Pathogenetic significance of endotoxins in infections with Gram-negative bacteria

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Pathogenetic significance of endotoxins in infections with Gram-negative bacteria

by

Bernhard Urbašek

With two Figures

Summary - A review with 76 references.
The ultrastructure of Gram-negative bacterial cells exhibits three separate cell wall units, which enclose the protoplasm (1; 2). The outer membrane consists of two dense layers, which are regarded as one membrane unit. That membrane consists of lipopolysaccharides, i.e. the endotoxin or O-antigen, respectively, and lipoproteins, while the solid membrane consists of muropeptides. The plasma membrane encloses the cytoplasm, and consists chiefly of lipids and proteins. The term endotoxin is based on the early view holding that endotoxin is a component of the inner portion of the cell (3).

Boivin and Mesboreanu, who in 1933 introduced the trichloroacetic acid procedure as method for extracting endotoxins (using *S. typhimurium*) (4; 5), already held the lipopolysaccharide moiety to be identical with O-antigen and endotoxin (6). Additional procedures for extracting endotoxins have been developed, for example, by Morgan (7) using diethylene glycol, by Raistrick and Topley (8) as well as by Tal and Goebel (9) and various other authors. The phenol-water procedure was described in 1940 by Palmer (10), and modified and improved in 1952 by Westphal and Luederitz (11).

The polysaccharide structures contain the antigen-determinant groups and represent the base for serological differentiation, and thus also for the Kauffmann-White schema. Microbiological science is greatly indebted to the subsequent cooperation of Kauffmann, Westphal and Luederitz, who have elucidated numerous antigen structures by chemical means, and correlated these structures with serological specificities. As has been outlined in a preceding review article, the latter are not associated with the pathogenic properties of the bacteria.

In the case of the Enterobacteriaceae, an additional antigen, namely the common antigen, is of interest, which has been described by Kunin (12), and elucidated further by Neter (13). Neter (14) has also investigated the distribution of that antigen.
While the investigations of the serological determinants, thus, proceeded with success, the structure of the moiety or portion responsible for toxicity is not adequately known. Detoxification procedures of various authors based on the removal of long-chain, esterified fatty acids from the macromolecule, indicate that the fatty acids as such represent the toxic principle. Other authors have discussed that the molecule becomes hydrophilic on removal of important lipid portions and is then less readily able to penetrate the cell membranes. Lipids penetrate membranes without carrier, while hydrophilic substances—like sugars, for instance—require a carrier for penetration. In front of the background of the classical toxoids from exotoxins, description of the detoxifying preparations as endotoxoids is not correct, since they, on the one hand, do not provide immunogenic protection and, on the other one, are not completely nontoxic.

The so-called endotoxins of the R forms of Gram-negative bacteria, as are described in literature, hitherto have contributed little to the promotion of our knowledge of the toxic determinants and, thus, to the principle of pathogenicity of Gram-negative bacteria. The description "endotoxin from R forms" is incompatible with the early realization of the identity of lipopolysaccharides from S forms and endotoxin, and the usage of employing both concepts in synonymous fashion. The fact that these extracts possess different properties—like Shwartzman activity, chick embryo toxicity, B-cell stimulation as well as nonspecific resistance to infections—i.e. properties possessed by the endotoxins, do not permit us to describe these substances as endotoxins. This is valid also with regard to the tumor-necrotizing activity, which can be explained by induction of nonspecific inflammations. It has been demonstrated that these preparations from the R forms are significantly less toxic in mice.
selected

Only certain mouse strains or mouse strains usually employed for determining acute toxicity (which strains are specifically conditioned) exhibited sensitivity. Glycolipid batches prepared by Nowotny et al. from R forms of Salmonella minnesota R 595 (15; 16) exhibited little toxicity in our laboratory in both mice and guinea pigs, and caused occasionally minor transitory changes in the terminal vascular system as demonstrated by vitalmicroscopic means. On the other hand, we have demonstrated with these glycolipids the induction of nonspecific endotoxin tolerance (17), which can be compared to the nonspecific endotoxin tolerance induced by detoxified endotoxins (18; 19; 20). The mortality rate of mice treated with the corresponding R strains, of course, was considerably smaller than after treatment with the corresponding S forms—a finding corresponding to previous knowledge in medical microbiology.

