Inflammatory Effects of Salmonella typhimurium Porins

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The inflammatory activity of porins purified from Salmonella typhimurium has been investigated. Porins (0.3 to 30 μg) injected into the rat paw induced a dose-related edema that was not due to lipopolysaccharide contamination and did not appear to be dependent on the activation of the complement system. The edema induced by 30 μg of porins was comparable to that caused by 1 mg of carrageenin and was inhibited by indomethacin (5 mg/kg) and dexamethasone (0.1 mg/kg). Porins (1 to 10 μg/ml) induced a concentration-related release of histamine from rat peritoneal cells. These results are discussed in the light of the possible pathogenic role of porins in infections.

The outer membrane of gram-negative bacteria contains some major proteins that are called porins because of their role in cellular permeability (14, 15). We carried out various studies on the immunobiological properties of these proteins. Salmonella typhimurium porins are toxic for macrophages in mice, and subtoxic concentrations of porins reduce phagocytosis and intracellular killing (27). Our studies show that porins inhibit phagocytosis by activating the adenylate cyclase system (4).

These proteins, therefore, induce the activation of the complement system by acting both on the classic pathway and on the alternative pathway (9); they bind to human polymorphonuclear leukocytes, decreasing some of their biological functions (29), and act as mitogens for B lymphocytes (24, 27). Furthermore, in rats the porins increase the toxicity of cardiotoxic molecules (8) and damage renal tubules (28).

Our results show that porins play a role as pathogenicity determinants. These proteins, whether directly secreted by bacteria (13) or derived from the lysis of the bacterial cells, are resistant to the action of proteolytic enzymes of the host and can therefore interact with host-mediated cellular and humoral systems.

The aim of the present study was to examine the inflammatory properties of porins and the possible dependence of porins on the activation of the complement system.

MATERIALS AND METHODS

Preparation of porins. S. typhimurium SH5014 served as the source of porins. The method described by Nurminen (16) was used to extract and purify the porins.

One gram of envelopes was treated with 2% Triton X-100 in 0.01 M Tris-HCl (pH 7.5) containing 10 mM EDTA; after the addition of trypsin (10 mg/g of envelopes), the pellet was dissolved in sodium dodecyl sulfate buffer (4% [w/v]) sodium dodecyl sulfate in 0.1 M sodium phosphate (pH 7.2) and applied on an Ultragel ACA34 column equilibrated with 0.25% sodium dodecyl sulfate buffer. The fraction enriched in protein, identified by A280, was extensively dialyzed and checked by gel electrophoresis by the method of Laemmli (11). All possible traces of lipopolysaccharide (LPS) were identified by means of gel electrophoresis and staining with silver nitrate as described by Tsai and Frasch (26) and by means of the Limulus amoebocyte lysate assay (Limulus test) (25) with as controls, endotoxin-free water and S. typhimurium SH5014 LPS extracted by the method of Galanos et al. (6) as described in a previous paper (7).

Preparation of LPS. LPS-R was isolated from S. typhimurium SH5014 with phenol-chloroform-petroleum ether as described by Galanos et al. (6). Briefly, liquid phenol (90 g of dry phenol plus 11 ml of water-chloroform-petroleum ether in volume ratio of 2.5:8) was added to 1 g of the dried bacterial extraction mixture. After homogenization for 2 min, the bacteria were centrifuged and extracted twice. The supernatant was filtered through filter paper and treated as described by Galanos et al. (6). LPS-S from S. typhimurium (Difco Laboratories) was also used.

Production of acute inflammation. Edema of the hind paw of male Wistar rats (140 to 160 g) was produced by injecting 0.1 ml of phosphate buffer containing 0.3, 3, or 30 μg of porins. Groups of at least five rats were used, and the volume of the paw was determined immediately after the injection with a differential volume measuring instrument (Basile, Milano) as previously reported (5). Subsequent readings of the same paw were carried out every hour for 5 h and compared with the initial reading. In some experiments the edema produced by injecting 0.1 ml of 1% lambda carrageenin (Sigma Chemical Co.) in saline was also assayed for comparative purposes.

