Virchows Arch [Pathol Anat] (1984) 404:197–211

Virchows Archiv A
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Identification of endotoxin-positive cells in the rat lung during shock

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Summary. Following an intravenous administration into rats of a shock-inducing dose of endotoxin (2 mg) the lipopolysaccharide (LPS) was demonstrated immunohistochemically (light and electron microscopy) and determined quantitatively (radio-labelled LPS) in the lung tissue and in isolated alveolar macrophages. At different times after LPS injection morphological investigations of the pulmonary tissue and alveolar macrophages were carried out.

One hour after endotoxin treatment 3% of the alveolar macrophages were already LPS-positive. The maximum extent of the immunoperoxidase reaction for endotoxin (100% cells involved) was observed on day 3, the vast majority (98%) of the alveolar macrophages being LPS-positive still on day 14. 0.9% of the injected radio-labelled LPS preparation was found to be associated with lung tissue on day 3. By this time 0.173 μg LPS/10^6 alveolar macrophages was detected. During the time of ultrastructural investigation endotoxin appeared in the lung only within cells. By their high capacity for storing endotoxin and their numerical superiority the mononuclear phagocytes are the leading LPS-positive cells in the lung, although granulocytes, endothelial cells, and alveolar epithelial cells were sometimes also involved.

The accumulation of a high percentage of activated macrophages in the lung seen in the late stage of shock could represent at least one of the main factors leading to damage of pulmonary tissue. The correlation between appearance of LPS-positive macrophages and histological signs of lung tissue injury in the present investigation is striking.

Key words: Endotoxin shock – Lung – Endotoxin-positive cells

It was recently shown in rats that, following injection of endotoxin (lipopolysaccharide = LPS) in a toxic dose, histological changes such as oedema of

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the alveolar wall and perivascular and peribronchiolar tissue with infiltration of neutrophils and mononuclear cells occur in the lung (Freudenberg et al. 1982). These morphological changes are parallelled by the appearance of endotoxin in the lung.

In the present communication the endotoxin-positive cells in the lung are identified by the immunoperoxidase method and investigated by light and electron microscopy in order to obtain more information on the mechanism underlying the development of the shock lung syndrome.

Material and methods

Lipopolysaccharide. Salmonella abortus equi S (smooth) form LPS was isolated from parent bacteria by the phenol-water method (Westphal et al. 1952) and purified by the chloroform-petroleum-ether procedure (Galanos et al. 1969). The mean lethal toxicity of the Salmonella abortus equi preparation used has previously been estimated to be 6.6 mg per rat (Freudenberg and Galanos 1978).

Antibodies. Antiserum to S. abortus equi was prepared by immunizing rabbits subcutaneously with the corresponding heat-killed bacteria in Freund's incomplete adjuvant (Freudenberg et al. 1980). Serum from non-immunized rabbits served as control. The antiserum and control serum were absorbed with washed rat-spleen cells and with rat plasma proteins coupled to AH-Sepharose 4 B (Pharmacia Fine Chemicals, Sweden) by the method of Avrameas (1969).

The IgG fractions of both antiserum and control serum were prepared as previously described (Freudenberg et al. 1982). For light microscopy the fractions were diluted to a protein concentration of 10 mg/ml with 0.1 M NaCl containing 15 mM sodium azide as preservative and stored at 4° C.

Fab'fragments coupled with peroxidase were prepared, using part of the anti-LPS IgG obtained above. The IgG was subjected to pepsin digestion and subsequent Sephadex G 100 filtration (Spielberg 1979) in order to obtain F $(ab')_2$ fragments. These were passed through an AH-Sephadex column coupled with S. abortus equi LPS as antigen (Nerkar and Galanos in preparation). LPS-non-specific F $(ab')_2$ fragments did not bind to the column and were washed off. They were collected and served for control experiments. The specific anti-LPS F $(ab')_2$ was dissociated from the antigen at low pH (0.1 M glycin-HCl buffer, pH 2.8). Both F $(ab')_2$ preparations were reduced to Fab' with dithiothreitol (Spielberg 1979). The Fab' fragments were coupled with horseradish peroxidase by the method of Nakane (1979). The Fab'-peroxidase conjugate was diluted to a protein concentration of 1 mg/ml with 0.1 M NaCl containing 1% BSA and 15 mM sodium azide, divided into small portions and stored at −20° C until required.

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

Normal swine serum was absorbed with the S. abortus equi LPS coupled to AH-Sepharose 4 B. It was diluted (1:20) with 0.1 M NaCl containing sodium azide (15 mM) as preservative and stored at 4° C.

Animals. AS_2 rats of both sexes, 3–5 months of age and bred under specific pathogen-free conditions, were supplied from the animal stock of the Max-Planck-Institut für Immunbiologie. They showed no anti-S. abortus equi antibodies in their serum as measured by the passive haemolysis test. The LPS (2 mg) was administered to the animals intravenously in the lateral tail vein in distilled water (0.5 ml).

Light and electron microscopic immunohistochemistry. The perfusion of the animals was performed under ether anaesthesia through the left ventricle with Tutofusin® (Pfrimmer and Co., Pharmazeutische Werke, Erlangen GmbH, FRG) containing 10.000 u heparin/l followed by the appropriate fixative (4% buffered formaldehyde for light microscopy or a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.3 for electron microscopy).

For light microscopic immunohistochemical investigation the animals were perfused at 7 h, 2, 3, 5 days, 2 and 4 weeks after the endotoxin administration. The lungs were removed and postfixed for 16 h in 4% buffered formaldehyde and embedded in paraffin in the standard way. From each lung 5 µm sections were taken. For routine light microscopy the sections were stained with haematoxylin and eosin. For light microscopic localization of LPS the indirect immunoperoxidase staining procedure was employed following a recently described method (Freudenberg et al. 1982).

