

INVESTIGATIVE REPORT

Intracutaneous Injection of Lysophosphatidylcholine Induces Skin Inflammation and Accumulation of Leukocytes

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Various cell stimuli act through activation of phospholipase A₂, which hydrolyses fatty acids from membrane phospholipids, resulting in the formation of fatty acids and lysophospholipids. One of the lysophospholipid classes, lysophosphatidylcholine, is a chemoattractant for monocytes and T-lymphocytes and induces the expression of adhesion molecules on cultured endothelial cells. The purpose of the present study was to determine whether lysophosphatidylcholine possesses proinflammatory properties *in vivo*. This was assessed clinically and histologically by intracutaneous injection of 200–800 nmol lysophosphatidylcholine in healthy volunteers. Lysophosphatidylcholine elicited a dose- and time-dependent local erythema and oedema. The erythema disappeared within 4 h, while the induration lasted for up to 48 h. HE-stained biopsies taken after 24 h showed a leukocytoclastic vasculitis in 2 of the 6 subjects. Microscopic examination of immunohistochemically stained biopsies taken 24 h after the injection showed a significant increase in the number of T-lymphocytes, monocytes and neutrophils, whereas the number of Langerhans' cells was unchanged after lysophosphatidylcholine injection. In addition, the number of intercellular cell adhesion molecule-1 and -3-positive cells was increased approximately 3-fold after injection of lysophosphatidylcholine. In conclusion, the phospholipase A₂ hydrolysis product lysophosphatidylcholine can induce erythema, oedema, a mixed cellular infiltrate and the expression of adhesion molecules. **Key words:** immunohistochemistry; phospholipase A₂.

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Eicosanoids are oxidative products of arachidonic acid liberated from cellular phospholipids by the action of phospholipase A₂ (PLA₂). While eicosanoids such as prostaglandin D₂ (PGD₂), 12-hydroxyeicosatetraenoic acid (12-HETE) and in particular leukotriene B₄ (LTB₄) have proinflammatory properties *in vitro* (1, 2) as well as *in vivo* (3–6), our knowledge of the actions of the PLA₂-generated hydrolysis products of phospholipids (lysophospholipids) is limited. *In vitro*, one of the major lysophospholipid classes, lysophosphatidylcholine (lysoPC), exhibits chemotactic activity towards monocytes (7) and T-lymphocytes (8). For T-lymphocytes, the length and number of double bonds influence the chemotactic ability of lysoPC compounds. It seems that compounds with a fatty acid length of 16 C-atoms without double bonds (lysoPC; 1-palmitoyl) are the most

effective chemoattractants (8). In addition, lysoPC induces the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) in cultured rabbit and human arterial endothelial cells (9). Because the migration of T-lymphocytes into the skin is modulated by adhesion molecules and chemoattractants, lysoPC might play a role in the induction and maintenance of skin inflammation. In support of this idea we recently found increased levels of lysoPC in lesional psoriatic skin (10).

The purpose of this study was to investigate the clinical and histological changes after intracutaneous injection of lysoPC in normal human skin.

MATERIALS AND METHODS

Monoclonal antibodies and reagents

LysoPC, 1-palmitoyl (99%), pyrogen-free and without organic solvents, and PLA₂ from the manufacturing procedure was purchased from Larodan (Malmö, Sweden). Monoclonal antibodies to cell surface markers and Mayers haematoxylin/eosin were purchased from DAKO, Denmark (Table I).

Toluidine blue, levamisol and 4-chloro-2-methylbenzene diazonium salt (fast red TR-salt) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Biotinylated-goat anti-mouse immunoglobulin G (IgG) and streptavidine alkaline phosphatase were obtained from Amersham Pharmacia Biotech, Horsholm, UK. Normal goat serum was purchased from Life Technologies, Roskilde, Denmark.

