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TIME COURSE OF CELLULAR DISTRIBUTION OF ENDOTOXIN IN LIVER, LUNGS AND KIDNEYS OF RATS

M. A. FREUDENBERG, N. FREUDENBERG AND C. GALANOS

From the Max-Planck-Institut für Immunbiologie Freiburg/Br. and Pathologisches Institut der Albert Ludwigs Universität, Freiburg/Br., Germany

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Summary.—The time course of distribution of 2 endotoxic lipopolysaccharides (LPS), *S. abortus equi* (S form) and *S. minnesota* R 595 (R form, Re), in liver, lungs and kidneys was studied by the immunoperoxidase method in rats.

After its uptake in the liver, both LPS were first detectable in Kupffer cells and granulocytes, the R form also in hepatocytes. A redistribution of the S-form LPS from Kupffer cells to hepatocytes was observed on Day 3 after injection. The detectability of both LPS was lost between Days 5 and 9 after injection.

In the lungs both LPS were detectable later than in the liver. Here the LPS were also found in alveolar and bronchiolar macrophages, which shows that they can also be eliminated through this organ.

The kidneys remained essentially free of LPS, small amounts being detectable here only in the first 24 h.

BACTERIAL ENDOTOXINS (lipopolysaccharides, LPS) injected into experimental animals induce a number of reactions both beneficial and harmful, leading on one hand to increased resistance to infections and to necrosis of tumours, and on the other to fever and the development of lethal shock (Galanos *et al.*, 1977*a*). Although a fair amount of information exists on the fate of endotoxin after its administration in the host, a complete understanding of its uptake and distribution among the various organs, and its possible degradation and elimination from the body is still lacking.

After i.v. administration, endotoxin was found to persist for some time in the blood (Braude, 1964; Chedid *et al.*, 1966). Studies on the distribution of LPS in the blood showed that circulating endotoxin is present predominantly in plasma (Braude *et al.*, 1955; Carey, Braude and Zalesky, 1958; Chedid, Skarnes and Parant, 1963; Mathison and Ulevitch, 1979). It was shown recently that endotoxin binds to plasma high-density lipoprotein and that it circulates in this complex form until it is cleared from the circulation (Ulevitch, Johnston and Weinstein, 1979; Freudenberg *et al.*, 1980). Some authors could detect also endotoxin in blood granulocytes, monocytes and thrombocytes (Fritze and Doering, 1959; Rubenstein, Fine and Coons, 1962; Herring *et al.*, 1963; Brunning, Woolfrey and Schrader, 1964).

It is generally accepted today that the cells of the reticuloendothelial system mainly the Kupffer cells of the liver, are responsible for the clearance of endotoxin from the blood (Braude, 1964; Mathison and Ulevitch, 1979). Recently it was possible to demonstrate that endotoxin uptake may also be effected through hepatocytes (Willerson *et al.*, 1970; Zlydaszyk and Moon, 1976; Ramadori *et al.*, 1980).

Very little information exists about the

Correspondence to: Dr Marina Freudenberg, Max-Planck-Institut für Immunobiologie, 78 Freiburg/Br., Stübeweg 51, Germany.

fate of endotoxin after its uptake by the reticuloendothelial system. Different authors have reported that endotoxin persists in the organism for long periods of time (up to weeks) (Braude, 1964). It has been assumed that during this time the endotoxin is detoxified and degraded, but conclusive evidence for this is still lacking. It is equally unclear by which route endotoxin is eliminated from the body. By immunological methods certain amounts of degraded endotoxin have been shown in the urine of mice (Chedid *et al.*, 1963) and by the use of ^{51}Cr-radiolabelled LPS it has been possible to show that small amounts of radioactivity are present in both urine and stool (Lipinska-Piotrowska, 1977) of rats collected up to 5 days after injection. In mice injected with ^{32}P-LPS, 15·6% of the radioactivity was excreted in the urine as inorganic phosphate during the first 3 days following injection (Howard, Rowley and Wardlaw, 1958). In a recent study in rats using biosynthetically labelled (^{14}C and ^{3}H) LPS, approximately 60% of the injected radioactivity was recovered in the faeces over a period of 7 weeks, while altogether only about 5% was detected in the urine (Kleine, 1980).