For ultrastructural immunohistochemical investigation lung specimens were obtained 18 h and 3 days after endotoxin administration, cut to the appropriate size and immersed in the combined fixative for 8 h at 4° C. After fixation the tissue was washed for 4 h in 0.1 M cacodylate buffer at pH 7.3 with 7% saccharose, left overnight in 0.1 M cacodylate buffer with 15% saccharose, and finally put into 0.1 M cacodylate buffer with 25% saccharose and 10% glycerol for 2 h at room temperature. Fifteen µm cryostat sections were then cut and mounted on glass slides. Endogenous peroxidase present in the tissue was inactivated by treating the sections with a fresh solution of 2.5% hydrogen peroxide in ethanol for 45 min at room temperature and washing with cacodylate buffer to remove residual ethanol. The direct immunoperoxidase technique was employed to localize the endotoxin. Each section was treated with 50 µl dissolved 0.1% glycin for 15 min and then washed with cacodylate buffer. The following steps were then carried out: Treatment of the sections with 1% bovine serum albumin (BSA) for 2 h to cover possible non-specific sites, alternate sections being covered with 50 µl diluted (1:10) anti-LPS Fab′-fragments peroxidase-conjugated, or with control Fab′-fragments, for 12 h at room temperature. After washing in cacodylate buffer the tissue was postfixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 for 30 min at 4° C. The staining for peroxidase activity was carried out with 0.09% H_2O_2 and 3.3′ diaminobenzidine in 0.05 M Tris buffer at pH 7.6. The specimens were then washed, postfixed with osmium tetroxide (2%, 30 min at room temperature), dehydrated in graded alcohols and embedded in epon. For this purpose epon-filled gelatinol capsules were put on the slides open end downwards to cover the sections. After polymerization of the epon the capsules with the included sections were broken off the slides.

Ultrathin sections were cut on a Reichert ultramicrotome. All micrographs were taken with a Zeiß-EM-9-A operating at 60 kV and at primary magnifications from 1,700 to 1,900.

Broncho-alveolar lavage (Guzman et al. in preparation). The broncho-alveolar lavage was performed under Inaktin®-anaesthesia at 1, 2.5 and 12 h, 3, 6, 7, 8 and 14 days and 4 weeks after LPS injection, one rat being used in each case.

Briefly: Insertion of a Abbocath-T® tube under sterile conditions into the trachea through a horizontal incision was followed by repeated injection/withdrawal of sterile NaCl solution (a total of 60 ml being injected and a maximum of 40 ml withdrawn). Isolation of alveolar macrophages was performed by 10 min centrifugation (1,000 r/min) – incubation of 500,000 cells for 1 h in ISCOV's medium with 10% fetal calf serum added, using a culture chamber attached to a glass slide (Lab-Tek®). The cells were cultured in 5% CO_2 at 37° C and washed with isotonic NaCl solution. They were then dried in air and underwent 5 min fixation with formaldehyde vapor, washing with distilled water and an indirect immunoperoxidase technique to localize endotoxin according to the method of Freudenberg et al. (1982).

In each immunohistochemical specimen 200 cells were counted in 10 different areas of the slide. For each observation the following features of immunostaining for LPS detection were described: negative, weakly positive, moderately positive, and heavily positive. In this way the percentages of LPS-positive cells and of the three intensities of endotoxin-positive macrophages were obtained.

Quantitative determination of the amount of endotoxin in lungs and in alveolar macrophages using radio-labelled LPS. Biosynthetically ^{14}C labelled LPS was isolated from S. abortus equi grown on N-acetyl-D (1-^{14}C) glucosamine (Amersham Buchler, Braunschweig, FRG) by the method described above for unlabelled LPS. The final preparation contained 1.5×10^6 ^{14}C-cpm/mg. ^{14}C measurement was carried out following oxidation of the samples by combustion in a Tri-Carb sample oxidizer (Packard Instrument Company Inc. Downers Grove, Ill, 60515) and measured in a Tri-Carb Liquid Scintillation Spectrometer (Packard) as described previously (Kleine, B., Freudenberg, M.A., and Galanos, C., submitted for publication).

The experiments with the radiolabelled LPS preparation were carried out on lung tissue and cultured alveolar macrophages on the 3rd day following endotoxin administration.

Results

Observations on pulmonary tissue

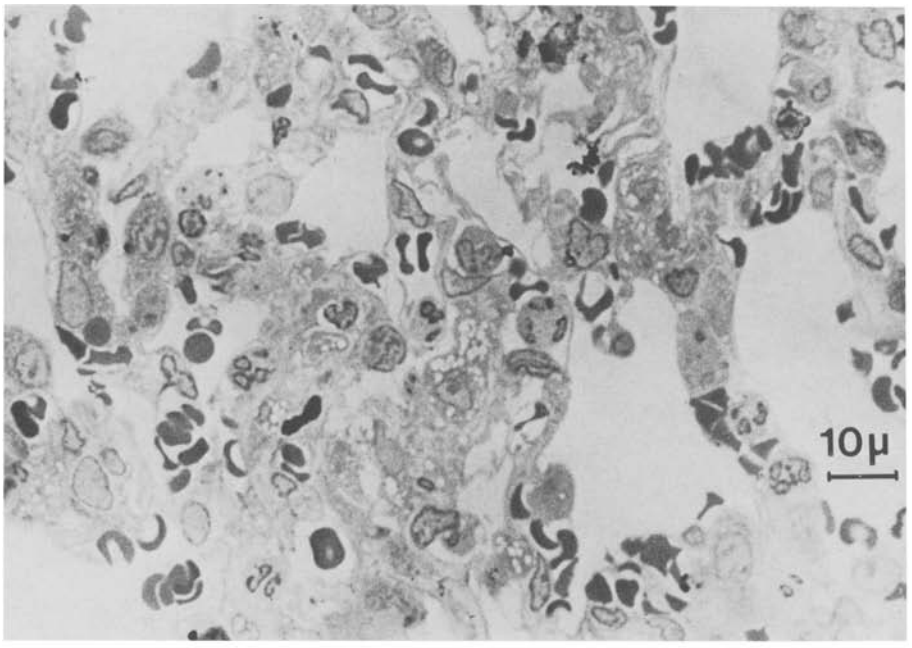
Groups of rats received 2 mg of Salmonella abortus equi LPS intravenously, a dose which causes an approximately 10% death rate between day 2 and 5 after the endotoxin injection. All treated animals developed acute signs of toxicity such as rough fur, immobility, haemorrhage into the mucous membranes of eyes and nose, and disinclination to eat and drink.

