

# The Proinflammatory Effect of Intra-articular Injection of Soluble Human and Venom Phospholipase A<sub>2</sub>

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*The proinflammatory effects of intra-articular injection of purified phospholipase A<sub>2</sub> from snake venom and rheumatoid synovial fluid were studied in rats. Purified soluble phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in concentrations ranging from 1000 to 20,000 units/ml, was injected intra-articularly. Histologic parameters examined were cell and protein content of synovial fluid, subsynovial cellular infiltration, synovial lining cell hyperplasia, bone erosion, and peri-articular soft tissue infiltration. Single intra-articular injections of PLA<sub>2</sub> resulted in an acute inflammatory infiltrate of the subsynovium with maximal changes seen 2 to 6 hours after injection. Acute inflammatory changes were dose-dependent. Joints injected repeatedly at 24-hour intervals showed prominent synovial lining cell hyperplasia, maximal at 96 hours. Human synovial and snake venom PLA<sub>2</sub>s were equipotent at inducing both the acute and chronic articular changes. These changes were not seen in joints injected with inactivated PLA<sub>2</sub>. It is concluded that soluble PLA<sub>2</sub> causes time- and dose-dependent acute inflammatory changes after a single intra-articular injection and synovial lining cell hyperplasia in response to repeated exposure to PLA<sub>2</sub>. The experimental proliferative synovitis in this model may correlate with features of acutely inflamed joints bathed in synovial fluids containing high levels of PLA<sub>2</sub> in patients with rheumatoid arthritis. (Am J Pathol 1989, 134: 807-811)*

been elucidated but it has been proposed that PLA<sub>2</sub> serves a role in the generation of pro-inflammatory biologically active lipids that, when sequestered in the joint space, contribute to the characteristic articular changes associated with inflammatory arthritides.<sup>4</sup> Indeed, high levels of PLA<sub>2</sub> have been documented in the synovial fluids of inflamed joints in patients with rheumatoid and psoriatic arthritis and the inflammatory variant of osteoarthritis.<sup>4,5</sup> This PLA<sub>2</sub> has been characterized and purified.<sup>6,7</sup> It is a neutral-active, calcium-dependent soluble enzyme with a molecular weight of 14,000. In patients with rheumatoid arthritis (RA), serum levels of circulating PLA<sub>2</sub> correlated significantly with PLA<sub>2</sub> activity in synovial fluid<sup>4</sup> and with both clinical and laboratory indices of disease activity ( $P < 0.001$ ).<sup>8</sup>

We have demonstrated recently that the intradermal injection of purified, soluble PLA<sub>2</sub> in rabbits resulted in a time- and dose-dependent inflammatory reaction in the skin.<sup>9,10</sup> Inactivation of PLA<sub>2</sub> with the active site-directed histidine reagent p-bromophenacyl bromide (pBPPB) before injection resulted in attenuation of the subsequent inflammatory reaction. Because inflamed joints of patients with RA are bathed in exudate containing high levels of endogenous PLA<sub>2</sub> and because the tissue effects of intra-articular injections of PLA<sub>2</sub> have not been described, we undertook a study of the response to intra-articular injection of PLA<sub>2</sub> in levels approximating those found in rheumatoid synovial fluids.

## Materials and Methods

Two preparations of purified soluble PLA<sub>2</sub> were used in these experiments, one from snake venom, the other from rheumatoid synovial fluid. Naja naja venom PLA<sub>2</sub> was obtained from Sigma Chemical Co. (St. Louis, MO)

Soluble phospholipases A<sub>2</sub> are secreted extracellularly by various cells types including stimulated osteoblasts,<sup>1</sup> chondrocytes,<sup>2</sup> and synoviocytes.<sup>3</sup> The biologic functions of extracellular phospholipase A<sub>2</sub> (PLA<sub>2</sub>) have not yet

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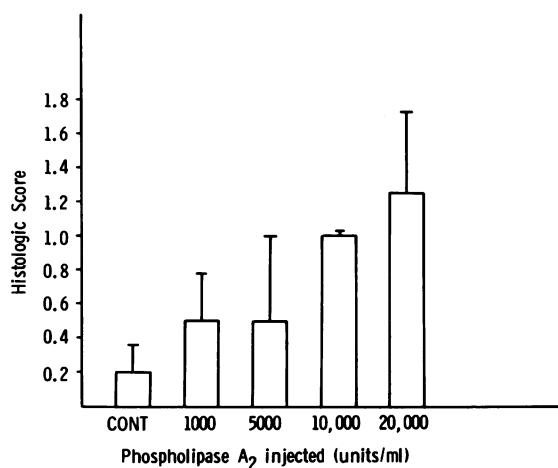