In the present study the time course of appearance, distribution and disappearance of endotoxin in the liver, lungs and kidneys of rats was followed by the immunoperoxidase technique. For this purpose an S-form (smooth) and an R-form (rough) LPS were employed.

MATERIALS AND METHODS

Lipopolysaccharides.—*Salmonella abortus equi* S- (smooth) form LPS was isolated from parent bacteria by the phenol–water method (Westphal, Lüderitz and Bister, 1952) and purified by the phenol–chloroform–petroleum ether procedure (Galanos, Lüderitz and Westphal, 1969). *Salmonella minnesota* R595 (Re) LPS was isolated from the bacteria by the phenol–chloroform–petroleum ether procedure. Both LPS were converted to the uniform triethylamine salt form as described earlier (Galanos and Lüderitz, 1975). The mean lethal toxicity of the *S. abortus equi* preparation was 6·4 mg/rat and of the *S. minnesota* R595 preparation 6·6 mg/rat (Freudenberg and Galanos, 1978).

For the passive-haemolysis and Ouchterlony tests, alkali-treated (Neter, 1965) preparations were used as antigens. These included the *S. abortus equi* LPS, *S. minnesota* R595 LPS and additionally the LPS obtained by the phenol–chloroform–petroleum ether method from R (rough) mutants of *S. minnesota* R60 (Ra), R345 (Rb), R5 (Rc) and R3 (Rd). For the measurement of anti-lipid A antibodies, free lipid A (from *S. minnesots* R345) in the triethylamine form was used.

Antibodies.—Antiserum to *S. abortus equi* and *S. minnesota* R595 LPS were prepared by immunizing rabbits s.c. with the corresponding heat-killed bacteria in Freund's incomplete adjuvant as described earlier (Freudenberg *et al.*, 1980). Serum from non-immunized rabbits served as control. The antisera and control serum were absorbed with washed rat-spleen cells and with rat plasma proteins coupled to AH–Sepharose 4B (Pharmacia Fine Chemicals, Sweden) by the method of Avrameas (1969). After this the IgG fractions of the sera were prepared (Harboe and Ingild, 1973). The control IgG was additionally absorbed with the *S. abortus equi* and *S. minnesota* R595 LPS coupled to AH–Sepharose 4B (Nerkar, D., Galanos, C., in preparation) to remove possible antibodies directed against the LPS. All purified fractions were diluted to a protein concentration of 10 mg/ml, with 0·1M NaCl containing sodium azide (15mM) as preservative and stored at 4°.

The antibody preparations were used for the detection of LPS in tissue. The LPS molecule contains in principle a number of antigenic specifities (O-, Ra to Re, lipid A) which may not necessarily be expressed in the intact molecule but might become exposed *in vivo*. It was therefore important to know which specificities were detectable by the above IgG preparations, as this would enable a better interpretation of the results. For this reason, all 3 IgG preparations were tested in the passive haemolysis test (Galanos *et al.*, 1977*b*) and in the Ouchterlony analysis for their anti-LPS activities. The anti-*S. abortus equi* IgG was directed against the O antigen of the LPS and showed no activity against any R specificities or lipid A present in the LPS molecule. The anti-*S. minnesota* R595 IgG recognized only the Re antigen and did not react with the lipid A antigen. The control IgG preparation was free of antibodies directed to any part of the LPS preparation used in the present study.

Peroxidase-conjugated swine anti-rabbit IgG was purchased from DAKO, Denmark, and was absorbed with rat spleen cells and rat plasma proteins before use.

Normal swine serum was absorbed with the *S. abortus equi* and the *S. minnesota* LPS

coupled to AH–Sepharose 4B. It was diluted (1:20) with 0·1M NaCl containing sodium azide (15mM) as preservative and stored at 4°.