Inflammation in decomplemented rats. In some experiments paw edema was induced by porins in complement-depleted rats.

To determine the drop in complement level of the animals, three male Wistar rats were injected intravenously with bovine serum albumin–anti-bovine serum albumin. The soluble complexes were prepared by precipitating antibody from the rabbit antiserum at the point of equivalence and dissolving the precipitate in excess bovine serum albumin (20 times the amount needed for precipitation at equivalence) (4). Complement activity of the serum was evaluated as described by Lachmann and Hobart (10).

Release of histamine and prostacyclin. Resident peritoneal cells (approx 5% mast cells) were recovered from male Wistar rats (200 to 250 g) as previously described (2). Cells were washed and suspended in Tyrode solution (pH 7.2), which contained 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl2,
1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 5.6 mM glucose.

Cell suspensions (final volume, 1 ml) were allowed to equilibrate at 37°C in a metabolic shaker with gentle agitation, and histamine release was initiated by the addition of the following agents (final concentrations): porins (1, 3, and 10 µg/ml), concanavalin A (ConA; 1, 3, and 10 µg/ml), compound 48/80 (0.1, 1, and 10 µg/ml).

Experiments with ConA were carried out in the presence of phosphatidylserine (50 µg/ml).

The release was terminated after 10 min by the addition of 2 ml ice-cold Tyrode solution.

Cells and supernatants were recovered by centrifugation (10 min, 150 × g, 4°C), and histamine concentrations in solutions and cells were quantified fluorimetrically (19).

Histamine release was calculated as a percentage of the total cellular content of the amine. All values were corrected for spontaneous release occurring in the absence of the inducer (approximately 5%).

Prostacyclin, measured as 6-keto-prostaglandin F₁α in the supernatants of cell suspensions incubated with porins, was determined by a specific radioimmunoassay after suitable dilution in radioimmunoassay buffer without prior extraction or purification (17).

Statistics. Comparisons between groups were done by the Student t; statistical significance was considered to be P < 0.05.

RESULTS

The purity of the porins preparation is shown in Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed two bands corresponding to porins with molecular weight of 34,000 and 36,000. The LPS contamination was evaluated on the same gel that was used to test porin purification. The gel was divided in two halves: one was stained with Coomassie blue and served for porin identification, and the other was stained with silver nitrate for LPS identification. With this procedure no detectable amounts of LPS were found in the porins fractions. The detection limits of the gel system were 1 ng with pure LPS of known concentrations. The Limulus test revealed traces of LPS (10 µg/10 µg of porins [final concentration]).

FIG. 1. Protein patterns of porins preparation made from S. typhimurium SH5014. Lanes: A, SH5014, 20 µg of protein in the sample; B, molecular weight standards (phosphorlase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100).

1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 5.6 mM glucose.

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FIG. 2. Rat paw edema induced by 0.3 µg (□), 3 µg (■), or 30 µg (○) of porins or by 1 mg of carrageenin (▲). Each point represents the mean ± standard error of the values from at least five rats.

Rat paw edema. Porins injected into the rat paw (0.3, 3, or 30 µg) induced a dose-related edema; a peak response occurred 2 to 3 h after the injection (Fig. 2). The curve for edema induced by 30 µg of porins was still evident after 5 h and was virtually superimposable on the curve for edema induced by 1% lambda carrageenin (Fig. 2). The edema induced by porins was not due to contamination by polysaccharides (LPS).

Thus two types of such compounds (LPS-R from S. typhimurium and LPS-S from Difco) injected into the paw (0.3 or 30 µg) failed to induce any detectable edema.

The decompartmentalized animals showed an 80% reduction in complement activity, which remained constant for approximately 12 h. The same degree of paw edema was observed when porins were injected in complement-depleted rats (data not shown).

The edema induced by porins was reduced by 50 to 60% in animals treated subcutaneously with indomethacin (5 mg/kg) or with dexamethasone (0.1 mg/kg) (Fig. 3).