Intracutaneous injections and skin biopsies

Normal healthy volunteers without a history of skin disease and taking no medication were recruited for the study. LysoPC in different doses (200 nmol, 400 nmol and 800 nmol) was dissolved in sterile isotonic saline (100 µl). A volume of 100 µl lysoPC dilutions (2×10^{-3} M, 4×10^{-3} M and 8×10^{-3} M) and saline alone were injected intradermally at the volar aspect of the forearm. The 4 injection sites were separated by 2 cm. Clinical reactions were recorded and the reactions were quantitated by measuring the greatest diameter of the oedema and erythema with a ruler placed over well-defined margins. If the erythema/oedema reaction was irregular, the measurement was taken so that satellite lesions could be avoided. Measurements were performed at 10, 30, 60, 90 and 120 min after the injection. Induration was assessed by physical examination at 4, 6, 8 and 24 h.

For histological evaluation, 4-mm punch biopsies were taken under lidocaine anaesthesia from the lysoPC injected site showing the strongest clinical changes and from the control site 24 h after the injection. The biopsies were cut in halves. One half was fixed in formal saline, embedded in paraffin and stained with haematoxylin and eosin. The other half was snap-frozen in liquid nitrogen together with the 2-mm punch biopsy taken from the control site and stored at

Table I. Monoclonal antibodies used to detect cell surface markers

Cell type or antigen	CD No.	Catalogue No.	Working dilution
Langerhans' cells	CD1a	M721	1:75
T-cells	CD3	M756	1:50
T-cells	CD4	M716	1:50
T-cells	CD8	M707	1:50
Monocytes	CD14	H1610	1:50
Neutrophils	CD15	M0733	1:50
LFA-1	CD18	M783	1:100
B-cells	CD20	M755	1:50
IL-2R	CD25	M731	1:50
ICAM-3	CD50	M7076	1:50
ICAM-1	CD54	M7063	1:50
Mouse Ig		E433	1:100

IL-2R: Interleukin-2R; ICAM: intercellular cell adhesion molecule; LFA-1: Leukocyte functional antigen 1

–80°C until sectioning and immunohistochemical staining. In order to evaluate the time-course of the reaction, biopsies were taken after 3 h and 24 h from a lysoPC (400 nmol) injection site and a control site in 2 volunteers and then processed as described above. In addition, the biopsies taken after 3 h and 24 h were stained with toluidine blue.

To assess the contribution of histamine release to the lysoPC-induced skin response, another series of lysoPC injections was given to the 6 subjects 2 h after oral administration of the antihistamine acrivastin 16 mg. Oedema and erythema were measured as described above. The study was approved by the local ethics committee according to the Helsinki II declaration.

HE staining

The biopsies were fixed for 24 h in 3.6% formaldehyde. The fixed tissue was embedded in paraffin, and 4 µm-thick sections were prepared and stained with haematoxylin 1%. This was followed by counterstaining with eosin 1%.

Immunohistochemistry

The distribution of cell markers was evaluated in sections of each biopsy. Mouse antibodies were detected by the streptavidine–alkaline phosphatase technique.

In short, 6 µm-thick cryostat sections from each biopsy were cut and fixed in pure acetone for 8 min. This was followed by blocking for 20 min in 10% v/v normal goat serum triphosphate buffered saline, pH 7.4. Sections were incubated with primary mouse antibodies in 1% v/v normal goat serum in Tris buffered saline overnight at 4°C followed by a biotinylated-goat anti-mouse IgG 1:100 and detected by streptavidine–alkaline phosphatase 1:300. The staining was developed with fast red-TR salt. Levamisole was added to inhibit endogenous phosphatases. The samples were allowed to incubate for 5 min before they were rinsed in Tris buffered saline. Counterstaining was done in Mayer's haematoxylin. Positive staining was visualized by red coloration.

Specific staining was verified by substitution of the primary antibodies with normal mouse IgG at the same concentration and by further dilution of the primary antibody, both resulting in negative staining.

The biopsies were examined with a Leica Dialux 20EB microscope at magnifications up to ×400. The number of infiltrating cells in epidermis and dermis were counted manually per total biopsy area (1.67 mm²) and expressed as number of infiltrating cells per total biopsy area (Table II).