Animals.—Female AS2 rats 3 months of age bred under specific-pathogen-free conditions were supplied by the animal stock of the Max Planck Institute für Immunbiologie. They showed no anti-*S. abortus equi* or anti-*S. minnesota* R595 antibodies in their serum as measured by the passive haemolysis test. The LPS (1 mg and 5 mg) was administered to groups of animals i.v. in the lateral tail vein in distilled water (0.5 ml). At different times (30 min, 2, 7, 24, 48 h and 3, 5 and 9 days) after injection groups of 3 animals were perfused under ether anaesthesia through the left heart ventricle with Tutofusin (Pfrimmer and Co., Pharmazeutische Werke, Erlangen GmbH) containing 10,000 u heparin/l followed by 4% buffered formaldehyde. Kidneys, lungs and liver were removed, fixed for 16 h in 4% buffered formaldehyde and embedded in paraffin according to standard methods. From each organ sections 5 μm thick were prepared.

For routine light microscopy, the 5μm organ sections were stained with haematoxylin and eosin. The liver sections were also stained with periodic-acid–Schiff as well as with Best's carmine for glycogen.

Immunoperoxidase staining procedure.—To localize LPS in tissue sections the indirect staining procedure was employed using peroxidase-coupled antibodies (Rose and Bigazzi, 1973).

Briefly: 5μm-thick paraffin sections were dewaxed with xylene and washed successively with 100%, 96% and 70% aqueous ethanol. Endogenous peroxidase present in tissue was inactivated by treating the sections with a fresh solution of 0·5% (v/v) hydrogen peroxide in methanol for 30 min at room temperature and washing with phosphate-buffered saline to remove residual methanol. Each section was treated with 50 μl diluted (1:20) normal swine serum for 30 min to cover possible nonspecific sites. The sections were then treated with 50 μl diluted (1:100) anti-LPS or control IgG for 60 min at room temperature. After washing in phosphate-buffered saline, 50 μl of diluted (1:20) peroxidase-conjugated anti-rabbit IgG was overlayered for 30 min. Excess conjugate was removed by washing with phosphate-buffered saline, and staining for peroxidase activity was carried out with hydrogen peroxide (0·09%) and 3·3% diamonobenzidine (Sigma) in 0·05M Tris buffer, pH 7·6. After peroxidase staining the cell nuclei were counterstained with haematoxylin. The sections were dehydrated and mounted in resin under coverslips.

RESULTS

Liver

Both S- and R-form LPS in the doses administered caused necrosis of liver tissue with haemorrhages. The damage was fully developed after 24–48 h, by which time strong leucocyte infiltration of necrotic areas was observed. With both LPS the degree of injury was higher with 5 mg of the preparations than with 1 mg. Glycogen staining revealed strong depletion of hepatic glycogen 2 h after injection, which lasted up to 48 h after 5 mg and up to 24 h after 1 mg LPS. Thereafter hepatocyte glycogen content increased continuously and on Day 9 exceeded the normal glycogen level of rat liver cells. By this time the liver damage was completely restored.

In animals receiving *S. abortus equi* LPS 2 h after injection, peroxidase-staining activity was present only sporadically, and associated with a few sinusoidal cells, indicating that at this early time the liver was still mainly free of LPS. Seven hours after injection distinct peroxidase staining was observed in the liver, indicating the presence of LPS (Fig. 1a). The LPS was associated primarily with sinusoidal cells, which had very often ingested one or more other cells (granulocytes, mononuclear cells, erythrocytes). In addition, some granulocytes showed deep brown peroxidase staining.

In contrast to sinusoidal cells the parenchyma, apart from a small number of hepatocytes containing a few faint brown granules, was essentially free of LPS. Only in necrotic areas was LPS staining present, in a diffuse pattern. There was no visible difference in the content and distribution of LPS in the periportal area and in the area of the central vein. The above pattern of LPS distribution persisted up to 48 h. On Day 3, however, the sinusoidal cells became less LPS positive and the LPS now appeared also associated with hepatocytes (Fig. 1b). A similar distribution of LPS was also observed on Day 5. On Day 9 after injection only very weak diffuse LPS staining was observed in the liver from animals injected with 5 mg and no staining at all was detected in animals receiving 1 mg

