Inflammatory Effect of Intradermal Administration of Soluble Phospholipase A_2 in Rabbits

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Extracellular phospholipase A_2 (PLA_2) has been found in association with inflamed sites in experimental animals and in humans. The tissue effects of soluble PLA_2 have not been defined. We studied the development of inflammatory changes in rabbit skin subsequent to intradermal injection of active and inactivated venom and pancreatic PLA_2, over a broad concentration range. PLA_2, at concentrations encountered in human disease, caused acute inflammatory changes characterized grossly by erythema and induration, and histologically by inflammatory cell infiltration, vascular and tissue damage, and abscess formation. Extracellular PLA_2 may be considered as one of the pathogenic factors in inflammatory reaction. *J Invest Dermatol 86:380–383, 1986*

Phagocytic cells, both mononuclear and polymorphonuclear, respond to specific stimuli with the extracellular release of lysosomal enzyme phospholipase A_2 (PLA_2) [1–5]. Extracellular PLA_2 has been identified in experimental delayed-type hypersensitivity [4], allergic uveitis [6], glycogen-induced peritonitis [7], and endotoxin shock [8]. Inflammatory diseases in humans, such as acute pancreatitis [9], gram-negative septic shock [10], and rheumatoid arthritis [11,12], have been associated with high extracellular PLA_2 activity. It was proposed that lysosomal PLA_2, released into the extracellular milieu, cleaves phospholipids of contiguous plasma membranes, giving rise to strongly proinflammatory fatty acids and lysophosphatides [4,11].

In fact, high levels of PLA_2 were documented in lesion-free epidermis of patients with psoriasis [13,14]. The highest specific activity of PLA_2 was noted in patients with pustular psoriasis, lesions of which are characterized by massive polymorphonuclear leukocyte infiltration [14].

Enhanced PLA_2 activities in both rheumatoid arthritis and psoriasis may be the result of autoimmune deregulation of this enzyme [12,14], allowing for the increased production of proinflammatory products. However, the proinflammatory effect of PLA_2 has been demonstrated only indirectly. The intratracheal instillation of cobra venom factor contaminated with PLA_2 caused a marked pneumonitis in rabbits which was abolished by prior inactivation of the PLA_2 contaminant [15]. Soluble PLA_2 has been identified in rheumatoid synovial fluids at levels greater than 21,000 units/ml, and in serum in acute hemorrhagic pancreatitis and septic shock at levels greater than 60,000 units/ml (Pruzanski, Vadas, Fornasier, unpublished observations). The tissue effect of purified PLA_2, at concentrations comparable to those found in humans in rheumatoid arthritis and septic shock, have not been defined. Herein, we describe the acute inflammatory effects of exogenous, soluble PLA_2.

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Abbreviations:

*p*BPB: *p*-bromophenacyl bromide

PLA_2: phospholipase A_2

MATERIALS AND METHODS

New Zealand white rabbits, 2.5–3 kg, of either sex were used. *Naja naja* venom PLA_2 and porcine pancreatic PLA_2 (Sigma) were dissolved in sterile, nonpyrogenic 0.9% saline. Enzyme activity was determined as described previously [1]. One unit of PLA_2 activity is defined as the hydrolysis of 56 pmol of *Escherichia coli* phospholipid in 15 min at 37°C.

Dose-Response Relationship The backs of rabbits were shaved and injected intradermally with 0.1-ml aliquots of PLA_2 in concentrations ranging 1,500–60,000 units/0.1 ml. The diameter of resultant erythema and increase in skin thickness (induration) were measured immediately before sacrifice. Animals were sacrificed 24 h after injection and the injection sites were biopsied and fixed in buffered formalin.

Time-Course of Inflammation The time course of development of lesions after PLA_2 injection was investigated. PLA_2, in concentrations of either 1,500 units/0.1 ml or 60,000 units/0.1 ml, was injected at times ranging from 0–48 h before sacrifice. The diameters of erythema and skin thickness of lesions were determined prior to sacrifice, and biopsies of injection sites were submitted for histologic examination. Sterile saline (0.9%) served as the control.