Table II. Number of cells stained positive in epidermis and dermis per total biopsy area. Biopsies were taken after 24 h. Values are mean ± SEM (n=6)

Treatment	CD number	No. of positive cells	p-values
LysoPC	CD-1a	150.5 ± 31.6	0.69
Control	CD-1a	145.2 ± 30.8	
LysoPC	CD-3	99.2 ± 30.4	0.046
Control	CD-3	36.2 ± 9.5	
LysoPC	CD-4	89.7 ± 31.0	0.12
Control	CD-4	24.5 ± 8.1	
LysoPC	CD-8	13.0 ± 5.3	0.46
Control	CD-8	7.8 ± 2.5	
LysoPC	CD-14	26.5 ± 8.4	0.046
Control	CD-14	5.0 ± 2.0	
LysoPC	CD-15	170.3 ± 32	0.028
Control	CD-15	6.0 ± 3.3	
LysoPC	CD-18	103.8 ± 37.9	0.068
Control	CD-18	41.3 ± 13.9	
LysoPC	CD-20	13.5 ± 2.5	0.046
Control	CD-20	3.3 ± 2.2	
LysoPC	CD-25	15.5 ± 3.9	0.016
Control	CD-25	5.3 ± 1.7	
LysoPC	CD-50	97.8 ± 16.3	0.028
Control	CD-50	33.7 ± 7.1	
LysoPC	CD-54	36.2 ± 7.7	0.028
Control	CD-54	14.2 ± 3.1	

Statistical analysis

If the data showed a parametric distribution, they were analysed using a paired *t*-test. If not, Wilcoxon's test for matched samples was used. A *p*-value below 0.05 (2-tailed) was considered significant.

RESULTS

Clinical evaluation

In all subjects, injection of saline produced a slight weal and flare reaction, which resolved completely after 10 min. At the lysoPC injection sites (200 nmol, 400 nmol and 800 nmol) erythema and oedema appeared after 5 min. The size of the erythema and oedema was dose-dependent. In addition, the response to the highest dose of lysoPC persisted for a longer period of time (Fig. 1).

The erythematous reaction was maximal at 10–30 min and disappeared within 2–4 h. During the observation period, the erythema became less intense, and after 4 h no erythema was left. The size of the oedema increased until 60 min after the injection. Thereafter it disappeared gradually and was replaced after 4–6 h by an induration (Fig. 2). This induration was still present at 24 h in 4 of 6 subjects and had disappeared completely after 48 h. Transient burning was noted after the lysoPC injections, especially after the highest dose. Two of the 6 subjects noted a loss of cutaneous sensation extending 2 cm beyond the injected area (800 nmol), which lasted for 6 h.

To assess whether histamine release contributed to the lysoPC-induced skin reaction, the antihistamine acrivastin was given perorally 2 h before injection of a new dose of lysoPC (400 nmol). In 4 of the 6 subjects, acrivastin diminished the diameter of the erythematous reaction by 50%. In the remaining 2 it was diminished, but to a smaller

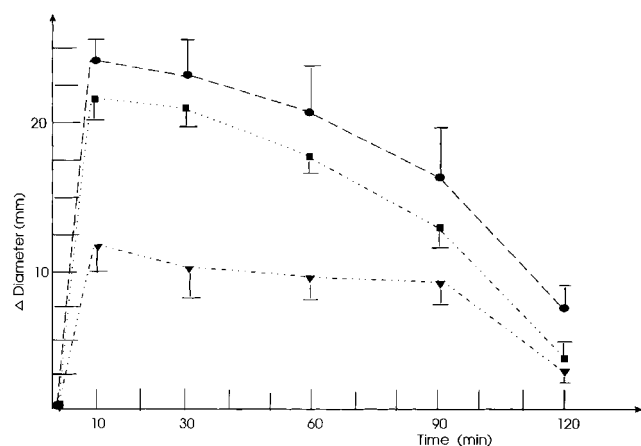


Fig. 1. Erythema occurring after intracutaneous injection of lysoPC 200 nmol (▼), 400 nmol (■) and 800 nmol (●). The control values have been subtracted from the greatest diameter of the lesion in each subject. Each point represents mean $\pm$ SEM of 6 subjects.

