

EJP 00687

## Effect on mouse peritoneal macrophages of orally administered very high dilutions of silica

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Received 26 June 1986, revised MS received 26 September 1986, accepted 6 January 1987

The activity of very high dilutions of silica, a substance cytotoxic for macrophages, was tested on the synthesis by mouse peritoneal macrophages of the inflammatory ether-lipid paf-acether and its precursor lyso paf-acether. C<sub>57</sub>Bl<sub>6</sub> female mice received for 25 days either  $1.66 \times 10^{-11}$  M silica (11 sil) or  $1.66 \times 10^{-19}$  M (19 sil) (final concentration) in the tap-water they were given to drink while control mice remained untreated. Isolated macrophages from mice treated with 11 sil produced 44.2 and 30.8% more paf-acether than cells from untreated mice in the presence of 50 and 200  $\mu$ g zymosan (Z)/ml respectively. When 19 sil was given to the mice, the respective increases were 67.5 and 38%. In an experiment with a blind design, the mice were either untreated or received 19 sil or saline submitted to the same dilution procedure (19 sal). After administration of 19 sil, paf-acether synthesis was 55.5 and 33.5% higher upon stimulation with 50 and 200  $\mu$ g Z/ml, respectively, than in the 19 sal group. In a third blind experiment, macrophages from mice that received 19 sil formed 61.3 and 28.6% more paf-acether upon stimulation with 50 and 200  $\mu$ g Z/ml respectively, as compared to mice receiving 19 sal or lactose submitted to the same dilution procedure (19 lac). There was no difference between the 19 sal and the 19 lac groups. The differences between control and silica-treated mice were highly statistically significant in all experiments. There was no effect on the synthesis of lyso paf-acether. These results demonstrate clear ex vivo cellular effect of high dilutions of silica, that cannot be explained in our present state of knowledge.

Peritoneal macrophages; Silica; Paf-acether; Inflammation

### 1. Introduction

Since macrophages are implicated in the immune response and inflammatory reactions, the modulation of their functions is of major interest (Cohn, 1978; Karnovsky and Lazdins, 1978). We and others have extensively studied the influence of the local microenvironment on lipid mediator synthesis by murine macrophages (Drapier et al., 1983; Mencia-Huerta and Benveniste, 1979; 1981; Roubin et al., 1982a,b; Rouzer et al., 1980a,b; 1982). The cytotoxic effect of silica is well known and its ingestion by macrophages is followed by

cell death (Kessel et al., 1963; Allison et al., 1966) and impaired resistance to bacteria (O'Brien et al., 1979). However, our preliminary experiments demonstrated a stimulating effect on the production of lipid mediators by macrophages when low concentrations of silica were administered in vitro to the cells. This effect was paradoxical in that it increased in parallel with the dilution of the compound. We thus systematically investigated in the mouse the effect of the administration per os of very high dilutions of silica on the synthesis of paf-acether (platelet-activating factor), a well-defined monokine (Roubin et al., 1983). We show for the first time that the ex vivo biosynthesis of paf-acether by mouse peritoneal macrophages was stimulated after ingestion of drinking tap-water

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containing hardly more than a few molecules of silica per milliliter.

## 2. Materials and methods

### 2.1. Silica dilutions

Silicon dioxide (Sigma, St. Louis, MO, USA), being insoluble in water, was first thoroughly mixed with lactose (1:99, w/w) by using a grinder. The mixture was mixed once more with lactose (1:99, w/w) and the latter mixture was then serially diluted 100-fold in saline 3 and 7 times by thorough mixing with a Vortex, the final dilutions corresponding to  $1.66 \times 10^{-9}$  M silica and  $1.66 \times 10^{-17}$  M, respectively. The controls were (1) saline diluted, as was silica, in lactose to explore the possible role of silica transferred from the glass walls of tubes into water during the shaking procedure and (2) lactose prepared exactly as above except that silicon dioxide was omitted. The dilutions used in the second and third series of experiments (see below) received an arbitrary code number given by an investigator foreign to the experiment and to INSERM U.200. The code was broken only at the end of the experiments.

