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Histopathological features and modulation of type IV collagen expression induced by *Pseudomonas aeruginosa* lipopolysaccharide (LPS) and porins on mouse skin

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Summary. Background: *Pseudomonas aeruginosa (P. aeruginosa)* is responsible for serious infections in the immunocompromised host. Many skin lesions induced by *P. aeruginosa* have been described. Few investigations have been performed on the local action of *P. aeruginosa* components. Objectives: To shed light on the "*in vivo*" activity of lipopolysaccharide (LPS) and porins extracted from *P. aeruginosa*, by verifying their effects after inoculation in mouse skin through the observation of histological changes and immunohistochemical expression of collagen IV. Results: Both substances were able to induce a similar inflammatory process and a characteristic reversible change in collagen IV distribution. Interestingly, a fibroblast increase was observed at 24 h in the skin treated with porins, while it appeared later in the skin treated with LPS. Besides these changes, porins particularly increased collagen edema, together with disgregation of hypodermal structures. Moreover "*in vitro*", porins were able to stimulate fibloblasts 3T3 to convert 72 kDa type IV collagenase into the activated 62 kDa form and to release the 92 kDa collagenase. Conclusion: LPS and porins, released by Gram-negative bacteria during cell growth and lysis, interact with the host at target cells, such as keratinocytes, fibroblasts and immunocompetent cells, thus contributing significantly to the pathogenesis of *P. aeruginosa* skin infections.

Key words: Bacterial components, *Pseudomonas aureginosa*, Inflammation, Type IV collagen

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Introduction

P. aeruginosa is responsible for serious infections in the immunocompromised host. Skin lesions caused by *P. aeruginosa* are above all folliculitis, gangrenous cellulitis and gangrenous ecthyma. Patients with diabetes, under steroid treatment or with neutropenia have deep skin infections.

The physiopathological mechanism in gangrenous ecthyma is believed to be a vasculitis. This is caused by bacterial invasion of the vascular bed, by circulating immunocomplexes or by effects of exotoxin or endotoxins. The last two mechanisms may explain why microorganisms are usually not isolated from the lesions (Greene et al., 1984; El Baze et al., 1985).

The biological effects of structural components from *P. aeruginosa* have been widely studied. However, few investigations have been performed on the effect of bacterial components on cutaneous inflammation physiopathology (Parmely, 1993; Hamood and Griswold, 1995; Miyazaki et al., 1995). Among them, lipopolysaccharide (LPS) from *P. aeruginosa* is considered less toxic than LPS extracted from enteric bacteria (Isidore and Bornside, 1989). This difference has been ascribed to the different chemical composition of the lipid A moiety of LPS purified from *P. aeruginosa* compared to that produced by the Enterobacteriaceae family (Sadoff, 1974). Besides LPS, the outer membrane of gram-negative bacteria contains major proteins called porins that are involved in cell permeability.

Several studies carried out with purified porins have demonstrated that these molecules stimulate the immune response in mice (Tufano et al., 1984) behaving as chemotaxins and chemotaxinogens (Tufano et al., 1989), thus activating the complement system (Galdiero et al., 1984). They thus induce release of soluble mediators with proinflammatory (Galdiero et al., 1990) and immunomodulatory activity (Tufano et al., 1992,

1994a,b; Galdiero et al., 1993; Cusumano et al., 1997).

In this report we studied the "*in vivo*" activity of LPS and porins extracted from *P. aeruginosa*, verifying their effects on mouse skin through histological alterations. In addition, we also described the immunohistochemical expression of collagen IV when LPS and porins were inoculated under the skin at different times.

To establish a correlation between "*in vivo*" activity of LPS and porins on perivasal collagen IV degradation and "*in vitro*" production of type IV collagenase, an experimental model using 3T3 mouse fibroblasts stimulated with LPS and porins was used. Evaluation of collagenase activity was performed demonstrating its gelatinolytic ability by zymography.

