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Activation of macrophages by silicones: phenotype and production of oxidant metabolites

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Abstract

Background: The effect of silicones on the immune function is not fully characterized. In clinical and experimental studies, immune alterations associated with silicone gel seem to be related to macrophage activation. In this work we examined *in vivo*, phenotypic and functional changes on peritoneal macrophages early (24 h or 48 h) and late (45 days) after the intraperitoneal (i.p.) injection of dimethylpolysiloxane (DMPS) (silicone). We studied the expression of adhesion and co-stimulatory molecules and both the spontaneous and the stimulated production of reactive oxygen intermediates and nitric oxide (NO).

Results: The results presented here demonstrate that the fluid compound DMPS induced a persistent cell recruitment at the site of the injection. Besides, cell activation was still evident 45 days after the silicone injection: activated macrophages exhibited an increased expression of adhesion (CD54 and CD44) and co-stimulatory molecules (CD86) and an enhanced production of oxidant metabolites and NO.

Conclusions: Silicones induced a persistent recruitment of leukocytes at the site of the injection and macrophage activation was still evident 45 days after the injection.

Background

Nowadays we are in permanent contact with silicones, synthetic polymers containing a repeating Si-O backbone and organic groups attached to the silicon atom [1]. Medical-grade silicones consist primarily of dimethylpolysiloxane (DMPS) and are widely used in devices including cardiac valves, intravenous tubing, intraocular lenses, digital joint arthroplasty prostheses, breast implants, syringes, needles, baby bottle nipples and many others products [1]. Depending upon the length of the polymer chains and the amount of cross-linking between chains medical-grade silicones can be found as fluids, gels or elastomers.

The effect of silicones on the immune function is not fully characterized. It has been shown that certain forms of silicone are immunologically active [2] and depending upon the molecular weight and the degree of cross-linking of the polymers, silicones are potent humoral adjuvants [3]. Several studies of the silicone-induced inflammatory response in patients and animals revealed histopathological

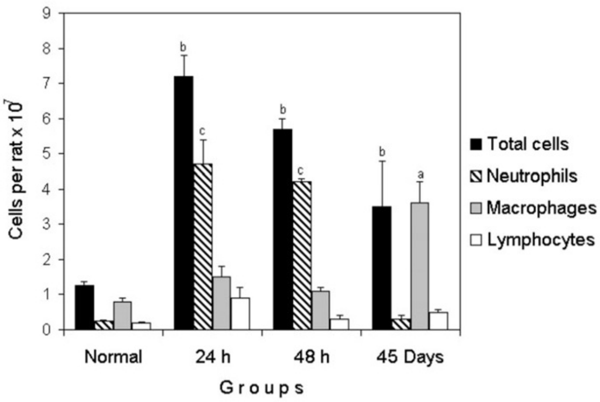

Figure 1
Silicone induces differential recruitment of leukocytes. Rat peritoneal cells (n = 4/group) were obtained after 45 days, 48 h and 24 h of the i.p. injection of 1 ml of silicone. Differential cell counting was assessed by microscopic observation of cytospin preparations stained with Giemsa. Standard errors of the means are depicted. A representative of three experiment performed is shown.[a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ vs. Normal. Mann Whitney U-test, and Student-Neuman-Keuls post test comparisons were used in these experiments.

findings instead of direct evidences of cellular activation [4–6].

The initial body's reaction to the implanted material is the inflammatory response that induces recruitment and activation of different cells [7]. The magnitude of any inflammatory response can be related to the level of activation of macrophages. This activation occurs both in inflammatory and in adaptive immune responses, and involves phenotypic and functional changes [8]. Criteria widely used for activation are the ability to inhibit intracellular proliferation of microorganisms, the increased production of reactive oxygen intermediates and the enhanced expression of MHC and co-stimulatory molecules [9,10]. Recently, Naim et al. showed that silicone elastomer preadsorbed with plasma proteins activated human monocytes *in vitro* to secrete pro-inflammatory cytokines [11]. Besides, silicone gels and oils activated macrophages in female A.SW mice: increased production of IL-6 and IL-1β was obtained from macrophages collected from silicone fluid- and silicone oil-treated mice when cultured with increasing amounts of lipopolysaccharide [12].