### 2.2. Animals and treatment

Female 8 week old  $C_{57}Bl_6$  mice ( $n = 252$ ) (Charles River, St-Aubin-lès-Elbeuf, France) were used in a first series of 12 experiments. For each experiment, 21 mice were randomly distributed into 3 groups of 7 mice. Since 2 experiments were conducted at the same time, the mice were housed 14 per cage. Two silica-treated groups received every day for 25 day 1 ml of either  $1.66 \times 10^{-9}$  or  $1.66 \times 10^{-17}$  M silica in 100 ml of the drinking tap-water, that is  $1.66 \times 10^{-11}$  and  $1.66 \times 10^{-19}$  final concentration, respectively (11 and 19 sil). The control group was also given 100 ml of the drinking tap-water daily except that silica was omitted. A second series of 11 experiments was done with 99 female 8 week old  $C_{57}Bl_6$  mice (Iffa Credo, L'Arbresle, France). For each experiment, 9 mice were randomly distributed into 3 groups of 3 mice. In this case, 4 experiments were conducted

simultaneously and the mice were housed 12 per cage. Two groups received as described above 1 ml of either 19 sil or 19 sal in 100 ml of the drinking tap-water whereas the third group was given only tap-water. A third series of 7 experiments was performed with 105 female 8 week old  $C_{57}Bl_6$  mice (Iffa-Credo). For each experiment, 15 mice were randomly distributed into 3 groups of 5 mice. In this last case, 2 experiments were conducted at the same time and the mice were housed 10 per cage. The different groups received as previously described 1 ml of either 19 sal, 19 lac or 19 sil in 100 ml of drinking tap-water. Analysis of tap-water revealed (mg/l): sodium 14.4, potassium 2.9, calcium 87.8, magnesium 3.7, chlorides 28.7, sulfates 28, nitrates 19.7, silicates 7.75, phosphates 0.19. After 25 days, the mice were etheranaesthetized, exanguinated and peritoneal macrophages were recovered.

### 2.3. Isolation of macrophages and synthesis of paf-acether and lyso paf-acether

The cells were collected by peritoneal washing with 5 ml RPMI 1640 (Flow Lab., Irvine, Scotland) buffered with 4.2 mM HEPES (Gibco Ltd., Paisley, Scotland) pH 7.4. The cells were pooled from 7, 3 and 5 mice in the first, the second and the third series of experiments respectively. The cells were pelleted by centrifugation ( $200 \times g$ , 7 min,  $4^\circ C$ ) and were suspended at  $2.5 \times 10^6$  in 1 ml RPMI 1640. One milliliter of the cell suspension was layered on 15 mm diameter plastic dishes (Costar, Cambridge, MA, USA) and after 2 h at  $37^\circ C$  the dishes were washed 3 times with RPMI 1640 to remove non-adhering cells. Peritoneal cells which were allowed to adhere for 2 h were over 95% macrophages as already described (Roubin et al., 1982a). These cells were incubated at  $37^\circ C$  for 45 min in 500  $\mu$ l RPMI 1640 containing 0.25% fatty acid-free bovine serum albumin (Sigma) (RMPI-BSA), with or without zymosan (Z, Sigma). In a dose-response preliminary experiment, a concentration of 200  $\mu$ g Z/ml triggered maximal synthesis of paf-acether in macrophages, whereas 50  $\mu$ g Z/ml yielded about one half of this amount. Unstimulated macrophages were used for the determination of the cell protein content. Paf-

acether was recovered at the end of the incubation period by addition of 2 ml 80% ethanol into the dishes. The ethanolic extract was collected 1 h later and centrifuged ( $1\,500 \times g$ , 20 min,  $4^\circ\text{C}$ ) and the supernatant was brought to dryness under an air-stream at  $40^\circ\text{C}$ . The dry residues were either suspended in RPMI-BSA and kept at  $-20^\circ\text{C}$  until paf-acether bioassay or processed further for lyso paf-acether determination as follows. The dry residues were acetylated overnight at room temperature with 100  $\mu\text{l}$  pyridine and 100  $\mu\text{l}$  acetic anhydride. Pyridine was then eliminated by mixing the samples with 1 ml chloroform. The samples were evaporated and the latter procedure was repeated twice. The dry residues were suspended in RPMI-BSA and assayed for paf-acether activity. The efficiency of the acetylation process averaged 80% (Jouvin-Marche et al., 1984).