At different times after injection lung tissue was examined for histological alterations and for the presence and distribution of LPS. Histological changes caused by the endotoxin treatment became detectable from the 7th hour onwards. Moderate oedema and infiltration of the pulmonary interstitial tissue by mononuclear cells and granulocytes were observed. At this early stage of shock LPS was detected in some of the interstitial cell infiltrations. When the condition of shock is fully developed (from 12 h until 3 days after endotoxin administration) both a marked cellular infiltration of the alveolar tissue (Fig. 1) and a striking interstitial oedema of the perivascular and peribronchiolar spaces (Fig. 2) occur. At this time intense immunoperoxidase staining can be seen in the alveoli, the walls of the pulmonary vessels and bronchioles as well as in the peribronchiolar and perivascular interstitium. Under the light microscope the LPS-positive cells in the above tissues could be identified as mononuclear cells, granulocytes and alveolar macrophages. An extraordinarily strong immunoperoxidase reaction was detectable in the tissue near the visceral pleura (Fig. 3).

In rats injected intravenously with radiolabelled ^{14}C-LPS, 0.9% of the injected preparation (average of 3 animals) was found to be associated with lung tissue on day 3. This value corresponds to 18 μg of original LPS injected.

The ultrastructural immunohistochemical investigation provided closer insight into the localization of endotoxin in the lung during the period between 18 h and 3 days after the administration of LPS. During the time of observation endotoxin appeared in the lung only within cells. The cells showing a positive endotoxin reaction were mostly mononuclear phagocytes (macrophages), although granulocytes, endothelial cells and alveolar epithelial cells were also sometimes involved.

Mononuclear phagocytes carrying LPS were demonstrable in the terminal blood stream, the interstitium and the inside of the alveoli. The reaction products ware held in phagocytic vacuoles of various sizes which are seen as moderate or coarsely granular electron dense structures embedded in an electron lucid matrix (compare Fig. 5). Depending on their localization in the lung, the LPS-positive mononuclear phagocytes show considerable morphological variation. In the axial stream of wider blood vessels one sees round or oval cells with a diameter up to 15 μm (Fig. 4). The remaining



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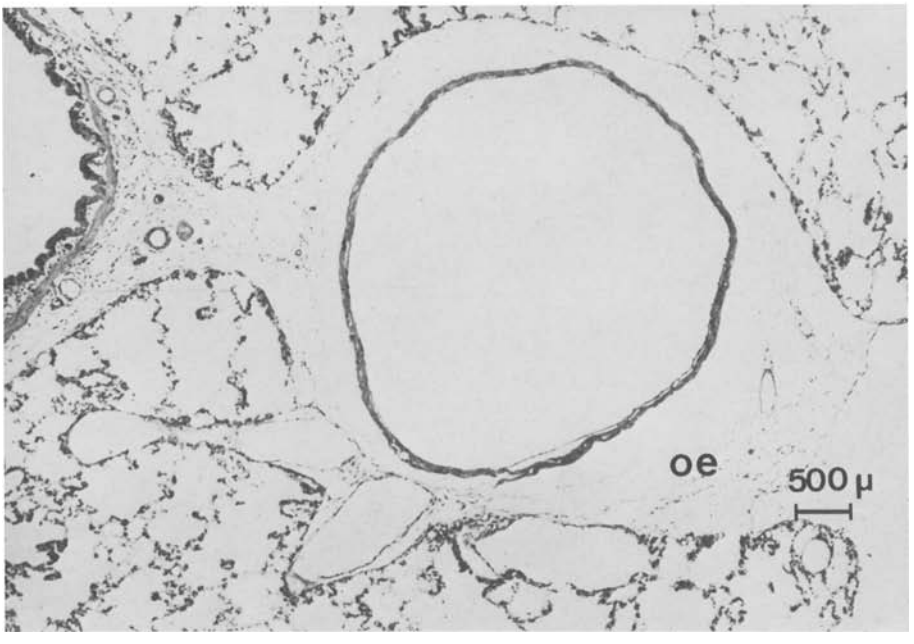

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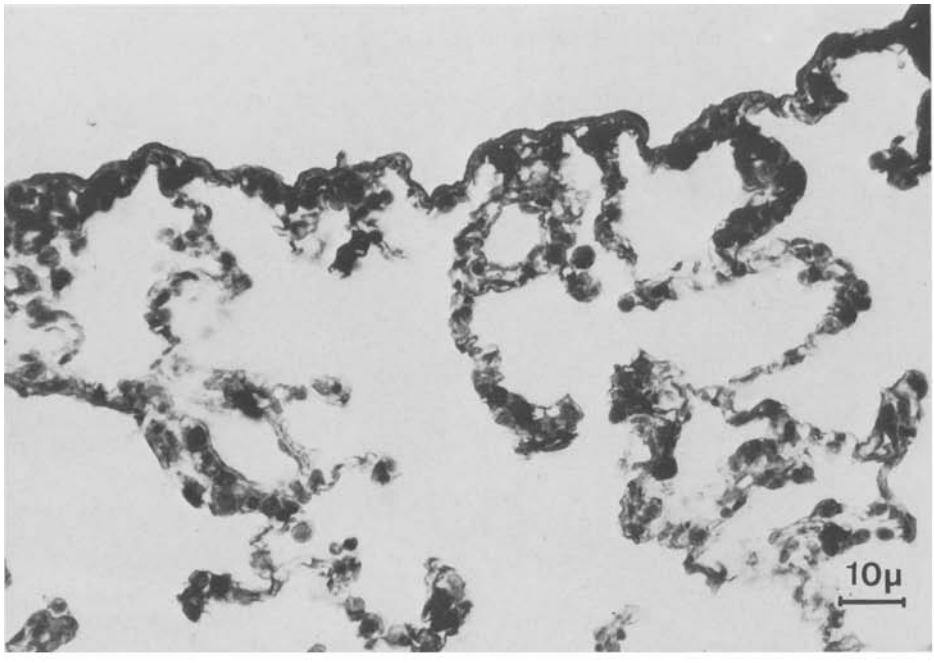
Fig. 1. Marked mononuclear cell infiltration and oedema of the alveolar tissue in the rat lung, 3 days after endotoxin injection. Semithin section. Toluidin blue staining