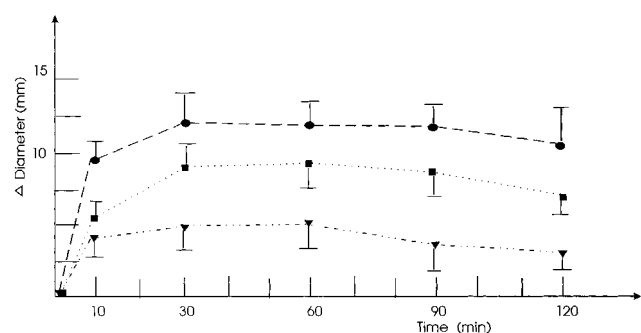


Fig. 2. Oedema occurring after intracutaneous injection of lysoPC 200 nmol (▼), 400 nmol (■) and 800 nmol (●). The control values have been subtracted from the greatest diameter of the lesion in each subject. Each point represents mean $\pm$ SEM of 6 subjects.

degree. When acrivastin was administered, the erythema disappeared within 3 h. The antihistamine had no significant effect on the size of the oedema (data not shown).

Histological evaluation

Skin biopsy specimens obtained 24 h after injection of lysoPC were taken from the saline-injected site and the lysoPC-injected site showing the most pronounced induration (800 nmol in 4 cases, 400 nmol in 2 cases).

Histological examination of the lesion evident at 24 h (HE stain) revealed a dermal cellular infiltrate around the venules and capillaries. The cellular infiltrate comprised principally of neutrophils in 4 subjects (Fig. 3a). In the remaining 2 it consisted mainly of lymphocytes (Fig. 3b). Oedema of moderate degree and dilated vessels were observed in all the volunteers. Four volunteers had vasculitis with endothelial swelling and a cellular infiltrate dominated by neutrophils. In addition, 2 of these volunteers had nuclear dust in relation to the cellular infiltrate, making it a leukocytoclastic vasculitis. One of the remaining 2 volunteers reacted with a superficial vasculitis dominated by lymphocytes. The other had a cellular infiltrate, but lacked vascular changes.

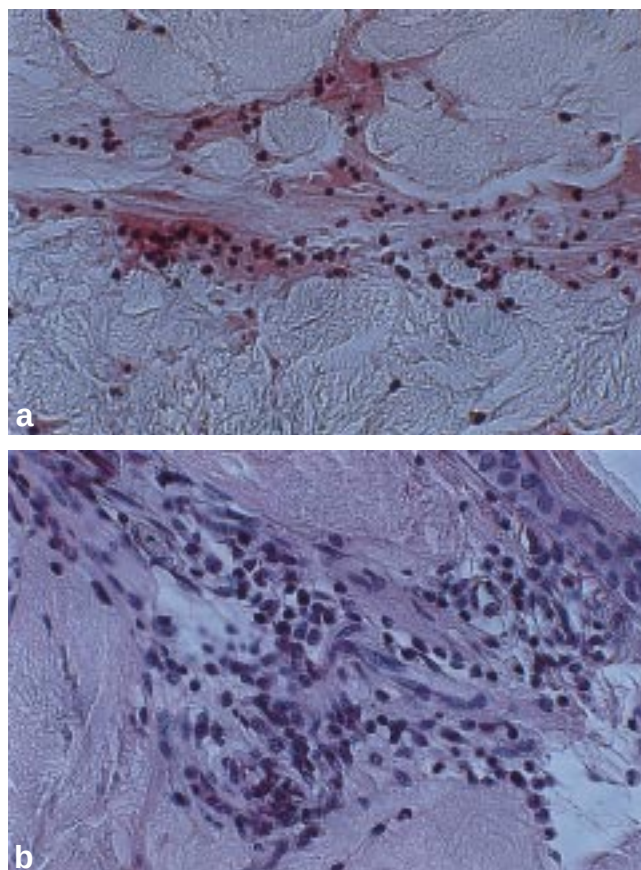


Fig. 3. Dermal changes at 24 h after intracutaneous injection of a) typical reaction pattern in 4 of 6 subjects showing vasculitis with a cellular infiltrate consisting mainly of neutrophils, and b) 2 of 6 subjects showing perivascularitis and infiltration with lymphocytes (4 μ m paraffin-embedded specimen. HE stain $\times$ 350).

In the biopsies stained with toluidine blue, we observed no secretion of mast cells after 3 and 24 h (data not shown).