Smith sees additional difficulties in the investigation of the determinants of pathogenicity in that phenotypes are induced due to the change of environment of in-vitro cultivated bacteria from infected macroorganisms, which phenotypes differ from those active in vivo (21). These phenotypes may miss either one or several virulence determinants, or other factors in virulence may appear, which are not present in vivo (22). There exists a multitude of papers showing that different bacterial species are different both in chemical terms and biological ones when they grow in the infected organism and grow in vitro, respectively (reference in 23). Losses of virulence following subcultivation in vitro and a rise of virulence following animal passages have been known for a long time, as we have observed in the case of Brucella abortus also with regard to endotoxin production (24). More recently, Formal et al. have been able to demonstrate that the growth conditions may also change, to a significant degree, the length of the O-sidechain of
the lipopolysaccharide of *Sh. flexneri*, with differences demonstrable even during growth in bouillon and growth on agar (25).

Investigations of the biological activity of the endotoxins encounter also other difficulties; among them, we find the fact that almost every parameter employed in changed in some way by endotoxins. In addition, there exists not a single endotoxin-specific detection procedure. As is generally known and demonstrated by a few examples listed in Figure 1, the Sanarelli-Schwartzman phenomenon, too, is not specific for endotoxins. Even the Limulus test is not endotoxin-specific, as has been demonstrated, for example, by McCabe (26), Kass (27) and other authors, since positive Limulus tests have been obtained also in patients with infections caused by Gram-positive bacteria. Wolff (28) has demonstrated that, for instance, polynucleotides and proteins also produce a positive reaction. Although the Limulus lysate reaction today represents the most sensitive test, a positive finding in suspected plasma samples, other liquids or extracts does not represent definite evidence for endotoxin activity. However, the Limulus test retains its significance in experimental work, as do certain sensitive immunological detection procedures. For instance, on the basis of the antigenic specificity of the endotoxins in the serum, we have previously demonstrated intake of orally administered endotoxins following intravenous administration of histamine (18).

A further difficulty is that animal-experimental findings cannot without reservation be applied to man, since there exist great species differences in both sensitivity and mode of response—a statement valid also with respect to the mediators of the endotoxins. Among the species investigated, man is by far the most sensitive one. To illustrate the order of magnitude: In a mouse weighing 20 g, the LD-75 amounts to 250 μg (12.5 mg/kg), while the same
endotoxin batch led in human volunteers given 1.5 ng per kg (at a body weight of 70 kg, approx. 0.1 μg) to chills, fever, headache, pain in the limbs, motor restlessness and vomiting, the well-known changes in thrombocytes and leukocytes, and yo disturbances of plasma coagulation parameters.

It has been found that certain extraction procedures for obtaining endotoxins are more suited than other ones in certain Gram-negative bacteria. For instance, we have been able to obtain toxic preparations from Brucella only with the aid of the trichloroacetic acid procedure. In endotoxin production after Boivin's procedure using E. coli—harvested from solid nutrient medium during the last third of the logarithmic phase—one obtains about 4.4 mg endotoxin from 300 billion cells.

Although the significance of the endotoxins in various pathological events can as yet not be definitely established, it is certain that endotoxic substances arise in vivo during disintegration of cells, which substances, on the basis of clinical and pathological findings—can be compared to the endotoxins extracted in vitro. The endotoxins produce their pathophysiological effects, once they reach the circulation in adequate quantities, independent of the bacterial species from which they had been derived; for instance, on the basis of an infected uterine cavity, a pyelonephritis, a peritonitis or in the course of a state due to burning or due to contaminated canned goods. The similarity between the manifestations of typhoid fever and the clinical consequences of an injection of bacterial endotoxins in man suggest the concept that endotoxins of S. typhi might be of decisive importance in the pathogenesis of typhoid fever. On the basis of experimental work using human volunteers, Greisman (29) concluded that the circulating endotoxins play no significant role in the pathogenesis of the course of fever and of the toxemia in cases of typhoid fever, but he did not exclude that the endotoxins might
contribute to the manifestation of typhoid fever, in that they bring about acute exacerbation of both the fever and the toxemia, and might enhance the local tissue injury by means of intensifying the inflammatory reaction, since endotoxins are potent inducers of inflammatory reactions. McGill, Porter and Kass (30) found no correlation between the endotoxin content of the blood and the occurrence of increases of temperature, decrease of blood pressure or death in patients with infections caused by Gram-negative bacteria.