Figure 1. Dose-responsive relationship of venom phospholipase A<sub>2</sub> concentration and histologic score. Data are expressed as mean ± SEM for n = 4 animals per group. Scores were assessed 24 hours after a single injection of PLA<sub>2</sub>.

and was purified to homogeneity by the described method.<sup>11</sup> Human soluble PLA<sub>2</sub> was isolated from synovial fluid obtained by arthrocentesis from rheumatoid joints. This PLA<sub>2</sub> was purified 4500-fold as described recently.<sup>7</sup> Purified phospholipases A<sub>2</sub> were diluted in sterile nonpyrogenic saline to the desired concentration. The hind legs of 200-g male Wistar rats were shaved and prepared using routine aseptic technique. Solutions were injected in 100 µl aliquots obliquely through the patello-tibial ligament into the knee joint space. Enzyme preparations were mixed with a suspension of carbon particles that, on histologic examination, confirmed the accuracy of injection. Some preparations of PLA<sub>2</sub> were inactivated using 10<sup>-4</sup> M pBPB for 60 minutes at 22 C as described elsewhere.<sup>10</sup> Injection of sterile non-pyrogenic saline mixed with carbon particles in the contralateral joint served as the control.

### Dose-Response Relationship

Knee joints of rats were injected with 100 µl aliquots of PLA<sub>2</sub> in concentrations ranging from 1000 to 20,000 units/ml. One unit of PLA<sub>2</sub> activity is defined as the hydrolysis of 56 pmol (ie, 1% of total) of *Escherichia coli* substrate in 30 minutes at 37 C.<sup>6</sup> The concentration range tested was comparable with that of PLA<sub>2</sub> activity found in rheumatoid synovial fluids.<sup>4</sup> Animals were killed 24 hours after injection and joints were excised, fixed in buffered formalin, and stained with hematoxylin, phloxine, safranin O, and alcian green (WHO stain).

### Time Course of Inflammation

In a second group of rats, soluble PLA<sub>2</sub> in concentrations of 5000 units/ml or 20,000 units/ml was injected repeat-

edly at 24-hour intervals and rats were killed at 24, 48, 72, and 96 hours after 1, 2, 3, or 4 daily injections, respectively. Joints were excised and histologic changes were quantitated as described below. Serial injections of PLA<sub>2</sub> were intended to approximate steady state levels seen in RA.