Fig. 2. Pronounced interstitial oedema (*oe*) of the perivascular and peribronchiolar tissue of the rat lung, 3 days following endotoxin injection. H & E staining



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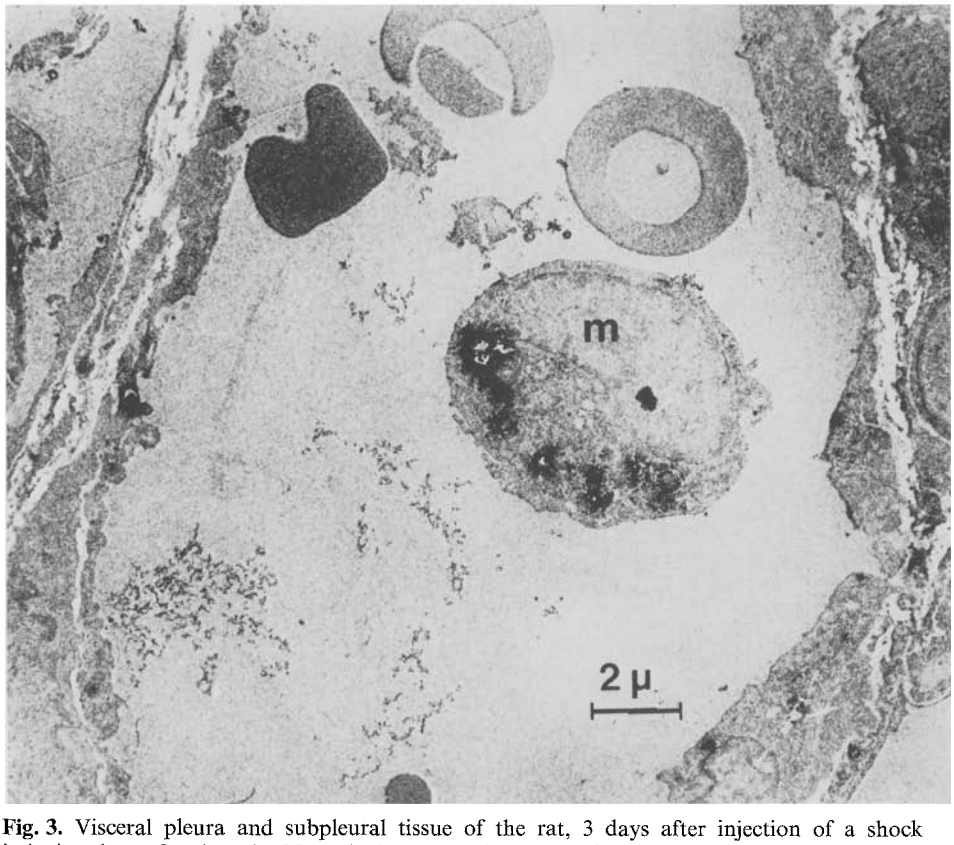

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Fig. 3. Visceral pleura and subpleural tissue of the rat, 3 days after injection of a shock inducing dose of endotoxin. Note the intense LPS storage of the tissue (dense black staining). Indirect immunoperoxidase method

Fig. 4. Endotoxin-carrying (black reaction product) mononuclear cell (*m*) in a small pulmonary vessel of the rat, 3 days following LPS administration. Direct immunoperoxidase reaction

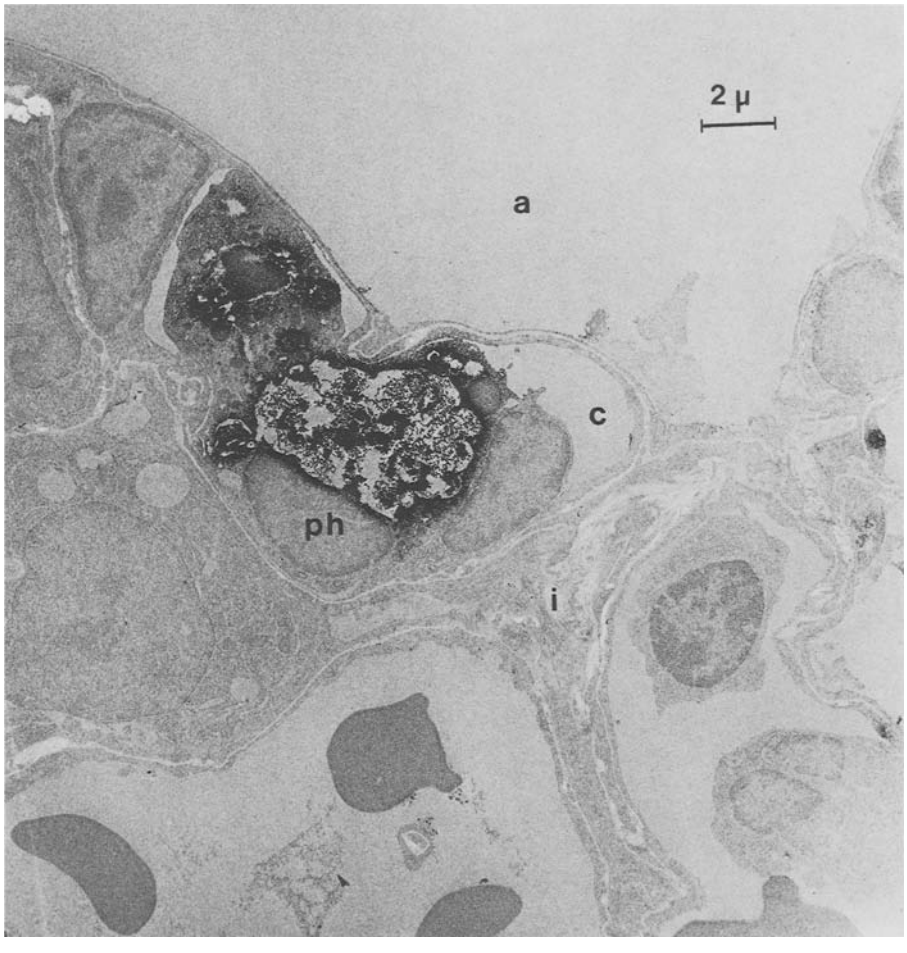


Fig. 5. EM immunohistochemical demonstration of endotoxin (granular black reaction product) in a heterophagocytic vacuole of a phagocyte (*ph*) in an alveolar capillary, 3 days after LPS injection. *c*, capillary; *a*, alveolus; *i*, alveolar interstitium. Direct immunoperoxidase method