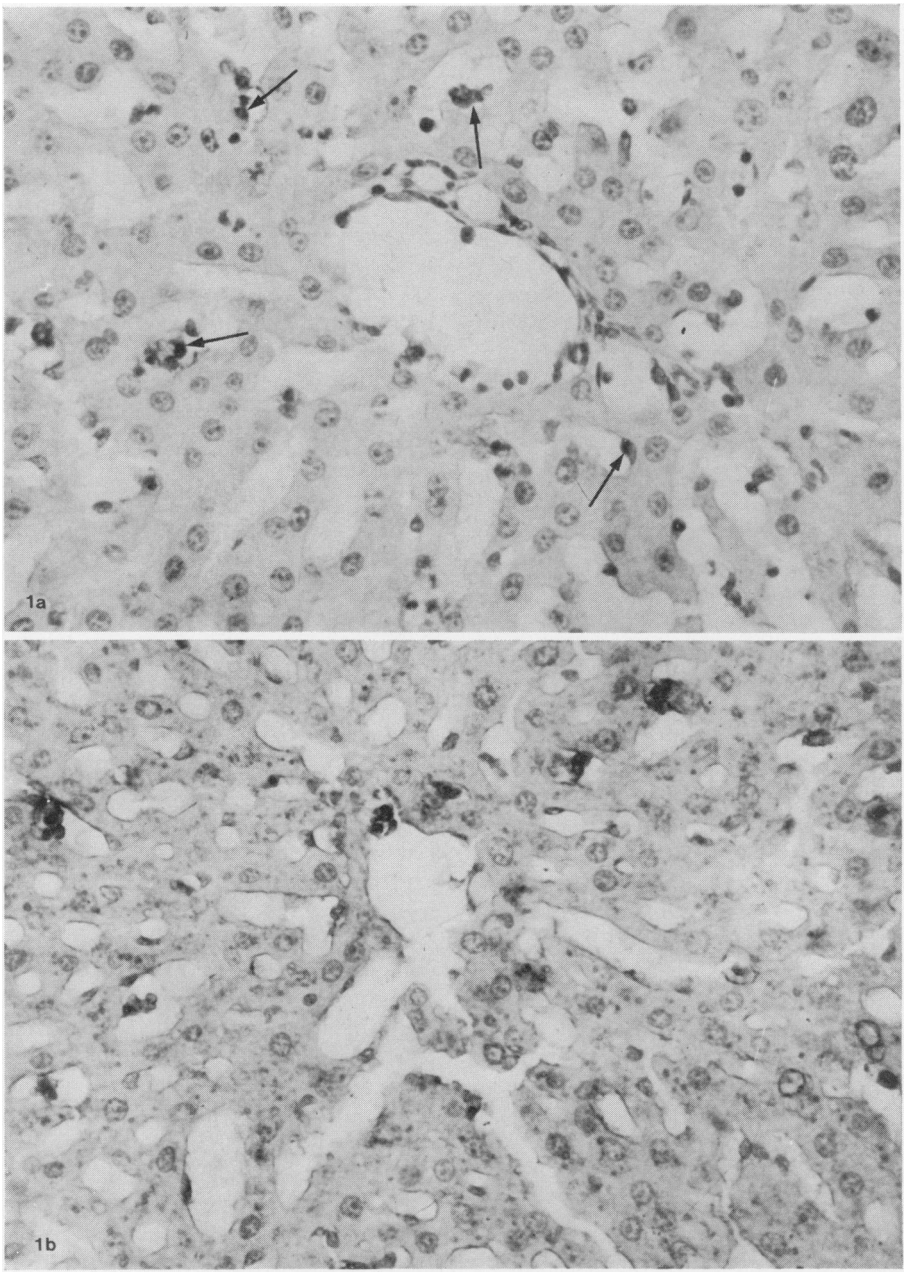

FIG. 1.—Indirect immunperoxidase staining of LPS in rat liver. (a) 7 h after injection of 5 mg *S. abortus equi* LPS showing LPS staining localized predominantly in sinusoidal cells (arrows). (b) 3 days after injection of 5 mg *S. abortus equi* LPS showing LPS staining distributed over the whole liver tissue. 5 μm paraffin sections. Peroxidase-staining procedure was performed as described in "Material and Methods", counter-staining with haematoxylin. × 490.