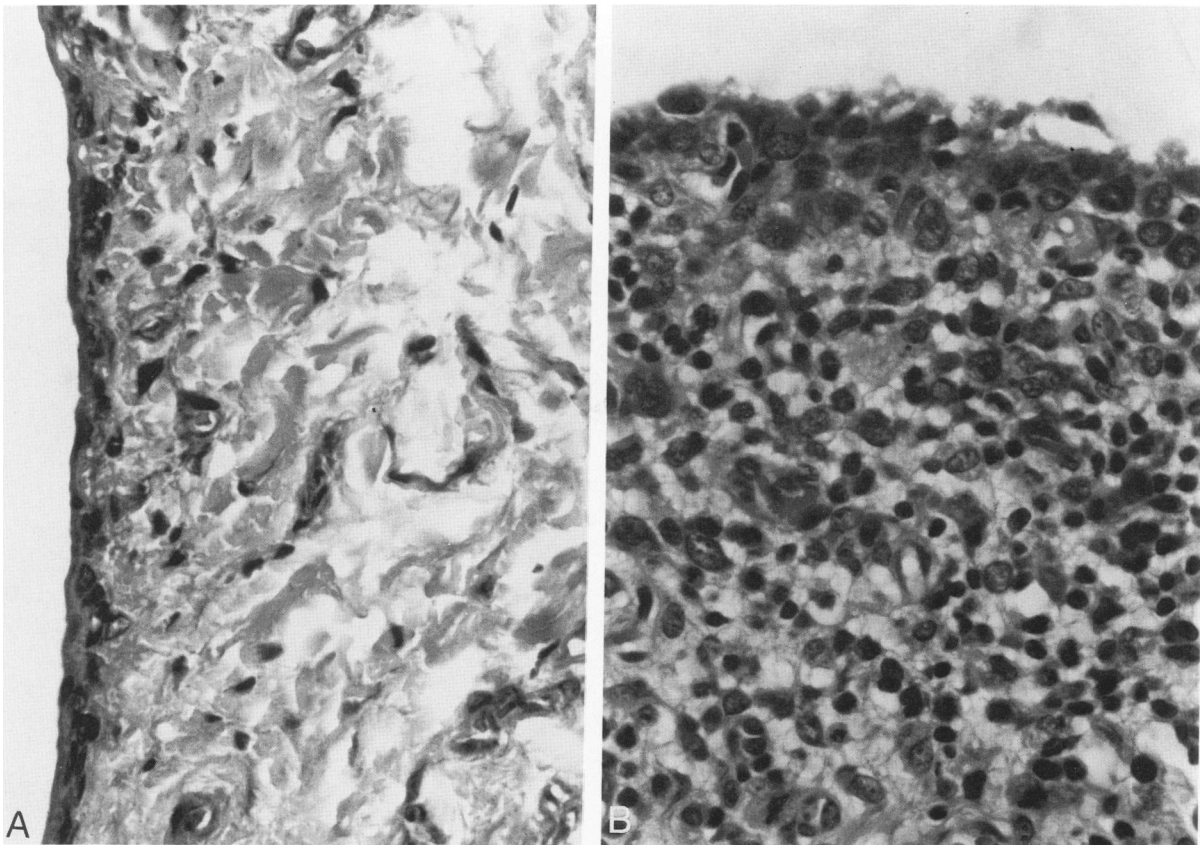
### Histologic Score

Inflammatory changes were scored in a blind fashion by a bone and joint pathologist (V.F.). Joints were fixed in 10% formal-saline and decalcified in 10% formic acid. After fixation, the tissues were stained with WHO stain. The severity of acute inflammatory changes was scored from 0 to 3 corresponding to normal, mild, moderate, or severe changes.<sup>12</sup> The following variables were assessed histologically: 1) cell and protein content of synovial fluid; 2) subsynovial cellular infiltration with polymorphonuclears; 3) synovial lining-cell hyperplasia; 4) bone erosion; and 5) periarticular soft-tissue cellular infiltration. The scores of each of the 5 inflammatory variables were added and the total divided by 3 to yield a maximum histologic score of 5. The scoring divided the synovium into surface exudate, lining cell layer, and subjacent connective tissue. Along the free surface, the presence and amount of fibrin and cellular emigration including polymorphonuclears, lymphocytes, and histiocytes were recorded. The lining cell layer was assessed by the presence or absence of a recognizable row of cuboidal cells and whether there was any evidence of layering or proliferation. In the supporting connective tissue, the presence of polymorphonuclears, lymphocytes, histiocytes, evidence of fibrinoid exudation, necrosis, vascularity, and fibroblastic proliferation was assessed. In sections that contained cartilage, the staining characteristics of the cartilage matrix and the presence of evidence of giant chondron formation, ie, proliferation of chondrocytes, were identified and quantitated. In sections that contained bone, particularly at the margins of the articular cartilage, the presence and rate of bone formation and remodelling were assessed with specific reference to periosteal resorption by osteoclasts.

Quantitation was done with 0 representing absence of the particular feature; 1 representing occasional presence of the feature requiring examination of multiple fields before it could be identified; 2 meaning the feature was present in at least one-half of the fields of examination; and 3 meaning the feature was present in all fields of examination and was a uniform observation of the entire area examined histologically.

### Statistical Analysis

All experiments were done in quadruplicate. The right knee joint served as the test joint whereas the left one



**Figure 2.** A: A control joint injected with sterile, non-pyrogenic saline 24 hours earlier. The lining cell layer is intact and almost devoid of any exudate or cellular infiltration. WHO stain; paraffin-embedded section; original magnification  $\times 400$ . B: Joint injected with venom phospholipase A<sub>2</sub> (20,000 units/ml) 24 hours earlier. Heavy infiltrate with lymphocytes and monocytes. Lining cell layer is composed of large cells which are irregularly layered and blend with the subjacent inflammatory infiltrate. WHO stain; paraffin-embedded section; original magnification  $\times 500$ .

served as the control in all animals. Results are expressed as mean  $\pm$  SE for N = 4 per group.