#### 2.4. Paf-acether assay and characterization

Paf-acether obtained directly or after chemical acetylation of lyso paf-acether was measured as previously described (Benveniste et al., 1972; Cazenave et al., 1979) by aggregation of washed rabbit platelets pretreated for 15 min at room temperature with 0.1 mM aspirin lysin salt (Aspegic<sup>®</sup>, Lab. Fgic, Amilly, France), a cyclooxygenase inhibitor. Aggregation was performed in an aggregometer (Icare, Marseille, France) using Tyrode buffer containing the ADP scavenger complex creatine phosphate (1 mM)/creatin phosphokinase (10 IU/ml), both from Sigma. The amounts of paf-acether were calculated using a calibration curve obtained with synthetic paf-acether, the cell protein content being determined by the method of Lowry (Lowry et al., 1951). All assays, including those from the first set of experiments, were conducted 'blind', that is the technician in charge was unaware of the origin of the samples.

Besides its ability to aggregate platelets independently of ADP and arachidonate metabolites, paf-acether was also characterized by its total hydrolysis resulting in complete loss of activity when it was incubated for 30 min at  $37^\circ\text{C}$  with 10  $\mu\text{g}/\text{ml}$  phospholipase  $A_2$  from hog pancreas (Boehringer, Mannheim, GFR) but not when in-

cubated overnight with 100  $\mu\text{g}/\text{ml}$  lipase  $A_1$  from *Rhizopus arrhizus* (Boehringer) (Benveniste et al., 1977). Platelet aggregation by paf-acether was specifically inhibited upon incubation for 1 min with 50  $\mu\text{M}$  BN 52021 (Nunez et al., 1986). To characterize paf-acether further, high performance liquid chromatography (HPLC) was performed using a method modified from Tencé et al. (1980). All paf-acether-containing supernatants were pooled and proteins were precipitated by incubating the samples for 30 min at  $4^\circ\text{C}$  with 4 volumes absolute ethanol. The dry ethanolic residue was recovered in chloroform/methanol (1:1, v/v) and HPLC (Waters, Milford, MA, USA) was performed using a Microporasil column (4.6 mm (ID)  $\times$  25 cm; Waters) eluted with dichloromethane/methanol/water (65:50:5, v/v) at a flow rate of 1.7 ml/min. One-minute fractions were collected, dried under negative pressure and bioassayed for paf-acether activity after recovery of the dry extract in RPMI-BSA. Phosphatidylcholine, sphingomyelin, lyso-phosphatidylcholine and synthetic paf-acether were used as standards. The platelet aggregating substance recovered from macrophages from treated or untreated mice eluted from HPLC between sphingomyelin and lyso-phosphatidylcholine with a retention time identical to that of synthetic paf-acether (fig. 1).

#### 2.5. Data analysis

All results are given in nmol paf-acether/mg protein (mean  $\pm$  S.E.M.). The data were compared statistically using Student's t-test for unpaired variates.

### 3. Results

#### 3.1. Effect of oral administration of silica on paf-acether production by mouse peritoneal macrophages

When stimulated with Z, macrophages from mice receiving 11 sil and 19 sil synthesized higher amounts of paf-acether than those from untreated mice (table 1, experiment I). Following 11 sil administration, the increase was 44.2% for Z = 50

TABLE 1

Effect of very high dilutions of silica on paf-acether synthesis in mouse peritoneal macrophages.

Experiment	Treatment <sup>a</sup>	Paf-acether (nmol/mg protein)		
		0 $\mu$ g Z/ml <sup>b</sup>	50 $\mu$ g Z/ml	200 $\mu$ g Z/ml
I (12) <sup>c</sup>	none	13 $\pm$ 4 <sup>d</sup>	92 $\pm$ 8	177 $\pm$ 10
	11 sil	13 $\pm$ 4	132 $\pm$ 14 <sup>e</sup>	232 $\pm$ 17 <sup>f</sup>
	19 sil	19 $\pm$ 5	154 $\pm$ 16 <sup>g</sup>	245 $\pm$ 17 <sup>g</sup>
II (11)	none	22 $\pm$ 7	126 $\pm$ 8 <sup>h</sup>	260 $\pm$ 20
	19 sal	29 $\pm$ 9	137 $\pm$ 19 <sup>h</sup>	264 $\pm$ 17
	19 sil	26 $\pm$ 12	214 $\pm$ 19 <sup>sh</sup>	352 $\pm$ 22 <sup>i</sup>
III (7)	19 sal	15 $\pm$ 4	124 $\pm$ 12	276 $\pm$ 23
	19 lac	24 $\pm$ 5	137 $\pm$ 13	267 $\pm$ 20
	19 sil	26 $\pm$ 6	200 $\pm$ 9 <sup>j</sup>	355 $\pm$ 11 <sup>f</sup>