From recent studies—carried out, inter alia, by Cottier (31), Field (32; 33; 34), Finkelstein (35; 36), Formal (37; 38; 39; 40), Neter (42; 42) and Smith (21; 43)—we know additional mechanisms of pathogenicity of Gram-negative bacteria. Not only Vibrio cholerae, but also certain pathogenic E. coli strains and Sh. dysenteriae produce enterotoxins, which unfold their activities in the small intestine. No enterotoxins have hitherto been demonstrated in the case of Salmonella. In addition, invasiveness and penetration represent essential components of the principle of pathogenicity, apart from the ability of Gram-negative bacteria to multiply in the macroorganism.

In animal experiments and in human volunteers, the working groups of Formal (44) and of Hornick (45), using Sh. dysenteriae, have demonstrated mutants, which had lost either the ability of penetration or the ability of enterotoxin production or both these properties of Sh. dysenteriae, and that only the mutants, which penetrate and form no enterotoxin, produce manifestations comparable to those caused by the starting form. On the basis of these findings, it has been concluded that penetration is a pre-condition for pathogenicity in Sh. dysenteriae as well as in Sh. flexneri and Sh. sonnei. The function of the enterotoxin in the pathogenicity of these bacteria remains unresolved. A further pre-condition for pathogenicity is the ability of
the bacteria to persist in the intestinal mucosa for an adequate length of time after penetration (46). Recent results obtained by Keusch (47) indicate that the Shiga neurotoxin is identical with the enterotoxin, while the cytotoxic property—the pathogenetic role of which has been discussed by Levine et al. (45)—can be separated by physical means. Formal has disproved Keusch's hypothesis (48)—according to which penetration is required for secretion of liquid—when he demonstrated that the mutants of Sh. dysenteriae, which had lost the property of penetration, produced a positive rabbit ileum loop test (44).

Several E. coli strains are able to produce pathological manifestations by means of two mechanisms also in adults, i.e. by means of production of a cholera-like enterotoxin and by means of Shigella-like penetration through the intestinal epithelial cell (49). It was demonstrated that the E. coli strains causing diarrhea in infants and adults contain a plasmid, which codes the enterotoxin production (50; 51). Ferguson (52) demonstrated in adult volunteers that high doses of enteropathogenic E. coli strains isolated from infants produce headaches, nausea, abdominal cramps and vomiting, i.e. symptoms that are comparable to those seen in human volunteers following administration of endotoxin. It was assumed that the enterotoxins unfold their activities solely in the intestines (53). Our own investigations of the microcirculation have shown that—in contrast to the endotoxins—enterotoxins on parenteral administration initially do not exert effects on the terminal vascular system.

An interesting fact is that enterotoxin-producing strains—like Vibrio cholerae and several E. coli strains—as well as invasive strains—like several types of S. typhimurium, E. coli and Shigella, for instance—cause enhancement of adenylcyclase activity (40). Formal has assumed that the
adenyl cyclase, in the case of the invasive strains, proceeds by way of prostaglandins synthetized in inflammatory reactions; however, synthesis of the prostaglandins may just as well be ascribed to the activities of the endotoxins.

One of the decisive factors in bacterial penetration through the intestinal epithelial cell, perhaps, is found in the chemical composition and structure of the lipopolysaccharide components of the S forms, as has been demonstrated by investigations following hybridization of Sh. flexneri with E. coli (54). Lipopolysaccharides may also bring about protection against cellular destructive mechanisms of the host following bacterial penetration, ensuring the further multiplication of virulent bacteria (54).