Materials and methods

Preparation of LPS and porins

Porins were purified from a strain of *P. aeruginosa* isolated in the Laboratory of the Institute of Microbiology, Faculty of Medicine, 2nd University of Naples, Italy and identified by conventional techniques. The method described by Nurminen (Nurminen, 1985) was used. Briefly, one gram of envelope was treated with Triton X-100 in 0.01M Tris-HCl (pH 7.5) containing 10 mM EDTA. After addition of trypsin (10 mg/g envelope), the pellet was dissolved in sodium dodecyl sulfate (SDS) buffer (4% [w/v] in 0.1M sodium phosphate [pH 7.2]) and applied on an Ultragel AcA 34 (Biospa) column equilibrated with 0.25% SDS buffer. The protein-enriched fraction, identified by A_{280}, was extensively dialyzed and checked by gel electrophoresis (Laemmli, 1970). Traces of LPS in our preparation were identified by gel electrophoresis (12%) and staining with silver nitrate, as described by Tsai and Frash (1982) and by means of Limulus amoebocyte lysate assay (Limulus test: BioWhittaker, Walkersville, Md) (Thye et al., 1972). Neutralization was performed with polymyxin B (PB: Sigma. St.Louis, MO), incubating porins (20 μg/ml) with PB (5 μg/ml) at a room temperature for 1h (Rifknd and Palmer, 1966; Galdiero et al., 1984; Tufano et al., 1989). A negative Limulus test was obtained when porins added with PB were used as a control. LPS of *P. aeruginosa* was purchased from Sigma Chemical Co. (St. Louis. MO) Serotype 10 cod. L 8643. Protein contamination was lower than 3% This was too low to stimulate any inflammatory response at the concentration used by us (data not shown).

Experiments "in vivo"

Two groups of mice (16 animals; weight 20-25 g) <3 months of age (Nossan, Milano, Italy) were injected under the back-skin respectively with 10 μg of porins in 0.2 ml of PBS and with 10 μg of LPS in 0.2 ml of PBS.

Preliminary experiments were performed to fix the most effective amounts both of LPS and porins.

Three groups of control mice (20 animals) were inoculated respectively with (i) 0.2 ml of PBS only, (ii) 0.2 ml of PBS containing SDS at the same minimal concentration (0.01 μg/20 μg porins) checked in porins preparation as contaminant or (iii) 0.2 ml of PBS containing 2.5 μg of PB.

Histology and immunohistochemistry

At 0, 3, 6, 12, 24, 48, 72 hours and 6 days, biopsies were taken from the back-skins of the animals injected with porins or LPS and from the controls. Skin samples were fixed in 10% neutral formalin and included in paraffin according to standard histological methods. The following staining techniques were carried out on 7 μm-thick microtomic sections: hematoxylin eosin; hematoxylin-van Gieson; P.A.S.; Alcian blue; Giemsa.

The study on type IV collagen was performed by staining the sections, included in paraffin, using the ABC technique (Vectastain, Burlingame, CA) according to Hsu et al. (1981). Anti-collagen IV antiserum DBA (DAKO, Carpinteria, CA) was used at a working dilution of 1:500 and normal mouse skin served as control.

Cell culture

3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, MA) supplemented with 10% fetal bovine serum (Eurobio), L-glutamine (2.5 mM) penicillin (50 U/ml) streptomycin (50 μg/ml) at 37 °C in 5% CO_2. Medium was changed and DMEM without serum was used when the cells reached 80% confluency. After 48h incubation at 37 °C with 5% CO_2, fresh medium with modulating agents LPS (10 μg/ml) and porins (10 μg/ml) was added. Conditioned medium was collected after 24h of incubation and centrifuged at 2,000 rpm/5 min to remove cells. The proteins were precipitated with cold ethanol at -20 °C overnight. The samples were centrifuged at 10,000 rpm/30 min at 4 °C, the pellet resuspended in 100 μl Tris-buffer 40mM pH 7.5 and then stored at -20 °C for gelatin zymography. Protein concentration in the sample was tested using the method described by Bradford (1976).

Gelatin zymography

Gelatinolytic activity was assayed by the method of Heussen and Dowdle (1980), adapted for a minigel format. 5 μl aliquots containing 1 μg of proteins were mixed with sample buffer and applied directly, without prior heating or reduction, to 10% (w/v) acrylamide gels containing 1 mg/ml of gelatin (Sigma). After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 30 min, the gels were incubated at 37 °C/18h under continuos stirring in 50mM Tris-HCl, pH 7.6, containing 0.2M NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Brij-35. Gels were stained for 30 min in 30% methanol/10% glacial acetic acid 0.5% (w/v) Coomassie

brillant blue and then destained in the same solution without dye. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin.