LPS. At all times investigated, the intensity of peroxidase staining obtained with the 5mg LPS dose was stronger than with the 1mg. Apart from this there was no other obvious difference in the cellular localization between the 2 doses of LPS.

In animals injected with *S. minnesota* R595 LPS, strong diffuse staining activity in the liver was present as early as 30 min after injection. In contrast to the *S. abortus equi* preparation at this early time, LPS was found both in sinusoidal cells and in hepatocytes. A few granulocytes were also LPS positive. The intensity of staining remained unchanged up to 24 h after injection and then decreased gradually with time. Five days after treatment, staining activity in the liver was weak in animals treated with 5 mg and no longer detectable in those treated with 1 mg LPS.

Lungs

Both LPS caused histological changes in the lung which became easily detectable at 24 h after endotoxin administration. Oedema of alveolar walls, perivascular and peribronchial tissue with infiltration of neutrophils and mononuclear cells were found throughout the entire lung. These alterations remained visible during the first 5 days after injection.

With both LPS preparations the lungs were found to be extensively free of endotoxin up to 7 h after injection. In the animals investigated 24 h after treatment with *S. abortus equi* LPS a strong immunoperoxidase reaction was observed in the lung tissue, the activity being stronger with 5 mg than with 1 mg and lasting up to 3 days after injection. LPS was detected in a number of granulocytes, in interstitial cells (probably macrophages), in capillaries of the alveolar walls, mononuclear cells migrating through the walls of pulmonary vessels and in some alveolar and bronchiolar macrophages (Fig. 2a–d). The space under the visceral pleura was also strongly LPS positive. Five days after treatment the peroxidase reaction in the lung was almost negative.

After *S. minnesota* R595 LPS treatment, similar results were obtained; however, the LPS staining in the lungs was much weaker in comparison to that obtained with *S. abortus equi* LPS.

Kidneys

In the kidneys, apart from enlargement of tubular lumina during the first 2 days after injection, no other histological changes were seen to follow treatment with both LPS.

Only low amounts of LPS were found in interstitial capillaries and occasionally in capillaries of renal glomerula during the first 24 h after injection. Beyond this time no LPS was detected in this organ.

DISCUSSION

The time course of distribution of *S. abortus equi* (S-form) and *S. minnesota* R595 (R-form) LPS in liver, lungs and kidneys of rats was determined, by examining organ sections by the indirect immunoperoxidase method. The difficulties always associated with such studies are connected with the purity of the LPS used. Endotoxin preparations isolated by the conventional procedures are always contaminated with other bacterial components, especially proteins and nucleic acids. Since the antisera used in immunohistochemical methods, in addition to antibodies against LPS, always contain antibodies to other bacterial antigens, the identification of the antigens reacting in the tissue becomes difficult. In the present study, to ensure that the immunoperoxidase method was identifying only endotoxin, pure LPS preparations (Galanos *et al.*, 1979) were employed.

The appearance of endotoxin in the liver was paralleled by alterations in liver histology. In agreement with previous studies, early glycogen depletion of hepatocytes and necrosis of liver tissue was observed (Levy, Path and Ruebner, 1967; Levy *et al.*, 1968; Boler and Bibighaus, 1967; Utili, Abernathy and Zimmermann, 1977).