It must be stressed at this point that the pathophysiology of the manifestations caused by Gram-negative bacteria is associated with numerous unresolved problems. On the basis of the findings outlined so far, it can, perhaps, be understood that also at the present time the mortality rate is reported to be extremely high in many places in cases of Gram-negative septic shock. Since there exist no specific interventions (including, for the time being, the immunoglobulins), the mortality rate can be reduced considerably by employing various nonspecific measures including, inter alia, extremely high glucocorticoid doses, as have been administered, for instance, in the large American shock-treatment centers and, for instance, by Hennemann.

Next, we will discuss several of the demonstrable effects produced by the endotoxins and, in particular, those seen during the initial phase. Among the well-known mediators of the endotoxins, we find during the initial phase histamine, serotonin and catecholamines as well as additional vasoactive substances. Histamine is liberated or generated, respectively, by the macromolecule endotoxin, which, in that respect, imitates an antigen-antibody
complex, if no corresponding antibodies are present. The morphologically identifiable substrate is found in the perivascular mast cells, which undergo degranulation following action of endotoxin (18). The granules contain also serotonin and heparin. Degranulation is preceded by complement activation by way of the alternate pathway with retention of the early components, as has been described by Mergenhagen (55) and other authors. Activated C5 and, quantitatively less, C3 correspond to the classical anaphylatoxin, indicating histamine activity.

The sensitizing action of endotoxins on biogenic amines described by us in vitalmicroscopical studies in the smooth muscles and in the terminal vascular system (18; 56) contributes to the explanation of the initial vehement effects of the endotoxins. In the presence of microgram quantities of endotoxins, threshold doses of histamine or serotonin are enhanced in their pharmacological action by up to a power of ten. Our own investigations indicate that this enhancing mechanism facilitates an additional uptake path for endotoxins from the intestines into the circulation. Zweifach (57) found a dose-dependent potentiation of adrenalin activity following administration of endotoxin.

In the acute experiment using a smooth muscle preparation, antihistamines inhibited competitively only the histamine amounts present, and the endotoxin-induced enhancing effect remained unaffected. This explains, on the one hand, the partial influencing of the endotoxin action by antihistamines and, on the other one, the surgeon's experience that mitigation of the course can be achieved in patients with severe burns (in whom high concentrations of histamine are initially released from the skin) only if antihistamines are administered during the first 30 minutes. It has been demonstrated that pretreatment with
antihistamines immediately prior to endotoxin administration inhibits significantly the endotoxin-induced lipid mobilization and hyperlipoproteinemia, and lowers mortality (58).

The activities of the prostaglandins as mediators have recently been discussed. These derivatives of prostanoic acid are synthetized by various noxious agents, including the endotoxins. Assignment with the mosaic of mediators is difficult, since the individual factors exert heterogeneous actions and are extremely rapidly broken down following their synthesis and liberation. While, for instance, PGE, which exerts a direct effect on the vessels, shows a dilatory action on pulmonary vessels and bronchial muscles, we know that PGE₂₉ has a vaso- and bronchoconstricting action. A permeability-enhancing and platelet aggression-inhibiting action has been ascribed to PGE₁.

The significance of the mediators in the endotoxic event is underlined by their synergism, as is known, for instance, for serotonin and histamine or for prostaglandins and histamine. Zweifach (59) has described, in the case of microcirculation, the synergism of threshold doses of serotonin and bradykinins, which are also liberated by endotoxin.

In experiments using swine, injections of endotoxin caused, inter alia, high vascular activity (60). In addition to diarrhea and vomiting, transitory reddening of the skin, cutis marmorata (marbled skin), edema of the eyelids, delatation of the ear vessels, postmortal petechial hemorrhages in both the endocardium and the pericardium, the stomach, the renal cortex and the adrenal gland, and deep red discoloration of the entire small intestine were observed. Vitalmicroscopical investigations following administration of endotoxins revealed severe disturbances of microcirculation as well as of vascular content, of the vascular wall as well as of the perivascular space of different species
(18; 20). In addition to the initial decreases of both leukocytes and platelets, these changes represent the earliest recordable endotoxic effects, the consequences of which mainly determine the subsequent course of events (61; 62)—a fact confirmed by the electron-microscopical investigations described next. The mediator role of the biogenic amines during the initial phase of endotoxin action is confirmed by the observation showing that comparable changes appear in microcirculation following administration of histamine or serotonin, which, however, set in immediately following injection.