LPS-bearing macrophages are significantly longer (up to 30 µm), show a cytoplasm rich in organelles as well as many cytoplasmic projections, and in the alveolar capillaries may often appear as polygonal cells. In the case of phagocytes with particularly large endotoxin containing phagosomes the greater part of the cytoplasm is sometimes squashed into a narrow crescent by the LPS (Fig. 5). Many endotoxin containing phagocytes can appear in all possible positions between the blood vessel and the respiratory space, thus showing their ability to migrate by ameboid movement (Fig. 6). Occasionally macrophages can be seen containing tissue detritus as well as endotoxin in their phagosomes (Fig. 7).

The demonstration of specific reaction products in the cytoplasm of cells lining vessels and alveoli excited our special interest (Fig. 6 and 8). On occasion LPS-positive regions of the cytoplasm of the lining cells of

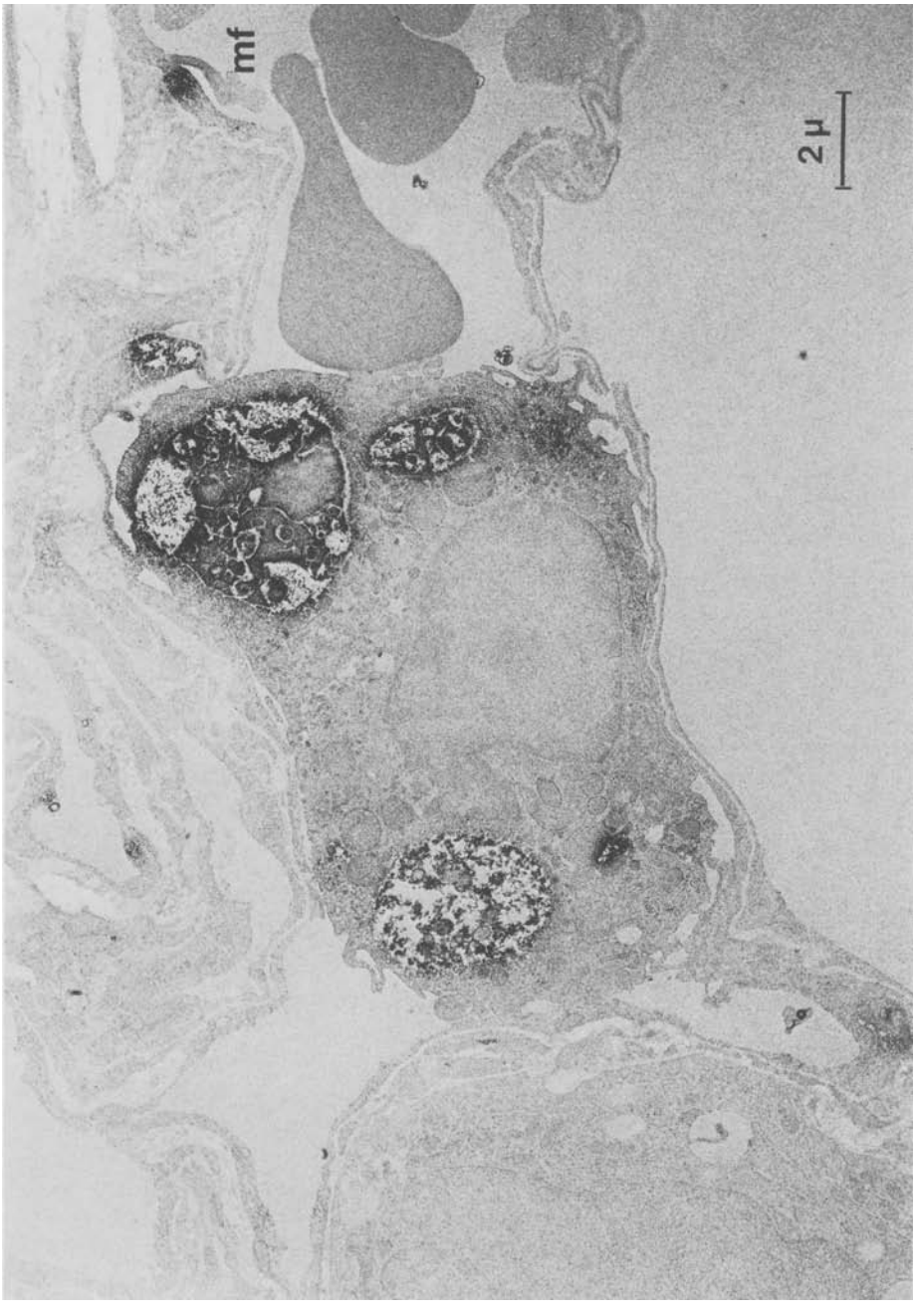


Fig. 6. EM immunohistochemical demonstration of endotoxin (granular black reaction product) in the lung of a rat, 3 days following LPS administration. *mf*, marginal fold in the region of an endothelial cell junction. Direct immunoperoxidase method