## Results

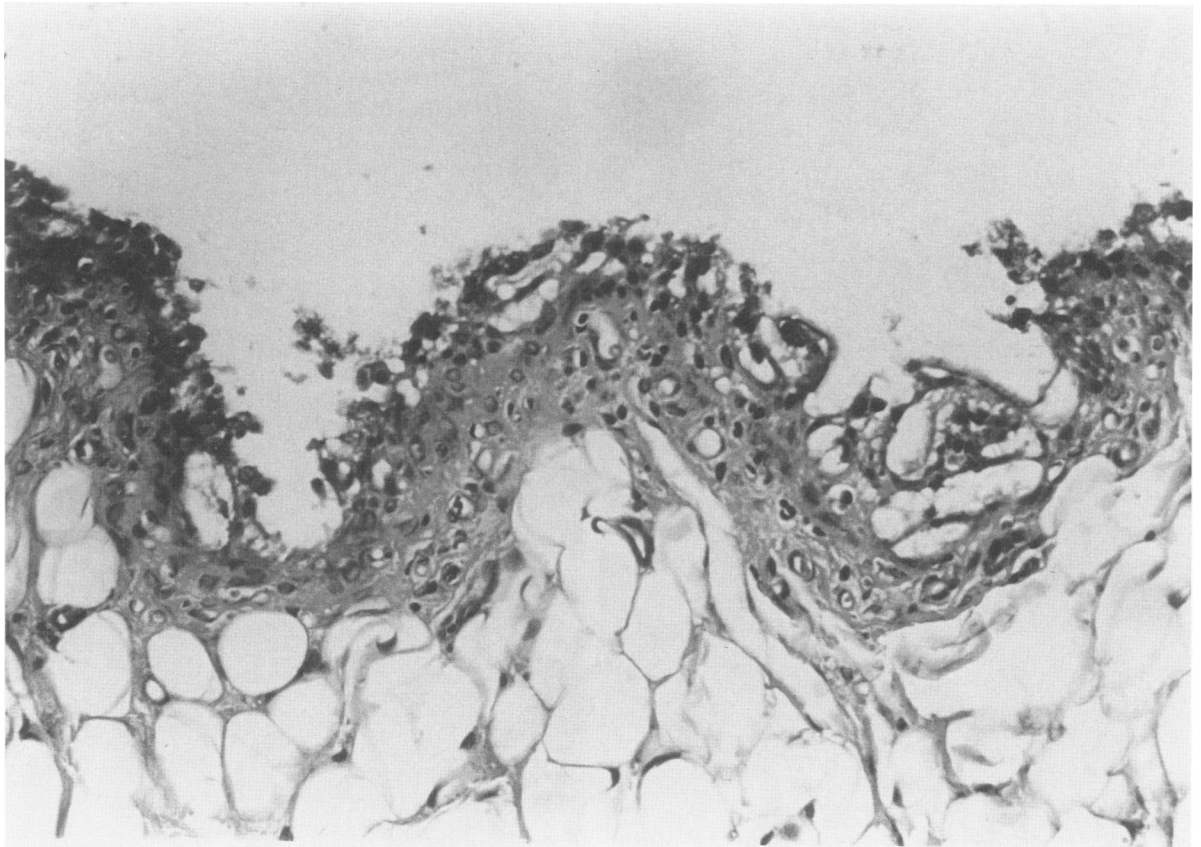
Soluble purified PLA<sub>2</sub> of both snake venom and human origin induced time- and dose-dependent inflammatory changes in the knee joint of the rat after a single injection. Injections of human and venom PLA<sub>2</sub> over the range of 1000 to 20,000 units/ml resulted in an incremental increase in the severity of the ensuing inflammatory reaction at 24 hours after injection as determined by the histologic score. The dose-response relationship of venom PLA<sub>2</sub> concentration and histologic score is shown in Figure 1. Similar changes were seen with human synovial fluid PLA<sub>2</sub>. Representative sections from control joints and PLA<sub>2</sub>-injected joints are shown for comparison in Figure 2.

Serial injections of sterile, nonpyrogenic saline or pB-PB-inactivated PLA<sub>2</sub> in control joints induced minimal dis-

cernible inflammatory changes. Exudate was present in small, localized amounts that appeared in the first 48 hours, peaked by 72 hours, and abated by the end of the experimental period at 96 hours. The synovial lining cell layer showed only minimal increase with enlargement of the cuboidal cells with focal layering seen only at 48 hours. The synovial supporting connective tissue contained small numbers of inflammatory cells that appeared by 12 hours, peaked at 72 hours and were greatly reduced by 96 hours. Basal modeling and remodeling activities were constant in bone and cartilage with no change in the cartilaginous matrix or in the bone turnover activity.