Compared with saline, immunohistochemical examination of lysoPC-injected skin showed a significant increase in neutrophils (CD-15-positive) (Fig. 4), T-lymphocytes (CD-3-positive) (Fig. 5), monocytes (CD-14-positive) and B-lymphocytes (CD-20-positive). The majority of these cells was localized in dermis. The B-lymphocytes were distributed diffusely in dermis. Monocytes and T-lymphocytes were predominantly found in perivascular cell aggregates. The T-lymphocytes in the lysoPC-injected biopsies included CD4-positive and activated interleukin-2 receptor-positive (IL-2R (CD-25)) cells, but their number was not significantly increased compared to control specimens. Neither did the activation marker for leukocyte functional antigen-1 (LFA-1) CCD-18 positive show an increased expression in the lysoPC-injected specimens.

Numerous neutrophils were present both in relation to the cell aggregates and throughout dermis. In contrast, no difference in the number of epidermal Langerhans' cells (CD-1a) was found between controls and lysoPC-injected specimens. In addition, ICAM-1 (CD-54-positive) and ICAM-3 (CD-50-positive) were expressed in increased amounts when lysoPC had been injected. Cells expressing these adhesion molecules were distributed diffusely throughout the specimen

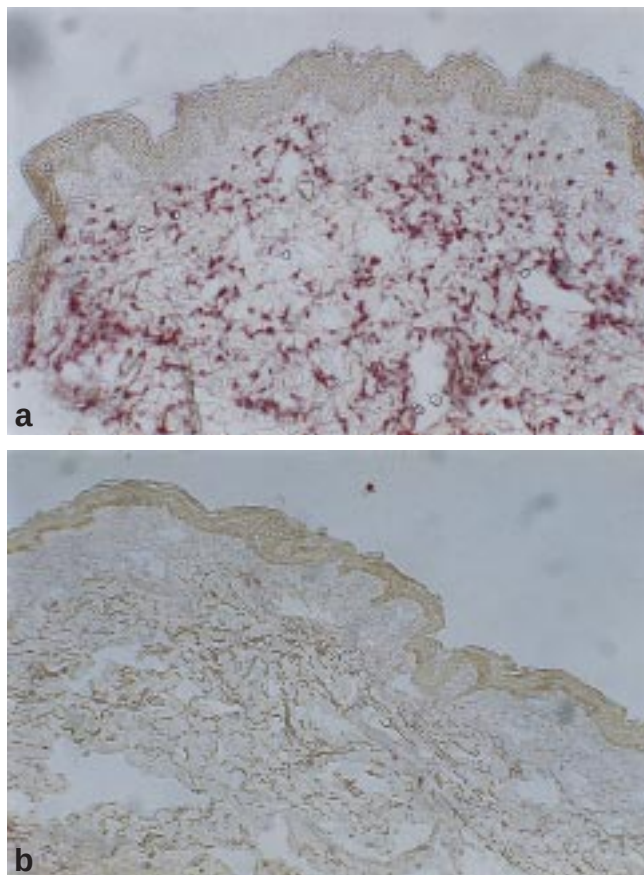


Fig. 4. CD-15-positive cells 24 h after injection of (a) lysophosphatidylcholine (400 nmol) and (b) saline (6 μ m cryostat section; stained with fast-TR salt; $\times 125$).

as well as in relation to vessels. Most were found in the dermis.

The clinical and histological changes paralleled each other in such a way that the subjects with the longest duration of oedema and induration also had the most pronounced inflammatory changes (data not shown). The degree of erythema (duration, maximal diameter) and the histological changes were not correlated in any way (data not shown).

To determine if the inflammation progressed more slowly in the volunteers with neutrophil predominance after 24 h, biopsies were taken at both 3 and 24 h in 2 volunteers injected with lysoPC (400 nmol). The person who had lymphocyte predominance at 24 h had very few neutrophils (CD-15-positive) at 3 h, and the person with neutrophil predominance at 24 h had virtually no cells at 3 h.