Results

Purity of porin preparation

Porins were purified from a strain of *P. aeruginosa* as described in the Materials and methods section. The protein pattern of purified *P. aeruginosa* porin is reported in Fig. 1. SDS-PAGE showed one band with a molecular weight of about 36-38 kDa. LPS was eliminated from porin preparations by gel filtration in the presence of SDS (Benz et al., 1978; Schindler and Rosenbusch, 1981). In final preparations after dialysis, SDS was only present in minimal traces (0.01 μg/20 μg of porins).

The controls submitted to SDS PAGE and subsequent silver nitrate staining demonstrated that the first fraction eluted contained no trace of LPS. The detection limit of gel system was 1 ng, using pure LPS at a known concentration. Only minimal traces of LPS (20 pg/ 10 μg porins) were revealed by Limulus tests (sensitivity, 0.125 endotoxin unit/ml corresponding to 10 pg of LPS/ml). In selected experiments, porin preparation incubated with polymixin B to neutralize the biological activity of LPS showed a negative Limulus test.

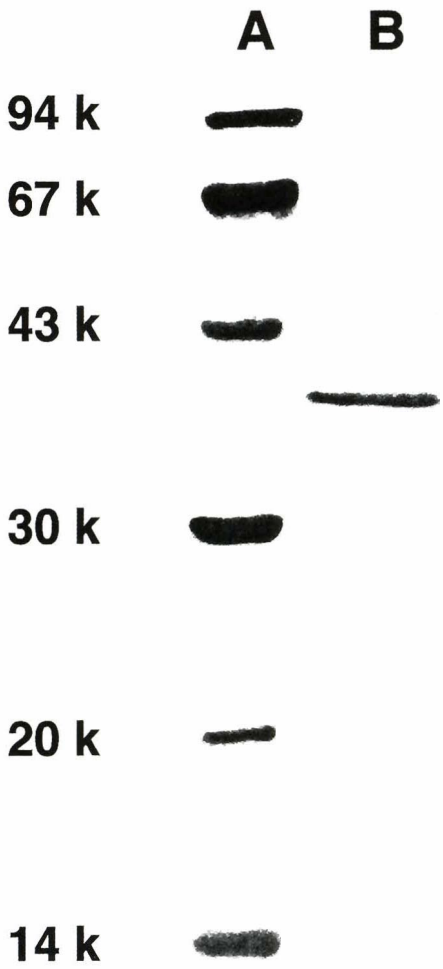


Fig. 1. Protein pattern of purified *Pseudomonas aeruginosa* porins (lane B). The SDS-polyacrylamide gel (12%) is stained with Coomassie blue. The monolecular mass markers, given in Kilodaltons, migrate in lane A.

Histopathological and immunohistochemical features

The findings from intradermal injection of either LPS or porins in mice, demonstrated that inflammation occurred within the subcutaneous region. This was especially evident in the muscles. The inflammation rarely reached or moved beyond the reticular and papillary layer of skin. Over a brief period (3 hours) there was little infiltrate and rare neutrophils. In the first 12 hours, a prevalence of PMNs (Fig. 2A) and eosinophils, reaching 80-90% was observed. The remaining 10-20% consisted mainly of lymphocytes and plasmacells. After 24 hours, PMNs and eosinophils fell to 20-30%, while lymphocytes, plasmacells, and histiocytes increased (Fig. 2B). After 48 hours the inflammatory exudate, consisting mostly of histiocytes largely disappeared. After 72 hours, a very mild lymphocytic and histiocytic infiltrate with fibroblast increase was observed in the treated mice. In the first 72 hours a similar mild inflammatory reaction was observed in the controls. After 72 hours, the histopathological features were similar in both treated animals and all the controls, consisting of scarce lymphocytic and histiocytic infiltrate below the dermis.

The mast-cells were always present. Their number did not seem to change. However, at about 24 hours, they showed more Giemsa-positive granules inside and outside the cytoplasm. In all the controls a similar mild phenomenon was evident (Fig. 2C,D).

Edema was present in the first 24 hours. Its intensity in treated animals matched all the controls. The maximum intensity occurred at 24 hours and decreased rapidly in the following periods.

The PAS-positive neutral mucopolysaccarides also increased in the interstitial spaces and in the exudate during 24 hours (Fig. 2E), then declined until baseline on the 6th day. They were always scarce in all the controls.

The Alcian blue-positive mucopolysaccarides behaved similarly. They increased in the exudate for up to 24 hours (Fig. 2F), remained high until 48 hours, then tended to disappear by the 6th day. They were practically absent in all the controls.