Serial injections of PLA<sub>2</sub> at a concentration of 5000 units/ml had no uniform histologically quantifiable effect on the production of exudate, on the proliferation of the lining cell layer, or on the presence of inflammation within the synovial connective tissue. Normal basal modeling activity was seen in both bone and cartilage.

In contrast, marked inflammatory and proliferative changes were evident after serial intra-articular injections of PLA<sub>2</sub> at a concentration of 20,000 units/ml. Substantial



**Figure 3.** Proliferative synovitis in a joint repeatedly injected with 20,000 units of venom phospholipase  $A_2$ . The joint was excised 24 hours after the fourth daily injection of  $PLA_2$ . Proliferation of synovial lining cell layer is seen with giant cells, a small number of inflammatory cells (mainly lymphocytes) and minimal exudate on surface. WHO stain; paraffin-embedded section; original magnification  $\times 400$ .

amounts of exudate were present along the synovial surface as early as 3 hours after injection, maximal at 72 hours, and still present at 96 hours. Intra-articular  $PLA_2$  induced synovial lining cell hypertrophy and proliferation within 12 hours of injection, to a depth of 7 to 10 layers at 48 hours. These changes persisted unabated until the termination of the experiment (Figure 3). Inflammatory infiltration of the subsynovium was evident by 3 hours after the first injection, with maximal cellular infiltration by 48 hours, followed by resolution of the acute inflammatory response by 96 hours. Both the bone and cartilage matrix showed constant modeling. The changes were indicative of an early exudative and inflammatory response to  $PLA_2$  that began to abate during the course of the experimental period. In contrast, the lining cell hypertrophy and proliferation appeared somewhat later and persisted through the duration of the experiment. The histologic scores representing inflammatory changes seen in  $PLA_2$ -injected joints, after subtraction of scores of corresponding controls, indicated that  $PLA_2$  induced a progressive and consistent proliferative synovitis.

## Discussion

Soluble  $PLA_2$  purified from rheumatoid synovial fluid preferentially cleaves membrane-associated phospholipid substrate,<sup>13</sup> resulting in production of pro-inflammatory lysophosphatides and free fatty acids, primarily arachidonic acid. In addition, exposure of various cells to  $PLA_2$  resulted in secretion of cellular products into the extracellular milieu suggesting that soluble  $PLA_2$  may act as a non-specific secretagogue.<sup>14</sup> Thus,  $PLA_2$ , by its enzymatic activity, may either directly or indirectly alter cellular function, induce the release of cellular or lipid mediators of inflammation, or disrupt cellular and tissue integrity leading to injury.

We now show that intra-articular injection of purified soluble  $PLA_2$  induces an acute inflammatory synovitis after a single injection and progressive synovial lining cell hyperplasia after repeat injections. Because exposure of both skin and joint tissues to exogenous  $PLA_2$ , in levels encountered in rheumatoid synovial fluid in humans, leads to characteristic acute inflammatory changes in both

structures,<sup>9,10</sup> and because joints of patients with RA are continually bathed in high levels of endogenous PLA<sub>2</sub>, the presence of the endogenous PLA<sub>2</sub> in the joint space may well contribute to the inflammatory changes associated with RA.

The histologic changes seen subsequent to intra-articular injection of PLA<sub>2</sub> in rats are similar qualitatively and temporally to those seen with other experimental models of inflammatory arthritis. After the intra-articular injection of carrageenan in rabbits, a pronounced subsynovial infiltrate was evident by 24 hours.<sup>15</sup> Marked proliferation of the synovial lining cell layer was seen 3 days after injection, and the intimal surface was grossly thickened with villous proliferation 1 to 2 weeks later. Similar histologic changes have been reported after exposure to arthritogenic strains of mycoplasma.<sup>16</sup> The synovial membrane changes seen secondary to exposure to carrageenan, mycoplasma, or phospholipase A<sub>2</sub> resemble changes of early RA.<sup>17,18</sup>

These data, in conjunction with those of previous studies, support our contention that extracellular PLA<sub>2</sub> in general, and synovial fluid PLA<sub>2</sub> in particular, is a pro-inflammatory enzyme, the intimate association of which with inflamed joints of patients with rheumatoid arthritis suggests a central pathogenic role in the development of destructive joint changes. The selective inhibition of synovial fluid PLA<sub>2</sub> activity may, therefore, be an efficacious therapeutic adjunct in the treatment of rheumatoid arthritis.

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