattern chiefly at the luminal side of smaller vessels. Fibrinogen deposits were more widespread among the elements of the vessel wall and at the periphery, a finding in accordance with plasma leakage through the injured wall.

These findings point to an immunological injury, mediated through antigen-antibody complexes and complement. The local tissue-damaging properties of antigen-antibody complexes prepared "in vitro" have been well established (McCluskey, 1971) and it is probable that they also play a similar role "in vivo". Moreover, the complement system can mediate damage by chemostatic attraction of leukocytes. In the vascular lesion of Rocky Mountain spotted fever neutrophils were easily demonstrated by both light and electron microscopy. Moreover, they appeared frequently with regressive cytoplasmic changes.

The mechanism by which leukocytes produce tissue damage may involve release of enzymes from lysosomes following phagocytosis of immune complexes. Other mechanisms by which they can bring about tissue damage may involve production of lactic acid, which would be expected to enhance the activity of the lysosomal enzymes, and release of a cationic protein or polypeptide which causes increased vascular permeability (McCluskey, 1971).

It is also possible that complement damages tissue in ways unrelated to a chemostatic effect on leukocytes. This might involve cell lysis, opsonization of complexes, or the effects of activated enzymatic components of complement (McCluskey, 1971).

As pointed out by Harrell (1940) and Lee Hand *et al.* (1970) widespread small vessel damage seen in Rocky Mountain spotted fever leads to increase capillary permeability and the resultant fluid loss causes hypovolemia and increased interstitial fluid. Endothelial damage is probably the etiology of the frequent thrombocytopenia and the less common consumptive coagulopathy seen in the disease. Most human fatalities occur during the time of most severe vascular damage.

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