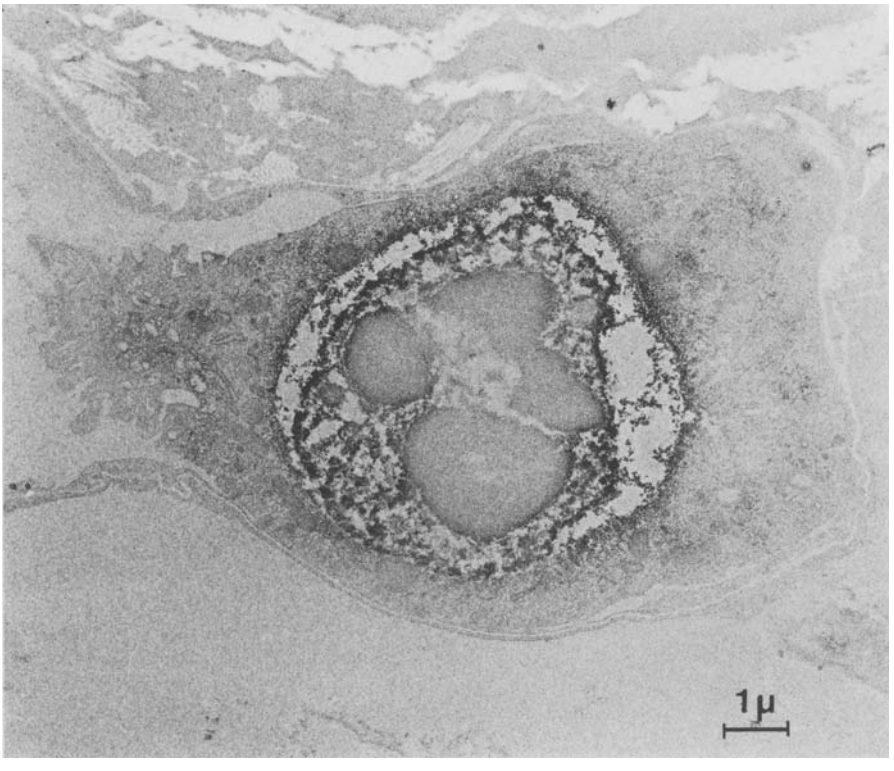


Fig. 7. Activated macrophage in the rat lung containing polymorphous debris (possibly cell remnants) as well as endotoxin (granular black reaction product) in its phagosome. 3rd day after LPS injection. Direct immunoperoxidase method

capillaries and alveoli can be seen without any clear limiting membrane, sometimes localized in swollen regions of a narrow strip of cytoplasm (Fig. 8), sometimes near to an easily recognizable cell boundary (Fig. 6). On the other occasion the reaction products can also be recognized in the perinuclear cytosomes of endothelial-like intimal cells and alveolar epithelial cells, mostly of type I (Fig. 8).

The lungs of animals which had recovered from endotoxin shock were investigated 14 days and 4 weeks after LPS injection. On the 14th day the cellular content of the peribronchiolar and perivascular pulmonary interstitium was still slightly increased. From the immunohistochemical point of view a moderate number of endotoxin-storing cells were found, the majority of these cells being alveolar macrophages. Four weeks following endotoxin injection only a few endotoxin-positive alveolar macrophages could be observed.

Observations on alveolar macrophages

Alveolar macrophages from LPS treated rats (2 mg i.v./rat), obtained at different times after injection were investigated and compared with normal alveolar macrophages.

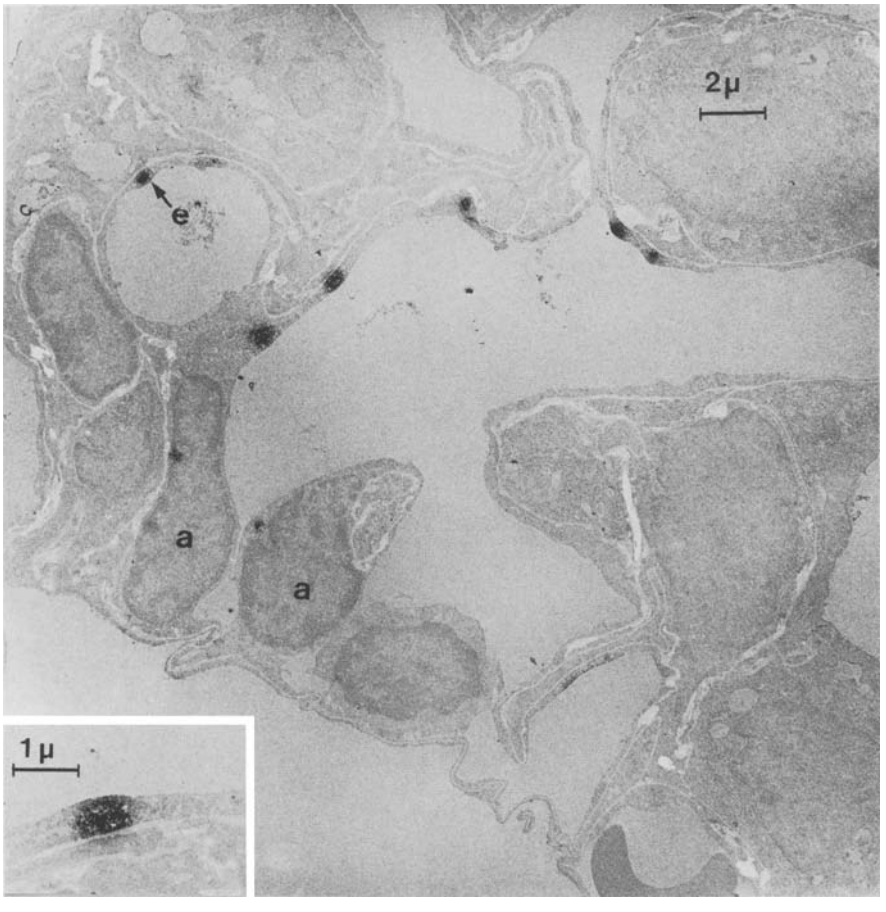
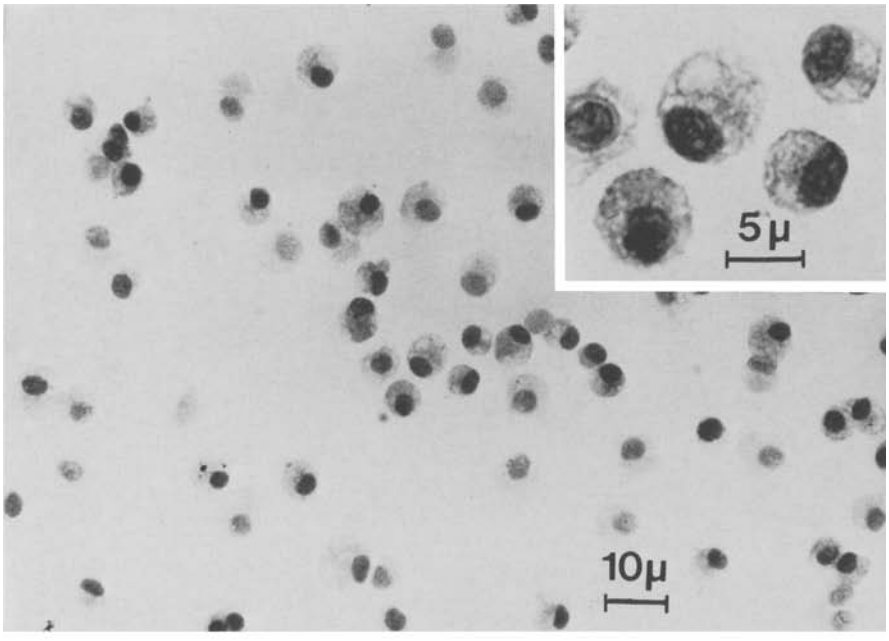