Aliquots of venom PLA_2 (60,000 units/0.1 ml) were inactivated with the active site-directed histidine reagent, *p*-bromophenacyl bromide (*p*BPB) as described elsewhere [16]. Briefly, PLA_2 was incubated with 10^{-4} M *p*BPB for 60 min at 22°C. The reaction mixture was dialyzed overnight at 4°C against 0.15 M NaCl. This removed unreacted *p*BPB, and resulted in greater than 99% inactivation of PLA_2. Skin sites injected with inactivated PLA_2 were biopsied 3 h and 24 h after injection.

Histologic Examination Skin biopsies were coded (for single-blind interpretation) and stained with WHO stain (hematoxylin, phloxine, saffron, and Alcian green). The following inflammatory variables were assessed: edema, hemorrhage, histiocytic and polymorphonuclear infiltrate, muscle and fat cell necrosis, collagen degeneration, and inflammatory changes in epidermis, der-

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mal appendages, and blood vessels. A histologic grade of 0 to 4 was assigned in each category, depending upon the degree of change observed, with grade 0 being normal and grade 4 the most severe [17]. The histologic score represents the arithmetic sum of the grades assigned to each category. Results are expressed as mean ± SEM for n = 3 rabbits per experiment.

RESULTS

Intradermal injection of venom PLA_2 in rabbits produced a profound inflammatory reaction characterized grossly by erythema and induration, and microscopically by inflammatory cell infiltration, abscess formation, vascular changes, and muscle and fat necrosis. These changes were both dose- and time-dependent.

Dose-Response Relationship In 24-h-old lesions, skin sites injected with increasing concentrations of venom PLA_2 demonstrated increasing histologic scores up to 6,000 units PLA_2 per injection site with little further increase (Fig 1). The diameters of erythema and induration continued to increase directly with the dose. The cutaneous connective tissue remained intact. Histologically, beginning with the 3,000-U dose, vascular distention with progressive edema of dermal tissue was detectable. Edema and acute inflammatory changes increased with PLA_2 concentrations of up to 30,000 U/site with little progression in the 60,000-U injection sites. No thrombosis of vessels was noted but focal hemorrhages were present in response to the 2 highest doses. The lesions were characterized by necrosis of muscle with associated inflammation. Loss of integrity of individual muscle fibers was seen in sites injected with 1,500 U PLA_2. The outline of muscle fibers was irregular and fragments of muscle fibers were enveloped by polymorphonuclear leukocytes. As the enzyme concentrations increased, the extent of myonecrosis increased. At the highest dose used, almost all muscle fibers were necrotic with basophilia, fragmentation, and separation by a massive infiltration of acute inflammatory cells. Similarly, fatty necrosis was evident in sites injected with 1,500 U and prominent at concentrations above 6,000 U. None of these changes was seen in the control sections.

Time-Course of Inflammation At relatively low concentrations (1,500 U per site), venom PLA_2 caused only transient changes, expressed mainly in induration and erythema which showed a rapid onset with maximal change at 3 h after injection, followed by a gradual resolution (Fig 2*A*). Histologic score was also greatest early after injection, with maximal microscopic inflammatory changes in 6-h-old lesions. Microscopic changes were still evident 48 h after injection.

Higher concentrations of venom PLA_2 (60,000 units per site) evoked a brisk, severe inflammatory response (Fig 2*B*). Induration and erythema increased rapidly over the first 6 h after injection, and these changes were sustained over 48 h. Histologic score also increased rapidly, with the highest scores noted 6–12 h after injection of PLA_2.