The fibroblast increase however was already present after 24 hours when stimulation was performed with porins, while it appeared later in the skin treated with LPS.

Collagen IV, analyzed by immunohistochemistry, appeared to be regularly distributed in the dermal-

epidermal junction basement membrane and in the vessel walls (Fig. 2G), maintaining a normal continuity with the perivasal collagen for the first 48 hours. At that time, it tended to decline within the interstitial space until it interrupted its continuity with the vessel walls. The *hiatus* was present also in the papillary microvasculature, in the superficial horizontal plexus, in feeder vessels and in deeper plexus. At the level of the dermal-epidermal junction basement membrane, the distribution of collagen IV showed only a little diminution in the expression level in this period. This hiatus persisted up to 72 hours (Fig. 2H) and completely disappeared after 6 days. In all the controls, collagen IV did not show relevant structural changes.

Gelatin zymographs

Gelatin analysis of 3T3 cell type IV collagenase (Fig. 10) revealed a single band of gelatinolytic activity with a molecular mass of 72 kDa in the control cells (lane 1). After incubation at 37 °C/24h in the presence of porins, but not LPS, the band of gelatinolytic activity of molecular mass of 72 kDa partially converted into another band of 62kDa; a 92kDa band was also demonstrated (lane 2).

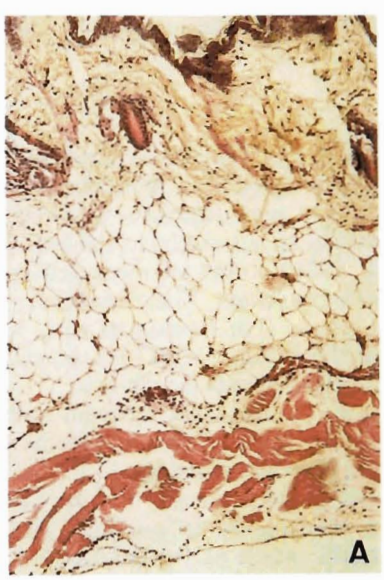
Discussion

The *P. aeruginosa* pathogenicity is connected with its opportunism. It relies on lowered humoral and cellular defense by the host, or on interruption of physical barriers, such as the skin and mucosa. Infection of healthy skin is rare. William and Mordan (1995) have recently reported 4 cases of healthy skin infection, with clinical involvement that varies from local to more serious infections (folliculitis and external ear infection) (Leyden et al., 1979) up to very severe diseases in the immune compromised host [malignant external ear infection, perirectal infections, ecthyma gangrenosus (Thomas et al., 1985; Ratnam et al., 1986; Johnson and Ramphal, 1990; Lang et al., 1990)]. It is of notable interest to identify the structural surface components of *P. areuginosa* which take part in tissue damage. Among these components, LPS and mucoesopolysaccharide (MEP) (Evans and Linker, 1973) stimulate the release of molecules "*in vitro*" that modulate biological responses, such as cytokines (Cusumano et al., 1997). Moreover, "*in vitro*" porins from *P. aeruginosa* can elicit the release of proinflammatory mediators, such as TNF-α (Cusumano et al., 1997). It has been shown, in fact, that skin microorganisms "*in vitro*" induce release of IL-1α from keratinocytes and IL-1α and IL-1ß from monocytes/macrophages (Walters et al., 1995). A large number of host mediators, such as blood monocytes, tissue macrophages, complement and neutrophils can be activated by LPS and porins (Tufano et al., 1989). Earlier studies have shown that human monocytes stimulated with structural bacterial components (Tufano et al., 1991; Galdiero et al., 1993) release cytokines, such as IL-1 and TNF-α. The latter is one of the key modulators of LPS effects, since TNF monoclonal antibodies can prevent septic shock during lethal bacteriaemia (Tracey et al., 1987).

Our experimental results, indeed, show that intradermal injection of LPS and porins from *P. aeruginosa* cause characteristic histopathological alterations. The inflammatory process is morphologically similar for both substances. It reaches its highest expression around 24-48 hours and completely disappears by the 6th day. The control animals injected with saline show a milder reaction that disappears by 72 hours. In the first hours of the inflammatory process, the exudate is mostly made up of neutrophils and eosinophils. These are later replaced by lymphocytes and plasmacells. Between 24 and 48 hours, a mild edema occurred with an increase of neutral and acid mucopolysaccharides and a greater mast-cell activity.