DISCUSSION

Our results suggest that lysoPC is capable of inducing an inflammatory reaction after intracutaneous injection. The size of oedema was dependent on the dose of lysoPC. This suggests that the applied lysoPC doses were not sufficient to saturate the processes, which led to increased venular permeability. Results obtained with antihistamine pretreatment indicate that histamine release partially explains the development of erythema. Histologically, only 2 subjects reacted with a leukocytoclastic vasculitis in response to

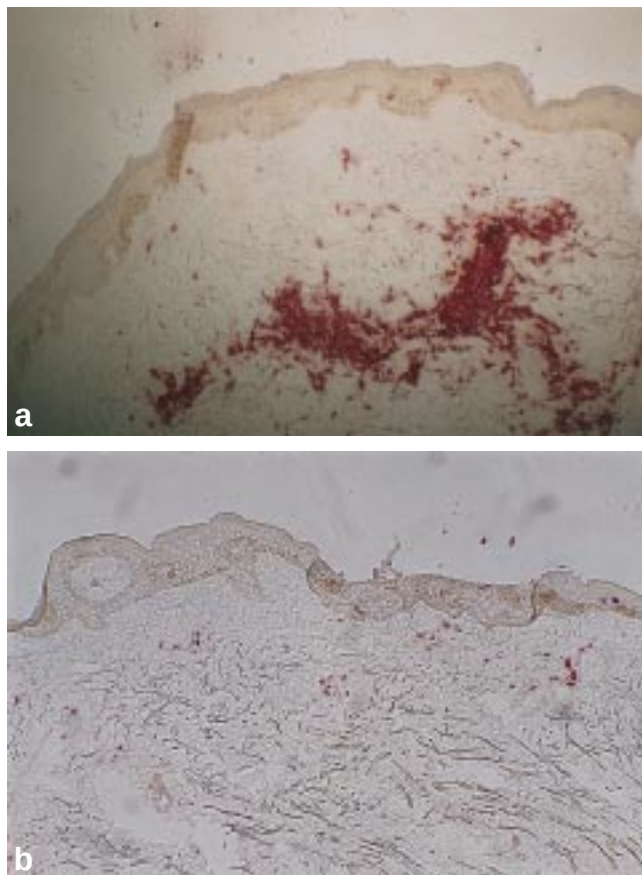


Fig. 5. CD-3 positive cells 24 h after injection of (a) lysophosphatidylcholine (400 nmol) and (b) saline (6 μ m cryostat section; stained with fast-TR salt; $\times 125$).

lysoPC injection. This is in accordance with the clinical findings showing an urticarial reaction lasting for more than 24 h and resembling urticarial vasculitis.

Regarding the time-course of the lysoPC-induced inflammation, we observed that the person with lymphocyte predominance at 24 h had very few neutrophils at 3 h, and the person with neutrophil predominance at 24 h had virtually no cells at 3 h. If it is assumed that the lysoPC-induced inflammation progresses through an early phase with neutrophils as the dominating cell type, to a later phase where the lymphocytes dominate, these data could be interpreted to result from differences in cell kinetics in the 2 individuals. Alternatively, the differences in composition of the cellular infiltrate could be explained by secretion of different chemokines or eicosanoids in different subjects, as lysoPC has been reported to stimulate secretion of chemokines (11, 12) and enhance the liberation of arachidonic acid *in vitro* (13).

In vitro lysoPC leads to degranulation and secretion of mast cells (14). The fact that we observed no secretion of mast cells in biopsies taken after 3 or 24 h (data not shown) does not contradict this hypothesis because the mast cells probably respond shortly after lysoPC injection, and this is difficult to observe in biopsy specimens.

The expression of the adhesion molecules ICAM-1 and ICAM-3 was stimulated by lysoPC. Results from studies of human endothelial cells incubated with lysoPC support the

observation that lysoPC can upregulate ICAM-1 (9). Regarding ICAM-3, it is worth noticing that no substance has been able to upregulate expression of ICAM-3 *in vitro*. Cofactors of importance for the upregulation are probably present *in vivo* because incubation of leukocytes with lysoPC failed to upregulate ICAM-3, as analysed by flowcytometry (unpublished results).

In conclusion, intracutaneous injection of lysoPC produces inflammation, which can be detected both clinically and histologically. If present in biologically active amounts, the naturally occurring phospholipid lysoPC might be one of the mediators participating in the generation and maintenance of the inflammatory process *in vivo*.

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