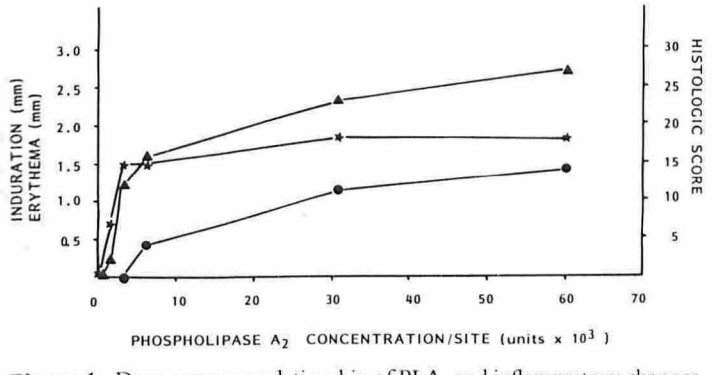


Figure 1. Dose-response relationship of PLA_2 and inflammatory changes in skin in rabbits. SE were less than 5% of the mean, n = 6. Induration, ●——●; erythema, ▲——▲; histologic score, ★——★.

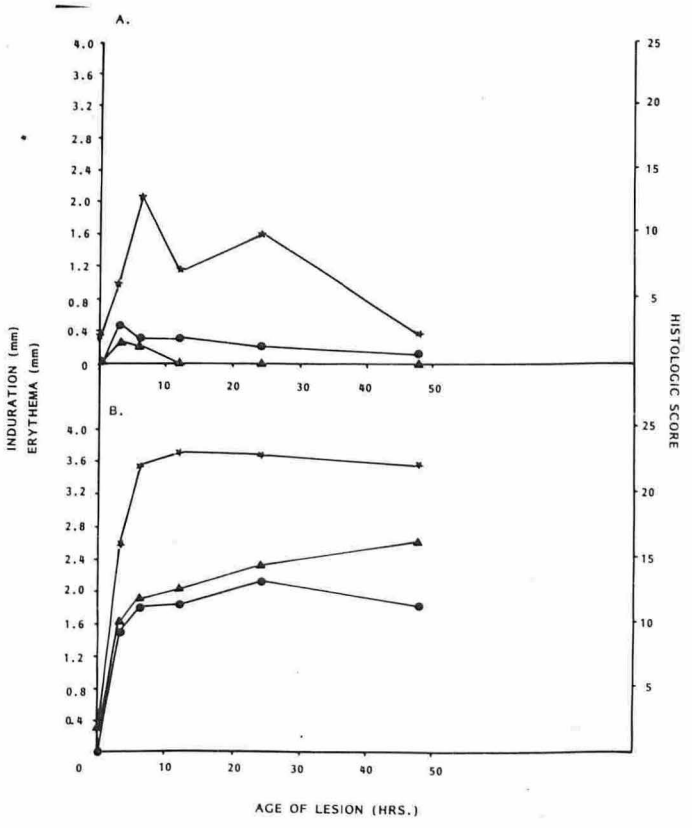
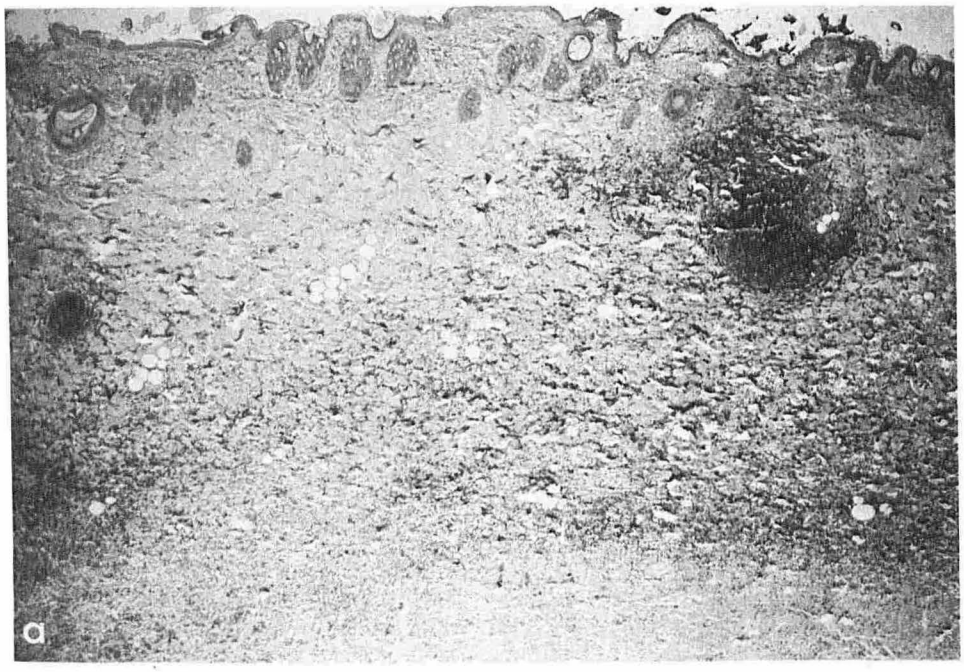