Histamine and prostacyclin release. Porins (1, 3, or 10 µg/ml) induced a concentration-related release of histamine from rat peritoneal cells (Fig. 4). The maximum release (about 90%) was observed after the cells were incubated for 10 min with 10 µg of porins; the effect was stronger than that of ConA and about equally as active as that of compound 48/80. At a concentration of 1 µg/ml, porins induced a weaker release as compared with those induced by ConA and compound 48/80.

In contrast, porins at any concentration (1, 3, or 10 µg) failed to modify the spontaneous release of prostacyclin by rat peritoneal cells. This release remained in the range of about 3 µg per 10⁶ cells even at the highest porin concentration.

DISCUSSION

Our results show that porins are clearly endowed with proinflammatory activity. In fact, when injected into the rat...
paw at concentrations between 0.3 and 30 μg, these porins induce dose-dependent edema with long-lasting effects. The highest dose injected (30 μg) induced edema comparable to that induced by 1 mg of lambda carrageenin. The inflammation produced by the porins is sensitive to both steroid (dexamethasone) and nonsteroid (indomethacin) anti-inflammatory drugs.

The in vitro studies carried out on peritoneal cells of the rat show that the porins at concentrations between 1 and 10 μg/ml are able to induce the release of histamine. At the highest concentration tested, the porins had an effect that was stronger than that of ConA and equal to that of compound 48/80.

Furthermore, in vitro experiments have also shown that porins do not seem to induce release of prostacycline, since the concentrations of its stable metabolite, 6-keto-prostaglandin F1-α, are not increased on incubation with porins. Porin-induced inflammation may depend on the release of histamine, even if we cannot completely exclude the participation of arachidonic acid metabolites. In fact, our in vitro results tend to exclude an increase of 6-keto-prostaglandin F6-α and consequent prostacycline release, whereas the in vivo results confirm both the prolonged duration of porin-induced edema and its marked inhibition by indomethacin.

Porin-induced inflammation was also observed in decomplented animals; therefore it is unlikely that the activation of the complement system plays a major role in the inflammation induced by porins.

Our results allow us to better understand previous data dealing with the interaction between endotoxins and inflammatory cells (19, 21, 22). Asboe-Hansen and Glick (1) first examined the in vitro response of rat peritoneal mast cell suspensions to an *Escherichia coli* endotoxin prepared by the method of Boivin. They were unable to detect any influence of endotoxin on mast cells. Sandusky et al. (18) obtained similar results with either *E. coli* O127:B8 Boivin or Westphal prepared endotoxin even at concentrations as high as 1 mg/ml. Morrison and Betz (12) demonstrated that, although endotoxins themselves were unable to stimulate rat peritoneal mast cells, the isolated protein component of the endotoxin was at a low concentration (1 to 10 μg/ml) and was able to initiate the noncytotoxic secretion of vasoactive amines.

Further experiments (18) demonstrated also that LPS from *Salmonella minnesota* RS95 enhanced the response of peripheral blood basophils obtained from allergic donors but not of basophils from nonallergic donors.

On the basis of our experimental results, we hypothesize that during natural infection bacteria accumulated in an infectious focus could stimulate an inflammatory process by way of products either secreted or released through lysis by the bacterial cells.

In the case of gram-negative bacterial cells, the porin concentration is approximately 2 × 10^{6} molecules per cell, covering an area of approximately 1.8 μm^{2} or roughly a third of the cell surface area. Therefore, lysis of about 10^{7} cells is sufficient for the release of porins in the range of amounts that in our experiments exhibited an inflammatory effect. This load is easily produced by bacteria. The activation carried out by the porins in our experimental model therefore is consistent with the possibility of porin-induced inflammation occurring in gram-negative bacterial infections.

This evidence of proinflammatory activity by porins further enlarges the field of action of protein-host interactions. These proteins intervene in nonspecific host defenses (4, 9, 29) and, as a consequence, in the specific immunologic response (27). Among surface components, therefore, porins can be considered another surface molecule, in addition to LPS, that may be an important inducer of biological activity in interactions with the host.
LITERATURE CITED