The vasodilatation and edema may depend on the proinflammatory activity of porins and LPS, which can activate the complement (Galdiero et al., 1984) and induce production of histamine or release of proinflammatory cytokines (Galdiero et al., 1990; Tufano et al., 1994a,b; Cusumano et al., 1997). Convincing evidence indicates a pivotal role for metalloproteinase of the matrix, in particular collagenase, in collagen degradation under rapid remodelling condition, e.g. inflammation (Everts et al., 1996). Previous studies on monocytes/macrophages, demonstrated an increased level of constitutive gelatinases of 67 and 72 kDa in prolonged skin inflammation and the appearance of a larger molecular

Fig. 2. A. Histopathology of mouse skin treated with *Pseudomonas aeruginosa* porins (12 h). Inflammation is evident on muscle level. Infiltration is mostly composed of leukocytes, neutrophils and eosinophils (Hematoxylin-Eosin, x 150). **B.** Histopathology of mouse skin treated with *Pseudomonas aeruginosa* porins (24 h). Inflammation is present above and below the muscle. Infiltration is mostly composed of lymphocytes, plasmacells and histiocytes. (Hematoxylin-Eosin, x 150). **C.** Histopathology of mouse skin treated with LPS (24 h) rich in metachromatic grains. Mast-cells are present in the interstice. (Giemsa, x 600). **D.** A detail of figure 4. Numerous mast-cells with intra- and extra-cellular metachromatic grains are evident. (Giemsa, x 1,500). **E.** Histopathology of mouse skin treated with LPS (24h). Neutral mucopolysaccharides are also present in inflammatory infiltration. (PAS, x 400). **F.** Histopathology of mouse skin treated with LPS (24). Acid mucopolysaccharides in mouse skin treated with LPS are present in the inflammatory process. (Alcian blue, x 150). **G.