Figure 2. Time course of development of lesions after injection of PLA_2. *A*, Injection of 1,500 units/site. *B*, Injection of 60,000 units/site. SE were less than 5% of the mean, n = 6. Induration, ●——●; erythema, ▲——▲; histologic score, ★——★.

Microscopically, the lower dosage showed only vascular engorgement and distention at 3 h. By 6 h, edema and separation of collagen fibers were evident, with polymorphonuclear leukocyte infiltration leading to involvement of the dermal connective tissue on the superficial side of the muscle. Muscle necrosis was extensive, with polymorphonuclear leukocyte infiltration in the dermis and around dead muscle fibers. Massive necrosis of muscle and the related acute inflammation appeared to have reached their maximum by 3 h, in sites injected with 60,000 units PLA_2. A wider extent of inflammation in the dermis and up to the epidermis was maximal at 6 h and persisted unabated in the 24-h-old lesions (Fig 3). It appeared that injection of PLA_2 caused an accelerated inflammatory response (by 6 h), with little further progression with time (up to 24–48 h). The response was dose related, with the more severe reaction occurring at the higher dose. The lack of progression suggested again an immediate reaction to the injection, far ahead of the usual response to necrosis or injury in which the polymorphonuclear infiltrate is maximal 24 h after injury. This observation suggests a possible chemotactic effect along with an immediate change in vascular permeability, with endothelial swelling occurring in the latter stages (24 h) of the experiment.

The purity of both venom and pancreatic phospholipases was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Minor high-molecular-weight contaminants were identified in *Naja naja* venom PLA_2, while porcine pancreatic PLA_2 was electrophoretically homogeneous. Inactivation of venom PLA_2 with the active site-directed histidine reagent, *p*BPB, resulted in significant reduction in the resultant inflammatory response in lesions of 3 and 24 h. However, residual inflammatory changes involving dermal appendages were apparent after injection of the



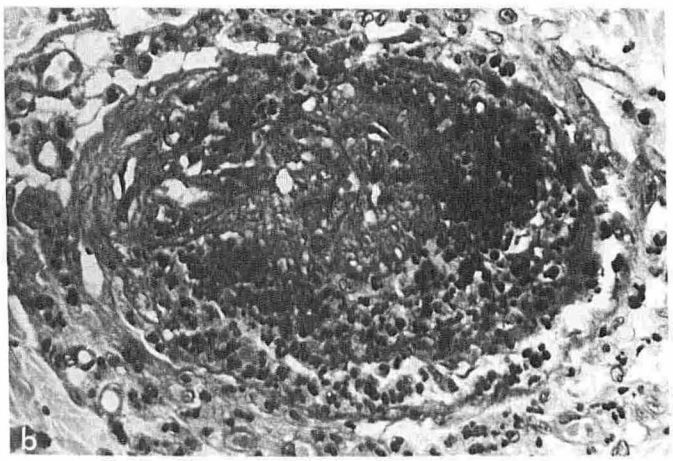


Figure 3. Photomicrograph of histologic appearance of site 24 h after injection of venom phospholipase A_2. *a*, Overview of skin with injection site at right upper corner associated with localized necrotizing inflammation. Diffuse inflammation extends throughout dermis. At left [enlarged in (*b*)] is a focus of necrotizing angiitis. × 25. *b*, Blood vessel with central core of prominent endothelial cells surrounded by acute necrotizing inflammation. Polymorphonuclear leukocytes infiltrating the connective tissue at the periphery of the blood vessel are representative of the acute inflammation present in the dermis. × 400.