Fig. 8. EM immunohistochemical demonstration of endotoxin in the rat lung, 3 days after LPS injection. Note the numerous electron dense accumulations of the specific reaction products in the alveolar epithelial cells (*a*) and capillary endothelium (*e*). Direct immunoperoxidase method. *Inset:* Higher magnification of an endotoxin-positive area in the cytoplasm of an alveolar epithelium

Normal alveolar macrophages were obtained from healthy untreated rats by washing them out through the trachea with sterile isotonic NaCl solution. In this way an average of 3×10^6 nucleated cells per normal rat could be obtained. The cells were allowed to attach themselves to glass slides and on examination showed the morphology of typical alveolar macrophages (Fig. 9) with a spherical shape, a diameter between 6 and 10 μm, a round or slightly elliptical eccentric nucleus, and foamy cytoplasm (Fig. 9, inset) which occasionally contained single anthracotic granules. As expected, the cells were completely LPS-negative to the indirect immunoperoxidase assay for endotoxin detection.

Light microscopy showed that during the first 12 h after injection of LPS no significant changes in the general morphology of alveolar macro-



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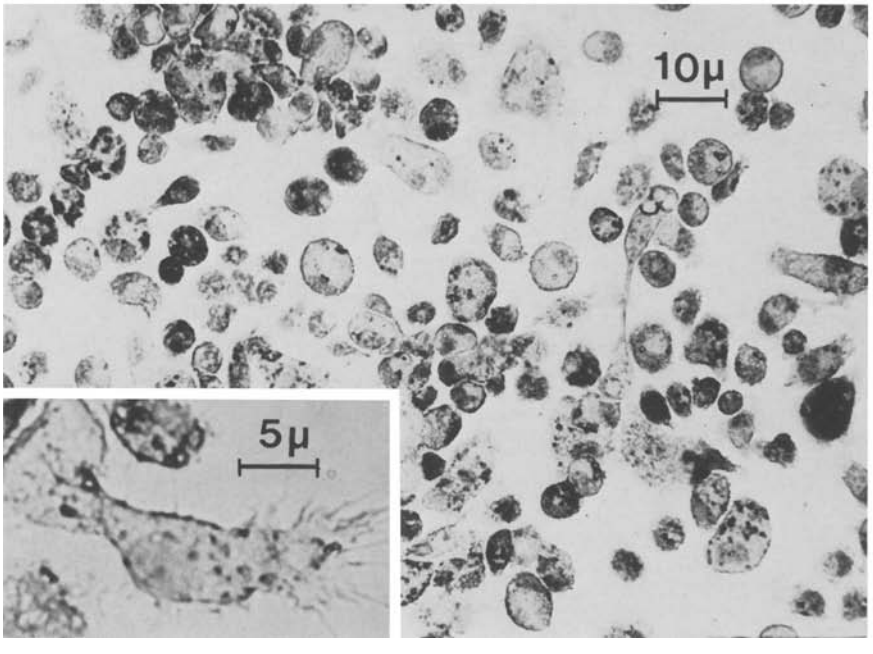

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Fig. 9. LPS-negative alveolar macrophages obtained from a rat by broncho-alveolar lavage. Indirect immunoperoxidase staining and hemalum counterstaining. *Inset:* Higher magnification of normal alveolar macrophages

Fig. 10. Activated alveolar macrophages from rat broncho-alveolar lavage, 8 days following 2 mg endotoxin injection. Note the intense storage of endotoxin (dark reaction product) as well as the enlargement of many cells and the high proportion of macrophages exhibiting cytoplasmic protrusions (Inset). Indirect immunoperoxidase reaction

Table 1. Time dependence of the percentage of alveolar macrophages showing a LPS-positive reaction and the degree of that reaction following endotoxin injection

Time after LPS injection	Percentage of alveolar macrophages showing LPS-positive reaction	Percentage thereof showing different degrees of immunostaining for LPS		
		Weak	Moderate	Heavy
1 h	3	100	–	–
2.5 h	20	100	–	–
12 h	60	8	58	34
3 days	100	10	17	73
6 days	100	12	66	22
7 days	98	8	48	44
8 days	98	33	39	28
14 days	98	30	38	32
4 weeks	<1	100	–	–

phages occur. On day 3 after LPS treatment 30% of the cells showed cytoplasmic protrusions, and a number of the macrophages had increased in size. Between the 6^{th} and 8^{th} day after the adminsitration of endotoxin the picture consisted of a significant increase in size of cells (up to 26 µm in length), of which about 50% showed marked cytoplasmic protrusions (Fig. 10, inset). On the 14^{th} day and at the end of 4 weeks after endotoxin administration the alveolar macrophages were almost exclusively round and of normal size.