** Immunohistochemical feature of collagen IV in mouse skin treated with *Pseudomonas aeruginosa* porins (24h). Collagen IV is well represented in vessel walls, in the dermal-epidermal junction basement membrane and was continues with the perivascular one. (ABC - Collagen IV, x 150). **H.** Immunohistochemical feature of collagen IV in mouse skin treated with porins (72 h) Discontinuity is present between collagen IV of vessel walls and of papillary microvasculature and the interstitial space. The symbols h indicate a *hiatus*. (ABC-Collagen IV, x 500). **I.** Immunohistochemical feature of collagen IV in mouse skin treated with porins (72 h). Discontinuity is present in the perivascular collagen IV distribution at the level of deeper plexus. The symbols h indicate a hiatus. (ABC-Collagen IV, x 500)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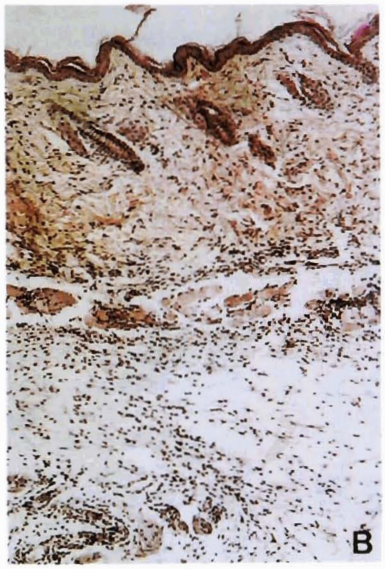
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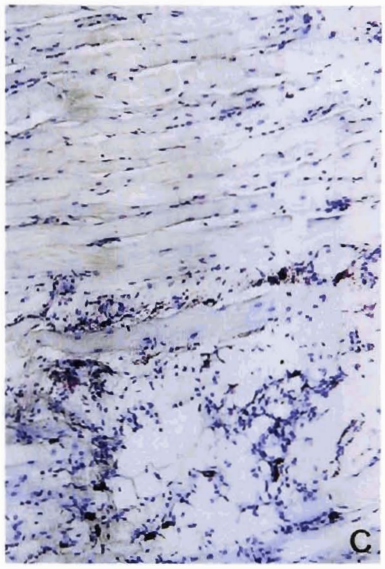
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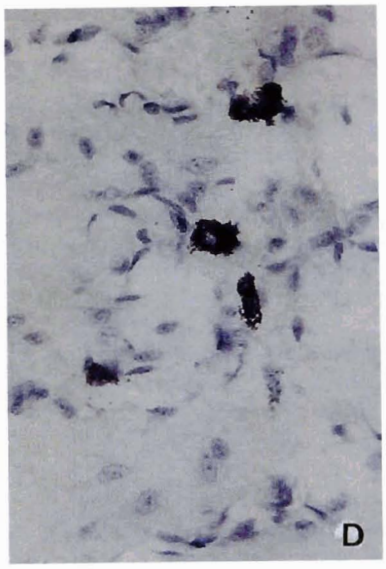
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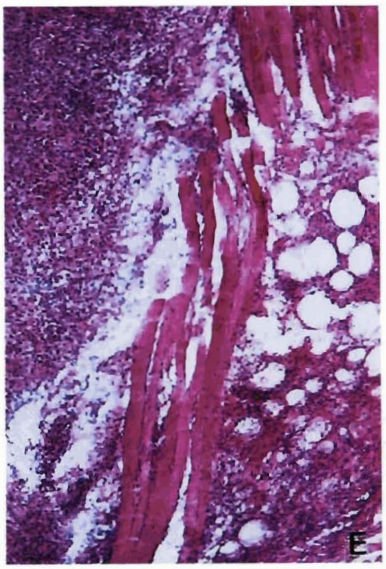
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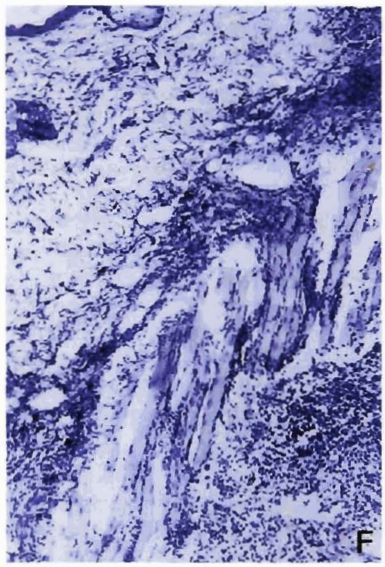
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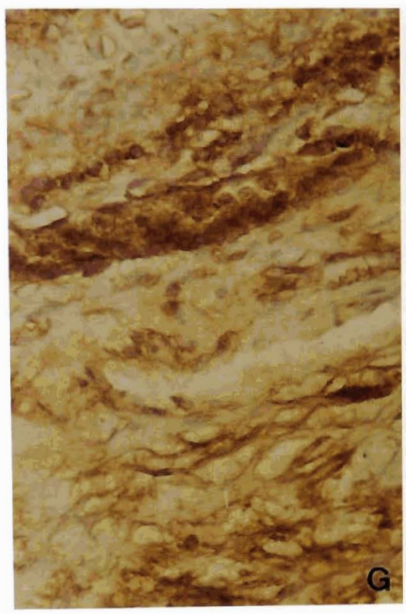
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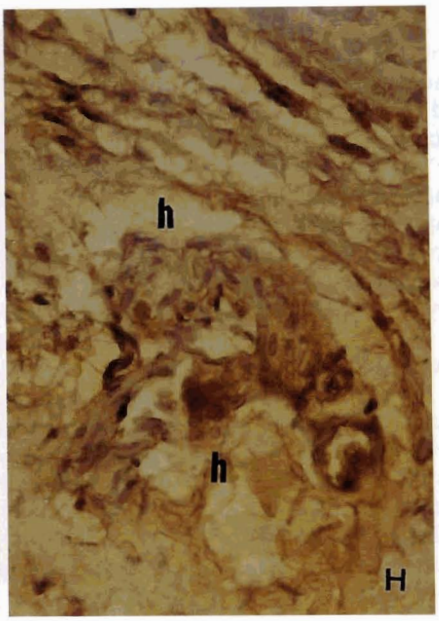
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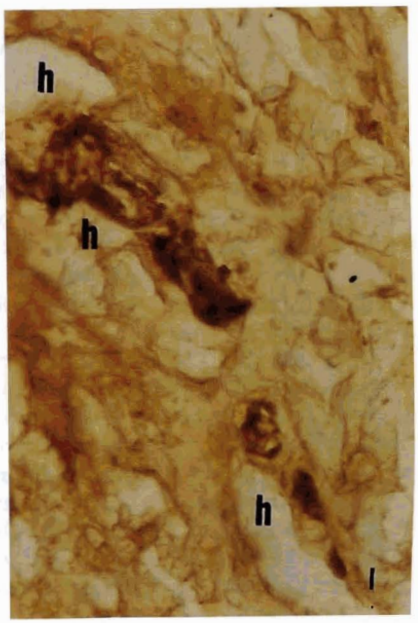
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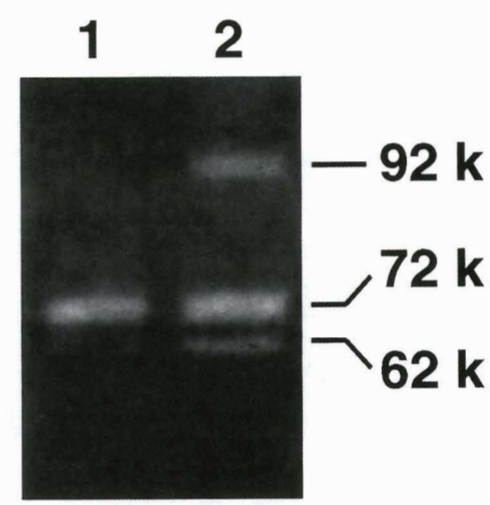