In this work we examined early (24 or 48 h) or late (45 days) after the intraperitoneal (i.p.) injection of the fluid compound DMPS, phenotypic and functional changes on peritoneal macrophages. We studied the expression of adhesion and co-stimulatory molecules and both the spontaneous and the stimulated production of reactive oxygen intermediates and nitric oxide (NO). The present work shows that silicones induced a persistent recruitment at the site of the injection and that cell activation was still evident 45 days after the injection. Activated macrophages exhibited an increased expression of adhesion (CD54 and CD44) and co-stimulatory (CD86) molecules and an enhanced production of oxidant metabolites and NO.

Results

In each experiment rats (n = 4/group) were injected i.p. with 1 ml of DMPS or 1 ml of PBS (normal group). Animals were killed 45 days, 48 h or 24 h after the DMPS injection and peritoneal cells were harvested to evaluate several parameters. The cell number increased significantly in all DMPS injected rats compared with normal group ($p < 0.01$) with a maximum 24 h post injection (Fig. 1). Differential cell counting showed a marked increase of polymorphonuclear neutrophils 24 h and 48 h post injection ($p < 0.001$) and a clear increase of macrophages on day 45 ($p < 0.05$). Lymphocytes peaked transiently 24 h post injection.

To assess the activated phenotype of peritoneal macrophages we studied the expression of CD54, CD44 and CD11b/c adhesion molecules and CD80 and CD86 co-stimulatory molecules by flow cytometry (Table 1). For each marker, we analyzed the percentage of positive cells and the density of the expression of this marker, evaluated as the mean fluorescence intensity (MFI). As can be seen, while the percentages of CD54 and CD44 positive macrophages were similar in all groups, CD11b/c positive macrophages decreased 24 h and 48 h after DMPS injection ($p < 0.001$). On the other hand, the MFI increased significantly for CD54 and CD44 molecules in DMPS injected rats ($p < 0.05$ for both markers) whereas for the CD11b/c molecule, the MFI diminished in 24 h and 48 h groups ($p < 0.001$) but increased 45 days after the DMPS injection ($p < 0.05$). Given the low expression of CD11b/c immediately after the injection, we considered it likely that active phagocytosis of silicone could contribute to a decreased level of this marker [13,14]. Consistent with this, a high number of clear vacuoles were observed in cytospin preparations of macrophages of the 24 h group (data not shown). No differences were observed in the expression of MHC class II molecules (data not shown). The percentage of CD80 positive macrophages decreased in 24 h and 48 h groups ($p < 0.01$) without differences in the MFI. For the CD86 molecule the percentage of positive macrophages was significantly higher only on day 45 ($p < 0.01$) but the MFI increased in all DMPS injected groups ($p < 0.05$).

Next we studied the production in macrophages of oxidant metabolites such as H_2O_2 using the probe 2,7-

Table 1: Surface molecule expression on peritoneal macrophages from silicone injected rats.

	CD54		CD44		CD11b/c		CD80		CD86	
Groups	Positive (%)	MFI	Positive (%)	MFI	Positive (%)	MFI	Positive (%)	MFI	Positive (%)	MFI
Normal	98 ± 1	39 ± 3	99 ± 1	146 ± 6	99 ± 1	328 ± 20	75 ± 1	6 ± 1	16 ± 5	2 ± 1
24 h	98 ± 1	<u>57 ± 6a</u>	100 ± 1	<u>200 ± 15a</u>	<u>80 ± 1c</u>	<u>66 ± 12c</u>	<u>39 ± 1b</u>	6 ± 1	18 ± 2	<u>4 ± 1a</u>
48 h	93 ± 2	<u>52 ± 2a</u>	100 ± 1	<u>202 ± 8a</u>	<u>84 ± 1c</u>	<u>70 ± 6c</u>	<u>27 ± 4b</u>	5 ± 1	14 ± 1	<u>4 ± 1a</u>
45 days	99 ± 1	<u>52 ± 6a</u>	100 ± 1	<u>209 ± 27a</u>	100 ± 1	<u>404 ± 40a</u>	76 ± 11	6 ± 1	<u>44 ± 6b</u>	<u>3 ± 1a</u>