The Table 1 exhibits the results of immunostaining for endotoxin detection in alveolar macrophages within the observation time from 1 h until 4 weeks after LPS administration. One hour after injection 3% of the cells showed weak LPS staining. The number of endotoxin positive cells increased with time, 20% being positive 2.5 h, 60% 12 h and 100% 3 days after LPS injection. The intensity of the immunostaining also increased with time, being weak in all cells during the first 2.5 h, predominantly moderate (58%) or heavily positive (34%) 12 h and mainly heavily positive (73%) 3 days after LPS treatment. Between the 7^{th} and 14^{th} day a very slight reduction in the number of LPS-positive cells to 98% was found (Fig. 10), although the proportion of the strongly positive macrophages was already significantly reduced to about half in comparison with the third day. Four weeks after LPS treatment less than 1% of the alveolar macrophages were endotoxin-positive and exhibited only a residual weak LPS-positive reaction.

On day 3, the time of maximal immunoperoxidase reaction for endotoxin, 0.173 µg LPS/10^6 alveolar macrophages were detected by means of radioactivity measurements following the injection of ^{14}C-LPS.

Discussion

It was shown recently in rats that during endotoxin-induced shock pathological changes in the lung develop which can be compared with shock-induced pulmonary alterations in man (Freudenberg et al. 1982). In this study the

natural history of the appearance of LPS in the lung, its localization and the accompanying changes in the lung morphology were followed in rats treated with endotoxin.

After parenteral injection endotoxin can be detected bound to various kinds of cells in the lung. The LPS-positive cells can be identified as mononuclear phagocytes (alveolar macrophages included), granulocytes, alveolar epithelial cells and vascular endothelium.

We were first able to demonstrate endotoxin in the alveolar macrophages, and already one hour after treatment a weak LPS-positive reaction could be seen in individual cells of the lavage. From this point on the endotoxin storage of the lung tissue increased continually and reached an immunohistochemical maximum on the third day after injection. The elimination of LPS from the lungs was not finished completely 4 weeks after endotoxin treatment.

Both on account of their higher capacity for storing endotoxin and their numerical superiority mononuclear phagocytes are the leading LPS-positive cells. In this connection our present findings agree well with earlier research which reported endotoxin-storing macrophages and granulocytes in the lung (Braude 1964; Mathison and Ulevitch 1979; Freudenberg et al. 1982). The detection of endotoxin storing cells in pulmonary vessels of different sizes on the third day after injection, suggests that a number of the LPS-positive macrophages seen in the lung have come from other organs and migrated into the lung as cells already carrying endotoxin. This suggestion is supported by the results of previous experimental investigations which strongly suggested that macrophages can pass from the liver to the lung after phagocytosis (Easton 1952; Freudenberg et al. 1982). Our ultrastructural findings have established that a considerable degree of migration of LPS-containing mononuclear phagocytes between blood vessels and air passages can take place during endotoxin shock.

A significant number of mononuclear phagocytes seen by us in the lung between the 2nd and 8th day after endotoxin application must be regarded as activated macrophages. Rabinovitch and De Stefano (1973) as well as Morland and Kaplan (1977) identified activated macrophages by their increased phagocytotic activity, increased lysosomal activity, extensive folding of the surface membranes and increase in size, and because of their greater tendency to spread and to adhere to surfaces. In the present study we were able to detect both the morphological signs of activated macrophages in the later stages of shock (from 3rd day onwards), such as increase in size, large number of cytoplasmic projections, and also increased phagocytotic activity for endotoxin, which showed its immunohistochemical maximum on the third day.

The accumulation of mononuclear phagocytes in the lung may represent at least one of the main factors leading to damage of lung tissue. It is assumed today that mononuclear phagocytes play a key role in the development of endotoxin shock. The interaction of LPS with macrophages leads to activation of these cells. The properties of activated macrophages and expecially their participation in tissue injury (i.e. due to their increased

production of catabolic enzymes such as elastase and collagenase) have been extensively studied and documented (for review see Tracey, 1983). In the present study the correlation between appearance of LPS-positive macrophages and histological signs of lung tissue injury is striking. In particular the interstitial oedema observed in the rat lung, which can be seen within 5 days after induction of the endotoxin shock, must be regarded as an typical pulmonary change due to that shock.

The fact that endotoxin remains in almost all alveolar macrophages for at least 14 days suggests two possibilities:

1. LPS can be removed from the alveolar macrophage only during the course of several weeks.

2. The endotoxin-loaded alveolar macrophages represent only a part of the mononuclear phagocyte system, so that the undegraded LPS appears in the lung over a long period. In this connection one must consider the possibility of elimination of the endotoxin through the respiratory passages.

By means of ultrastructural immunohistochemical investigation we were also able to localize the endotoxin precisely in the cells lining vessels and alveoli. In the alveolar epithelial cells and in the capillary endothelium of the lung LPS can be demonstrated in two different forms and in two different areas of the cytoplasm: on the one hand *diffusely* in the neighbourhood of cell junctions, and on the other *membrane-bound* in cytosomes. The demonstration of endotoxin without a clear membrane demarcation in the region of cell junctions suggests that this area of the cytoplasm has an affinity for LPS. So far as we know, the uptake of endotoxin in cytosomes of alveolar epithelial cells has not hitherto been reported. The proof that endothelial-like intimal cells of small blood vessels in the lung can store endotoxin in their cytosomes confirms the observations of Rubenstein et al. (1962) who demonstrated LPS in the endothelium of small vessels using an immunofluorescent method and the light microscope. These findings suggest the existence in the lung of special "resident" intimal cells capable of phagocytosis.

Acknowledgments. We express our thank to our colleague Dr. F. Steel for discussions on the preparation of the English manuscript. We are grateful to U. Pein, H. Stübig and U. Wiehle for expert technical assistance. The study was supported by a grant from the Deutsche Forschungsgemeinschaft.

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Accepted May 28, 1984