The changes in the terminal vascular system during the initial phase following administration of endotoxin—which changes have been described by vitalmicroscopical and scanning-electronmicroscopical means—have been investigated by the present author in cooperation with Forssmann (64) using the electron microscope. The subject of these investigations was the terminal vascular system in the rabbit ear chamber (initially involving vitalmicroscopic observations). In experiments, carried out together with J.W. Irwin, ear chambers were implanted in swine and rabbits. The implantation in the swine has hitherto been unsuccessful.

Following intravenous administration of 10 μg/kg E. coli endotoxin (batch ETU 106), the endothelial cells showed swellings, while the intracellular connections and the basement membrane occasionally were still intact. Micropinocytotic vesicles appeared in large numbers. Other areas, in the case of progressing pinocytosis, exhibited mitochondrial swelling of the endothelial cells; pericytes also exhibited swelling and corresponding mitochondrial changes. In addition, we observed capillaries with disintegrated endothelial cells in part broken into small fragments with discontinuous basement membrane and fibrinoid deposits in the perivascular space. Figure 2 shows disintegrated
endothelial cells, which, in part, have been replaced by platelets located on the partially disintegrated basement membrane. Several platelets contain empty vesicles as morphological substrate of past liberation responses. The changes in the terminal vascular system described here are comparable to the symptoms occurring at the onset of the acute inflammation—a finding supporting the view that various noxious agents, in principle, produce similar or identical responses (65).

The endothelial injury of large vessels—the aorta—has been described by McGrath and Stewart (66) as well as by Gaynor et al. (67). Gaynor (68) has investigated the question whether the granulocytes adhering to the endothelium cause intima injury following administration of endotoxin, or whether the adherence to the wall was a consequence of the intima injury. In the aorta of granulocytopenic rabbits, the latter author was able to demonstrate that that tissue was changed in the same fashion after administration of endotoxin as in normal rabbits following administration of endotoxin. These changes can be explained by the activities of the mediators and, in particular, of histamine and serotonin.

Springer has described an endotoxin-specific receptor on erythrocytes and leukocytes (69; 70; 71), so that the question after specific receptors on other cells—in particular, endothelial cells—has been raised anew. The binding of endotoxin to erythrocytes is known since Neter introduced the method of passive hemagglutination with lipopolysaccharides (72).

On the basis of the initial disturbances of the terminal vascular system there develop additional dose-dependent metabolic—including the blood coagulating system—changes, which may lead to shock fragments or shock. On the basis of investigations using the mouse, Berg et al. have described certain
metabolic changes following administration of endotoxin and, in particular, changes in the carbohydrate metabolism (73; 74; 75). The clinical and chemical data obtained in the dwarf swine in the course of endotoxemia and during the state of nonspecific endotoxin tolerance will be reported elsewhere (76). At this time, we already stress the importance of the glucose metabolism in this shock form. Following primary, adrenalin-induced hyperglycemia, the dwarf swine exhibited hypoglycemia with rise of lactate, while dwarf swine in the state of nonspecific tolerance, which survive, do not exhibit these changes.
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<thead>
<tr>
<th>Sensitizing Injection</th>
<th>Challenging Injection</th>
<th>Author</th>
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<tbody>
<tr>
<td>Colchicin</td>
<td>Pregnancy</td>
<td>Galton, 1964</td>
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<td>Cortison or iron oxyd-saccharat</td>
<td>A streptococci</td>
<td>Stetson, 1956</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>Staphylococci</td>
<td>Zdrodowski, 1928</td>
</tr>
<tr>
<td>A streptococci</td>
<td>Streptolysin O</td>
<td>Schwab et al., 1953</td>
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<tr>
<td>A streptococci</td>
<td>A streptococci filtrates</td>
<td>Schwab et al., 1953</td>
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<tr>
<td>A streptococci</td>
<td>Meningococci toxin</td>
<td>Thomas et al., 1952-1953</td>
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18. Mechanism of action of bacterial endotoxins and influences exerted on that mechanism. Dissertation submitted for the certificate of habilitation, Faculty of Medicine, Heidelberg University.

60. Unpublished results.
76. In preparation.