The mean ± SEM of a least 4 rats is given. MFI, mean fluorescence intensity. Significant differences vs. normal controls are underlined. [a]$P < 0.05$, [b] $p < 0.01$,[c] $p < 0.001$

dichloro-dihydrofluorescein(DCFH) as described previously [15]. The oxidation of DCFH by unstimulated or phorbol myristate acetate (PMA)-stimulated macrophages was assessed by flow cytometry; macrophages were selected and gated by light scatter characteristics and fluorescence was expressed as MFI (Fig. 2). Unstimulated macrophages obtained 48 h and 45 days after DMPS injection showed an increased MFI ($p < 0.01$ and $p < 0.001$ vs. normal, respectively). In all groups PMA stimulated an increase of the oxidative burst; however, a higher production of oxidant metabolites was observed after 45 days of DMPS injection compared with normal rats ($p < 0.05$).

Finally, the production of NO by purified macrophages was determined after 48 h of culture with or without LPS stimulation (Fig. 3). As can be seen unstimulated macrophages from 48 h and 45 day groups showed an increased release of NO ($p < 0.01$ vs. normal). While LPS stimulated the NO production in cells of the 24 h group ($p < 0.01$ vs. 24 h unstimulated), the production was not further enhanced in 48 h and 45 day macrophages (p=NS vs. unstimulated). The NO productions by LPS-stimulated macrophages derived from silicone-injected groups were higher than LPS-stimulated normal macrophages ($p < 0.05$). In addition, the NO synthesis inhibitor aminoguanidine (AG) effectively suppressed the LPS-stimulated NO release in all groups and the silicone-induced NO production in 48 h and 45 day groups ($p < 0.001$ vs. LPS or MEDIUM).

Discussion

Inflammation represents the body's local reaction to tissue injury and with biocompatible materials this step should not be long lasting. Moreover, it has been suggested that with DMPS this phase is not prolonged because the polymer is not providing a stimulus for continued inflammation [7]. In this study we show a persistent recruitment of leukocytes after DMPS injection in the peritoneal cavity. Besides, in peritoneal macrophages, activation markers were up-regulated and the spontaneous release of oxidant metabolites and NO was enhanced still 45 days after the injection.

In silicone breast implants, chronic inflammation seems to be the most relevant process with accumulations of lymphocytes and monocytes [16]. Even if a silicone gel filled breast implant does not rupture, small amounts of low molecular weight fluid DMPS may permeate (bleed or sweat) out of the implant into the surrounding tissue [17]. Hydrophobic materials such as silicone do not migrate well and are coated instead with host proteins [18], and within one hour elastomers are at least 70 % covered with host proteins [19]. Apparently, recruited inflammatory cells do not respond to DMPS itself but to adsorbed, partially denatured plasma proteins such as IgG, albumin, fibronectin and complement components [20]. It has been suggested that liposome-like structures can be formed within the body of an implant, involving water-soluble and hydrophobic constituents [5]. When administered *in vivo* liposomes interact almost exclusively with the mononuclear phagocytic system [21] and in an i.p. injection resident macrophages take up liposomes in large quantities and monocytes can be recruited from the general circulation [22,23]. This could account for the persistent stimulation that we found in our study, after several days of DMPS in the peritoneal cavity. Perhaps the association of vesicular and lipoidal structures with host proteins could facilitate this strong stimulatory capacity. However the activity of "naked" DMPS should also be considered. Previous work support the stimulatory capacity of silicone *in vitro* without the influence of plasma proteins: when peritoneal macrophages are cultured on silicone-coated plates, their cytotoxic activity on cancer cells is markedly augmented and the activity of antigen presenting cell is enhanced [24].