Fig. 3. Gelatin zymography. Lane 1: control cells. Lane 2: 3T3 cells stimulated for 24 h with porins (10 μg/ml).

form of 92kDa. These results confirmed the hypothesis that these metalloproteinase have an early and basic role in the inflammatory mechanism (Maillard et al., 1995).

A substantial difference is due to dissociation of type IV collagen distribution, which appears only in animals treated with porins and LPS. This result is particularly interesting, knowing that type IV collagen regulates interstitial fluid diffusion and vessel absorption and that any change causing fluid-electrolyte imbalance in the interstitial space improves the start of a more intense inflammatory reaction. In the first 24 hours, the type IV collagen does not appear to be substantially changed, at least morphologically. It is likely that during this time it undergoes structural and functional changes that cannot be demonstrated by immunohistochemistry. Around 48 hours and up to 72 hours the *hiatus* appears, as already described in the result section. The redistribution of type IV collagen coincides with rapid regression of the reaction process, which completely overlaps with the mild one seen in the controls after saline solution. The return to normal type IV collagen could be favored by the fact that the reaction described occurs in a well defined and limited area surrounded by healthy tissue, from which type IV collagen could came to reintegrate or substitute the fibers modified by porins/LPS. The reintegration of type IV collagen explains why the process does not become chronic and also the lack of sclero-hyalin scar tissue, as previously described by Baldi et al. (1994, 1996).

72 kDa and 92 kDa type IV collagenase (Liotta et al., 1981; Salo et al., 1983; Collier et al., 1988; Wihelm et al., 1989) are synthesized and secreted as a latent proenzyme 72 kDa form requiring the removal of an 80 amino acid-terminal domain resulting in the conversion to a 62 kDa activated form of the enzyme. In our experimental model of 3T3 mouse fibroblasts, porins (10 μg/ml), unlikely from LPS (10 μg/ml), can stimulate the release of 62 kDa and 92 kDa collagenases IV. A specific role of porins in the inflammation mechanisms through gelatinolytic activity is, thus, highlighted by our results. Moreover, some investigations showed that under specific experimental conditions (Tufano et al., 1989; Galdiero et al., 1990) porins are more active than LPS in inducing inflammations. The fibroblast burst seen at 24 hours in mouse skin inoculated with porins and at 72 hours in skin inoculated with LPS, reveals the influence of surface components in the fibrosis.

These findings can be clinically relevant because LPS and porins are released by Gram-negative bacteria during cell growth and lysis (Morrison et al., 1976). As a consequence, these components already interact with the host in the phases leading up to the infection by involving target cells, such as keratinocytes and fibroblasts, as well as immunocompetent cells, and significantly contribute to the pathogenesis of *P. aeruginosa* skin infections

Microorganisms and bacterial products, released at infected skin sites and wounds, could therefore induce cytokine release from keratinocytes (Walters et al., 1995), as well as from monocytes-macrophages that co-operate in the induction of the inflammatory process and on fibroblast stimulation by producing profibrotic mediators. Further studies using immunohistochemical methods are needed to shed light on the appearance, kinetics and cytokine-producing cell type induced by porins and LPS in the skin.

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