It was found that the *S. abortus equi* LPS

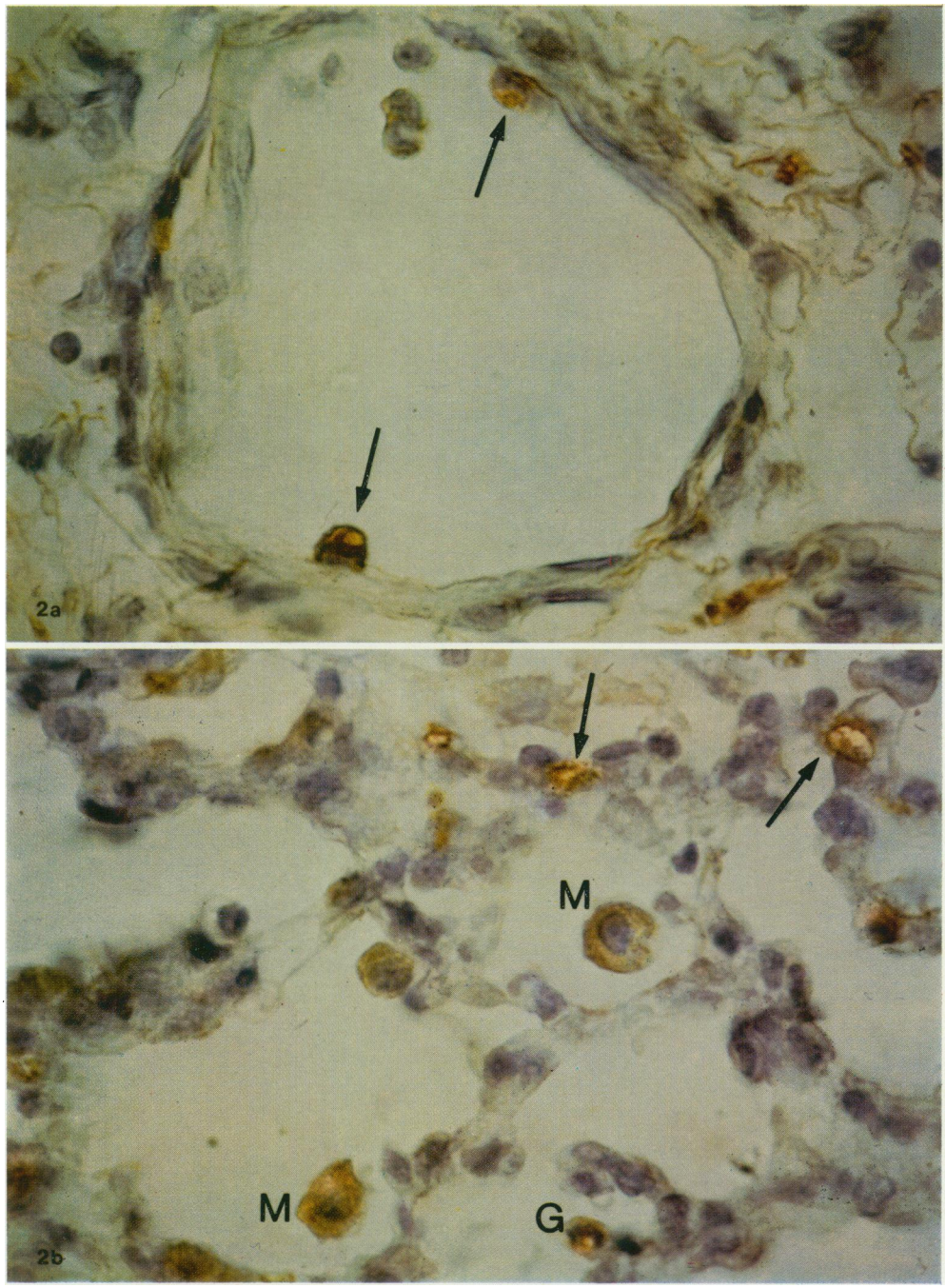

FIG. 2.—Indirect immunoperoxidase staining of *S. abortus equi* LPS (5 mg) in rat lung. (a) LPS$^+$ cells (arrows) adhering to the intima of a small pulmonary vessel 48 h after injection (× 600). (b) LPS staining in alveolar macrophages (M), granulocytes (G) and in the interstitial tissue (arrows) 3 days after 5 mg LPS (× 600). (c) LPS positive cells (arrows) in the interstitial space in the vicinity of a medium-sized pulmonary vessel (× 600). (d) LPS positive macrophages (arrows) attached to the bronchiolar epithelium (× 1000). 5 μm paraffin sections. Peroxidase and haematoxylin staining.