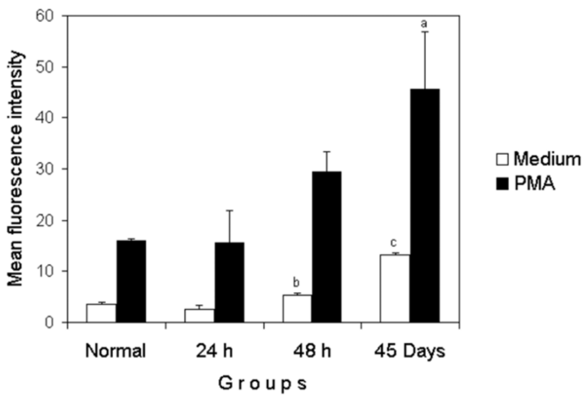

Figure 2
Silicone enhances the production of oxidative metabolites by Mϕ. Rat peritoneal cells (n = 4/group) were loaded with DCFH-diacetate, and the oxidation of DCFH by unstimulated and PMA-stimulated cells was assessed by flow cytometry. Macrophages were selected on the basis of their characteristic forward scatter (FWS) and side angle scatter (SS). Standard errors of the means are depicted. A representative of two experiments performed is shown. DCFH: dichlorodihydrofluorescein. [a] $p < 0.05$; [b] $p < 0.01$ and [c] $p < 0.001$ vs. Normal. ANOVA test and Student-Neuman-Keuls post test comparisons were used in these experiments.

The activation of macrophages is an important event involved in inflammation, T cell activation and adjuvanticity. Adhesion and co-stimulatory molecules are up-regulated with key implications on antigen presentation and T cell priming [8]. In rats, strong stimulatory capacity for primary immune response is associated with the expression of the co-stimulatory ligands CD80 and CD86 [25]. After DMPS injection in peritoneal macrophages the MFI for CD54, CD44 and CD86 increased significantly in all injected rats (24 h, 48 h and 45 days after injection) providing evidences of cellular activation status.

After activation, cytokines, reactive oxygen intermediates and NO that belong to the molecular repertoire of activated macrophages are up-regulated [8]. The cytokine stimulating capacity of silicone has been already demonstrated [26]. Macrophages layered on DMPS and silicone rubber with or without protein adsorption produce variable levels of IL-1β, IL-6 and TNF-α depending on the polymer and adsorbed protein [12]. Moreover, chronic loading of macrophages with silicone particles derived of dialysis tubing results in augmented release of IL-1 [26].

Inappropriate stimulation of NADPH oxidase and NO pathways are associated with chronic inflammation. Here we showed the ability of DMPS to stimulate the production of reactive oxygen intermediates and NO up to 45 after injection. We found that silicone primed macrophages for the production of both H_2O_2 and NO. In fact we observed 1) enhanced production of H_2O_2 after PMA stimulation and 2) NO production beyond only constitutive levels present in control samples. Measurement of H_2O_2 by inflammatory cells adherent to the surface of silicone elastomer 2 or 7 days after the implantation has been used to evaluate biological reactions against biomaterials [27]. On the other hand, our data are in agreement with a previous report showing that children breast fed by mothers with silicone implants have increased urinary excretion of NO metabolites and neopterine [28]. Spontaneous NO release involves the *in vivo* activation of the NO synthase, as frequently observed in macrophages isolated from infected animals [29]. Moreover, the NO production in macrophages grown on silicone in vitro is up to 60 % higher than controls [28].

With liposomes, the effective uptake and the initial activation of macrophages determine the prolonged stimulatory effect [22,23,30]. The similarities between silicone and lipid adjuvants [21] could partly explain the results we described here. Considering the widespread exposure to silicone in the environment and the expanding use of this material in the world, basic research addressing the immunobiology of silicone will help to understand the effects of the interaction between silicone and the immune system and to define its role in health and disease.

Conclusions

In this work we examined early (24 or 48 h) or late (45 days) after the i.p. injection of the fluid compound DMPS, phenotypic and functional changes on rat peritoneal macrophages. We studied the expression of adhesion and co-stimulatory molecules and both the spontaneous and the stimulated production of reactive oxygen intermediates and NO. We show here that silicone induced a persistent recruitment of leukocytes at the site of the injection and that macrophage activation was still evident after 45 days of injection. Activated macrophages exhibited an increased expression of adhesion and co-stimulatory molecules and an enhanced production of oxidant metabolites and NO.

Materials and methods

Rats

8- to 12-week-old female Wistar rats were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba, in accordance with institutional guidelines.