<sup>a</sup> Mice were either untreated or orally treated with 11 sil, 19 sil, 19 lac or 19 sal every day for 25 days as described in Methods section. Experiments II and III were blind. <sup>b</sup> Peritoneal macrophages were either unstimulated (0  $\mu$ gZ/ml) or stimulated in vitro with 50 or 200  $\mu$ g Z/ml. <sup>c</sup> (n) = number of experiments in duplicate. <sup>d</sup> Values are means  $\pm$  S.E.M. <sup>e</sup> Value significantly different from that for untreated mice ( $P < 0.05$ ). <sup>f</sup> Values significantly different from those for untreated (Experiment I) or 19 sal-treated (Experiment III) mice ( $P < 0.02$ ). <sup>g</sup> Values significantly different from those for untreated mice ( $P < 0.005$ ). <sup>h</sup> Number of experiments = 7. <sup>i</sup> Value significantly different from that for untreated mice ( $P < 0.01$ ). <sup>j</sup> Value significantly different from that for 19 sal-treated mice ( $P < 0.001$ ).

$\mu$ g/ml and 30.8% for Z = 200  $\mu$ g/ml. With the high dilution of silica (19 sil) the increases were 67.5 and 38.0%. The amounts of mediator synthesized by the 2 silica-treated groups were significantly different from those of the untreated group, especially after 19 sil treatment ( $P < 0.005$ ). In contrast, no effect on lyso paf-acether could be observed (results not shown).

In the second set of experiments, the synthesis of paf-acether was significantly increased after administration of 19 sil: by 55.5% for Z = 50  $\mu$ g/ml and by 33.5% for Z = 200  $\mu$ g/ml as compared to mice receiving 19 sal, (table 1, experiment II). There was no significant difference between untreated mice and those receiving 19 sal. No effect was observed on the synthesis of lyso paf-acether.

In experiment III, the synthesis of paf-acether was significantly increased only after administra-

tion of 19 sil: by 61.3% for Z = 50  $\mu$ g/ml and by 28.6% for Z = 200  $\mu$ g/ml as compared to mice receiving 19 sal (table 1, experiment III). No effect was observed on the synthesis of paf-acether when mice received 19 lac. Unstimulated macrophages synthesized negligible amounts of paf-acether in all experiments.

### 3.2. Characterization of paf-acether

The substance recovered from macrophages was characterized as paf-acether by using the criteria described in the Methods section. These results indicated that the paf-acether obtained from silica-treated mice did not differ qualitatively from that synthesized by untreated mice macrophages or from synthetic paf-acether. Moreover, the higher production of paf-acether by silica-treated vs. untreated mice peritoneal cells could be observed again when the paf-acether activity of the HPLC fractions was assessed (fig. 1).

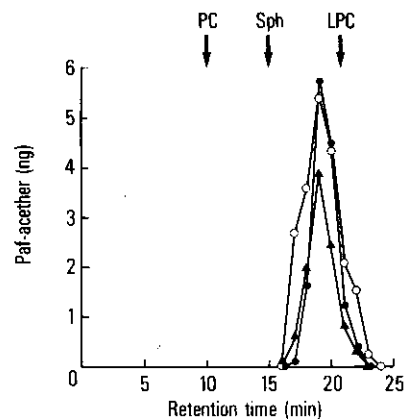


Fig. 1. HPLC elution pattern of paf-acether obtained from Z-stimulated macrophages from untreated mice ( $\blacktriangle$ ) and from mice receiving either 11 sil ( $\bullet$ ) or 19 sil ( $\circ$ ). Paf-acether from 12 experiments in each group was pooled, then extracted with 80% ethanol in water. After centrifugation and evaporation of the supernatant in an air stream, paf-acether was recovered in dichloromethane/methanol (1:1, v/v) and chromatographed as described in Methods. Column, microporasil; solvent, dichloromethane/methanol/water (60:50:5); flow rate, 1.7 ml/min. Arrows indicate retention times of phosphatidylcholine (PC), sphingomyelin (Sph) and lysophosphatidylcholine (LPC) as detected by refractometry. Paf-acether was measured using platelet aggregation as described in Methods.

#### 4. Discussion

After oral administration to mice of minute amounts of silica for 25 days the synthesis by peritoneal macrophages of paf-acether, a newly formed lipid mediator, but not of its precursor lyso paf-acether, was significantly increased. Further studies are necessary to find possible effects of the same procedure on other macrophage functions.