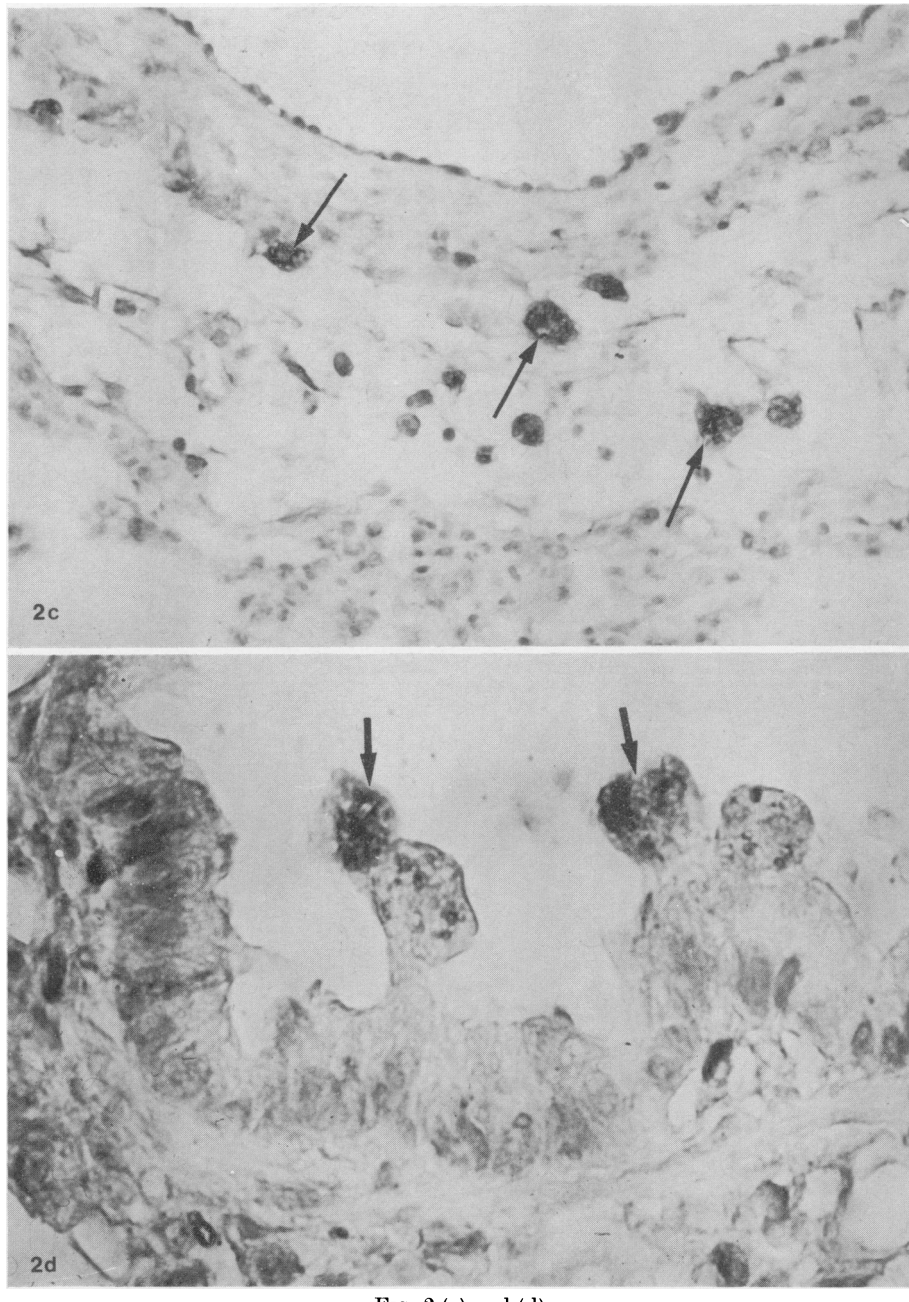

FIG. 2 (c) and (d).

appeared in the liver later than the *S. minnesota* R595. This is in agreement with earlier findings showing that the rate of blood clearance of this S form is slower than that of the R form (Freudenberg *et al.*, 1980). During the first 2 days after injection, the *S. abortus equi* LPS was predominantly detectable in the phagocytic sinusoidal cells, the Kupffer cells. In addition, a number of granulocytes were

LPS positive. The appearance of LPS in Kupffer cells and granulocytes is in accordance with numerous studies in the past which showed that LPS is primarily taken up by phagocytic cells. Kupffer cells were frequently found to contain ingested erythrocytes and, in addition, granulocytes and mononuclear cells that appeared to be LPS positive. Phagocytosis of blood cells has been confirmed in the mean time in a separate study by electron microscopy (Freudenberg, 1980). A similar phenomenon was observed earlier to follow endotoxin injection in dogs—however, only in combination with lead acetate treatment (Hoffmann *et al.*, 1974).