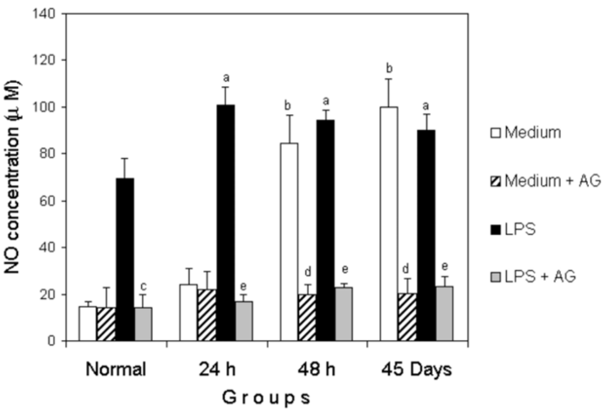

Figure 3
Silicone induces the production of NO by Mϕ. Adherent peritoneal cells (n = 4/group) were cultured with medium, AG, LPS or AG plus LPS for 48 h; the NO production was measured by a colorimetric assay using the Griess reagent. Standard errors of the means are depicted. A representative of three experiments performed is shown. [a] $p < 0.05$ vs. Normal, [b] $p < 0.01$ vs. Normal, [c] $p < 0.01$ vs. LPS, [d] $p < 0.001$ vs. Medium, [e] $p < 0.001$ vs. LPS. ANOVA test and Student-Neuman-Keuls post test comparisons were used in these experiments.

Monoclonal antibodies

PE-conjugated anti-rat CD11b, anti-rat CD44, anti-rat CD54, anti-rat CD80 and anti-ratCD86, and FITC-conjugated anti-rat MHC class II (IA) monoclonal antibodies were purchased from PharMingen (San Diego, CA). The isotyping control monoclonal antibodies were obtained from Sigma (MO, USA)

Silicone treatment and cell preparation

In each experiment rats were housed together and assigned to the 45 day, 48 h, 24 h and normal groups. Animals (n = 4/group) were injected i.p. with 1 ml of DMPS (viscosity (25°C) 5 centistokes, Sigma) or 1 ml of PBS (normal group). We started the treatment injecting the 45-day group; after 43 days of this injection, rats of the 48 h group received 1 ml of DMPS and 24 h later, 24 h and normal groups received 1 ml of DMPS or PBS respectively. The next day, all rats were killed by cervical dislocation, and peritoneal cells were harvested and prepared as described previously [10,30]. Viability was assessed by Trypan blue exclusion test. Differential cell counting was assessed by microscopic observation of cytospin preparations stained with Giemsa. Experiments were performed two to three times.

Flow cytometry

After blocking with mouse serum, peritoneal cells were stained with conjugated mAb as described previously [10,30]. All the staining steps were performed at 4°C in HBSS containing 5 mM EDTA and 1 % bovine serum albumin. After extensive washing cells were treated with 2% formaldehyde-PBS and 10.000 events per sample were analyzed using a Cytoron Absolute cytometer (Ortho Diagnostic System, Raritan, NJ). Debris was gated out on the basis of low forward scatter and low side angle scatter. Macrophages were selected and gated by light scatter characteristics.

DCFH oxidation assay by flow cytometry

Peritoneal cells (2×10^6 cells/ml) were loaded with 2,7-dichloro-dihydrofluorescein (DCFH, Sigma) (1 mM final concentration) in a water bath in the dark (15 min., 37°C). Loaded cells were incubated with or without phorbol myristate acetate (PMA, Sigma) for 15 min at 37°C [15]. After the incubation, tubes were placed on ice and green fluorescence was measured on a Cytoron Absolute cytometer (Ortho Diagnostic System, Raritan, NJ).

Measurement of NO production

Peritoneal cells were washed twice, diluted with RPMI-5% heat-inactivated calf serum, plated in 96-well tissue culture plates (1×10^6 cells/well) and incubated for 2 h at 37°C. Non-adherent cells were removed by extensive washing with RPMI and adherent cells were cultured with medium alone, medium containing 1 mM aminoguanidine (AG, Sigma) to inhibit the NO synthase activity [31], medium with LPS (1 μg/ml, Sigma) or LPS (1 μg/ml) plus 1 mM aminoguanidine. After 48 h supernatants were collected and NO was measured as nitrite using the Griess reagent by a microplate assay [32].

Statistics

Statistical analysis included descriptive statistics, Mann Whitney U-test, ANOVA test and Student-Neuman-Keuls post test comparisons.

Authors' contributions

PI and SGC contributed equally to this work. NS participated in the cell isolation and differential counting of peritoneal cells. CMR actively participated in interpretation of data, writing and revision of the manuscript. PI and SGC conceived the study, participated in its design and coordination, including interpretation of data and drafted the manuscript. All the authors read and approved the final version.

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