molar equivalent of 60,000 units of inactivated PLA_2 protein. It is unlikely that low-molecular-weight proinflammatory components were inadvertently removed by dialysis, since the control preparation of venom PLA_2, incubated and dialysed without *p*BPB, retained full proinflammatory activity.

Pancreatic PLA_2 also evoked an inflammatory response whose dose dependence and kinetics were similar to those observed for venom PLA_2. However, while the response to venom PLA_2 was characterized by microabscess formation, myonecrosis, and edema, the response to pancreatic PLA_2 was comparatively attenuated, characterized only by an acute inflammatory infiltrate in the 3- to 6-h-old lesions, which had largely resolved by 24 h. The polymorphonuclear infiltration of dermal connective tissue and dermal appendages did not progress to microabscess formation. Blood vessels remained intact, as did muscle fibers. At the concentrations tested, there was no accompanying erythema or induration.

DISCUSSION

The intradermal injection of soluble venom PLA_2 at levels comparable to those found in synovial fluids aspirated from actively inflamed joints of patients with rheumatoid arthritis evoked a brisk inflammatory response. The severity of the resultant lesions was both time- and dose-dependent. The reactions were characterized by early polymorphonuclear cell infiltration, followed by a preponderance of histiocytes. Higher concentrations of PLA_2 caused frank abscess formation with central liquefaction. Blood vessels were engorged but intact in early lesions, corresponding with the grossly erythematous appearance in vivo. The kinetics of development of erythema resembled those for hyperemia induced by PLA_2, as quantitated with radiolabeled microspheres [2,18]. Later lesions were increasingly hemorrhagic, reflecting ongoing vascular damage, concomitant with the appearance of fat and striated muscle necrosis. The findings were similar to those observed in the lungs following exposure to exogenous venom PLA_2 [15].

Interestingly, although the inflammatory response evoked by pancreatic PLA_2 was definite, its was considerably less prominent than that caused by venom PLA_2. Unlike venom and epidermal PLA_2 activities [14], pancreatic PLA_2 is not inhibited in vitro by albumin [19]. Therefore, it is unlikely that serous exudate contributes to the reduced inflammatory response evoked by the pancreatic enzyme. The attenuated response is, however, consistent with the relative inability of pancreatic PLA_2 to cleave intact tissue as compared with the venom enzyme [20,21]. Pancreatic PLA_2 hydrolyzes phospholipids of intact tissue at only one-tenth the rate of venom PLA_2 in the absence of bile salts [20]. Significantly, the resultant inflammatory responses seen in our study were similar quantitatively and with respect to kinetics, and differed only in magnitude.

Hydrolysis of phospholipid substrate by PLA_2 results in the generation of 2 potent biologically active lipids, free fatty acids and lysophosphatides. These lipids are surface-active, cytotoxic, chemotactic for neutrophils, increase vascular permeability, and enhance phagocytosis [11,22]. The proinflammatory effects seen subsequent to injection of PLA_2 may reside in the activities of one or both reaction products of PLA_2. However, PLA_2 may induce inflammation independently of its enzyme activity, perhaps as a consequence of its net cationic charge [23]. In fact, several studies have demonstrated a dissociation between the enzymatic activity of PLA_2, and its pharmacologic actions [23,24].

Soluble PLA_2 is a powerful proinflammatory enzyme capable of inducing acute inflammatory changes upon intradermal injection. The association of high levels of endogenous PLA_2 with inflamed sites in vivo, and the proinflammatory effect of exog-

enous PLA_2 suggest that the release and accumulation of extracellular PLA_2 in sequestered sites, such as joints and skin, may contribute to the resultant inflammatory changes.

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