The hepatocytes which remained up to 48 h essentially free of S-form LPS became on Day 3 strongly LPS positive. It is unlikely that this LPS represents material taken up by hepatocytes directly from the blood, since by this time the LPS was largely removed from the circulation (Freudenberg *et al.*, 1980). The appearance of staining activity in hepatocytes was paralleled by reduced activity in Kupffer cells. Therefore we assume that a redistribution of LPS from Kupffer cells to hepatocytes had taken place.

In contrast to the *S. abortus equi* LPS, the *S. minnesota* R595 preparation was found as early as 30 min after injection distributed equally between hepatocytes and Kupffer cells. A similar observation was made very recently in mice (Ramadori *et al.*, 1980). Therefore we assume that this LPS preparation, unlike the *S. abortus equi* preparation, is primarily taken up by hepatocytes as well as by phagocytic cells. Since in the present study only one S-form and one R-form LPS were tested, it cannot be decided whether the above difference in uptake of the 2 LPS is typical for the 2 classes of LPS. It has been reported, however (Mathison and Ulevitch, 1979) that in rabbits injected with ^{125}I-labelled *S. minnesota* R595 LPS hepatocytes, in contrast to phagocytic cells, remained free of endotoxin during the first 3 h after treatment. This discrepancy may be due to differences in the LPS preparations used or to the different animal species employed in the 2 studies.

Five to 9 days after treatment neither LPS could be detected. The antibodies used for the detection of LPS in the present study were specific for the sugar part of the respective LPS. We were therefore able to detect the LPS in the tissue only as long as the sugar part of the molecule remained unaltered. The above loss of LPS staining could be due to the removal of the LPS from the liver or to an alteration in its antigenic specificity, so that it could no longer interact with the antibodies used for its detection. The present data support findings that LPS associates with hepatocytes, suggesting that (at least in rats) the LPS trapped in the liver may enter hepatocytes *via* Kupffer cells or directly from the blood. There is good evidence that the LPS or its altered products leave the hepatocytes *via* the bile duct and enter the gut. It has been reported that radioactivity was found in the gallbladder of rabbits injected with ^{125}I-LPS (Mathison and Ulevitch, 1979). In rats treated with biosynthetically labelled *S. abortus equi* LPS, approximately 60% of injected counts were found in faeces, collected over the 7 weeks after injection (Kleine, 1980).

In contrast to the liver, the lung parenchyma was free of LPS up to 7 h after injection. In the animals investigated at 24 h, the lungs contained a large number of LPS positive cells. The LPS was also present in alveolar-wall capillaries and in the space under the pleura (probably in the lymphatic vessels). As in the liver, the appearance of endotoxin in the lungs was paralleled by pathological changes detectable by light microscopy. It cannot be determined from the present study whether the uptake of internalized LPS took place in the lung or whether the LPS positive cells originated from the liver or other organs of the reticuloendothelial system already carrying LPS. Strong evidence that macrophages that had phagocytized Thorotrast passed from the liver to the lung were found has been

reported earlier (Easton, 1952). It is further evident that LPS positive cells can be excreted in the lung, and this is supported by the finding of a number of LPS positive alveolar and bronchiolar macrophages.

In the kidneys only insignificant amounts of LPS were detectable and this only during the first 24 h after injection. Thereafter the kidneys were free of LPS staining activity. It is therefore unlikely that larger amounts of antigenically active LPS are excreted through this organ. In the same strain as that used in the present study, it was shown recently that less than 5% of ^{14}C counts were found in the 7-week urine of rats injected with biosynthetically labelled *S. abortus equi* LPS (Kleine, 1980).

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