Paf-acether is a well-recognized marker of macrophage stimulation (see review in Roubin and Benveniste, 1985) and the levels measured in the present study were of the same order of magnitude as those previously reported (Roubin et al., 1982a,b; 1983). The variation in paf-acether synthesis by untreated mice macrophages that we observed from the first to the second experiment is a frequently encountered phenomenon. Since macrophages are very sensitive to the local micro-environment (Roubin et al., 1983), their activity varies according to the source of the animals, the time of the year, and, most probably, the degree of microbial infestation of the mice. Nevertheless, the variation within each group was very small so that the effect of treatment could be detected with a high degree of significance. However, these effects were unusual in that very high dilutions of silica were given to the animals, 12-15 mice receiving 1 ml of either  $1.66 \times 10^{-9}$  or  $1.66 \times 10^{-17}$  M silica daily. We calculated that a mouse drinking the 19 sil solution would receive approximately 1000 molecules of silica per day.

Relatively few authors have explored the biological effect of very highly diluted substances by using rigorously controlled, up to date experimental design. Serum thymic factor injected to 6 week old mice at a dose as low as  $10^{-12}$  pg per mouse significantly suppressed the immune response as assessed by the direct hemolytic plaque-forming cell assay (Doucet-Jaboeuf et al., 1982). Murine  $\alpha, \beta$ -interferon ( $8 \times 10^{-10}$  IU intraperitoneally) caused a significant increase in the mouse humoral immune response whereas  $16 \times 10^{-10}$  IU significantly stimulated their cellular immune response (Bastide et al., 1985). In our laboratory, we have observed in vitro effects of various substances at very high dilutions on human basophil degranula-

tion (Poitevin et al., 1986; Beauvais et al., submitted). Our results and those found in the literature indicate that some compounds could exert their biological effects at concentrations well below those generally admitted as being active, i.e. in the range of the drug concentration used in homeopathic medicine. However, our present results cannot be interpreted as sustaining the value of this therapeutic approach.

Since the results we are reporting are likely to be highly controversial, we went to extreme lengths to insure the scientific validity of our protocol: (1) We used a very well-defined cellular system with which our laboratory has accumulated a great deal of experience (Mencia-Huerta and Benveniste, 1979; 1981; Mencia-Huerta et al., 1982; Roubin and Benveniste, 1985; Roubin et al., 1982a,b; 1983; 1986). (2) State-of-the-art technology enabled us to carefully characterize the mediator, paf-acether, the synthesis of which was monitored in our study. (3) Experiments II and III were performed blind, i.e. the experimenters did not know which group of mice received the silica and which one received the saline or the lactose. The code was held by an investigator foreign to our laboratory and was only disclosed at the end of the experiments. (4) The second experiment showed that the silica from the glass walls of tubes that may have been dissolved into water during the shaking procedure was irrelevant to the results observed since there was no difference between the mice receiving no treatment and the mice receiving saline buffer that underwent the same procedure as silica. Experiment III showed that the lactose used in the first steps of the silica dilution procedure did not interfere in the modulation of macrophage paf-acether synthesis and that the effect observed was due only to silica. (5) The specificity of the effect observed was also ascertained by the following experiment. Once the positive results had been obtained for silica, a substance cytotoxic for macrophages, we did an identical experiment using a compound (*Gelsemium sempervirens*) devoid of any cytotoxic effect on macrophages and diluted following the same procedure as for 19 sil. The latter experiment was entirely negative (results not shown). This indicates that the effect of the molecule is specific,

even at such very low concentrations. This specificity was also found in other studies (to be published by our group) showing that slight variations in the molecular structure of a compound abolished the effect observed at very high dilutions even when in all probability only a few molecules, if any, were physically present in the solution tested.

The result of these first well-controlled experiments demonstrates a definite biological effect for very high dilutions of silica. This is very puzzling considering the very small number of molecules of silica given to the mice (1 000 or less per day for 19 sil) compared to the billions of possible target cells. It is clear that studies using highly sophisticated physico-chemical means should be launched to try to understand what is happening when high dilutions are performed in water with vigorous shaking with only a few molecules being present in the solutions.

In conclusion, we present for the first time data obtained using very high dilutions of silica in *ex vivo* experiments performed according to the rules of up to date experimental pharmacology. These results clearly demonstrate an as yet unexplained cellular effect that calls for more studies on the biological and physico-chemical aspects of this phenomenon.

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