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In vitro assessment of lymphocytes response following re-exposure to silicone

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Currently, the use of silicone-filled devices, mainly in plastic surgery for breast reconstruction or augmentation, is being debated by the scientific community in connection with the risk to the patient. In this study the response of whole blood or isolated peripheral blood lymphocytes from patients with silicone-gel-filled breast implants was assessed *in vitro*, in order to verify the hypothesis of silicone material acting *in vivo* as a sensitizing agent. Both quantitative and qualitative changes of lymphocyte subpopulations of patients carrying silicone devices were assessed and compared with healthy subjects. Upon 24–72 h *in vitro* re-exposure of patients' lymphocytes to silicone extract, lymphocyte surface antigen expression was monitored by flow cytometry, and the functional response of lymphocytes was measured by radioactive tracer uptake and biochemical changes.

1. Introduction

The term silicone actually encompasses a large family of polymers where silicon and oxygen atoms are the recurrent units. The medical grade silicone rubber (PDMS, polydimethylsiloxane) was specifically developed for medical application and has remained substantially unchanged since its first development in 1940. It has, indeed, been extensively applied also for long-term implants in humans, being considered nearly totally inert in the human body. The adoption of silica-free polydimethylsiloxane as primary reference material for biomaterials testing, as suggested by the National Heart Lung and Blood Institute-Division and Technology Branch (NHLBI-DTB), is a proof of its inertness.

According to recent reports from some authors, it has become apparent that in a few cases, silicone implant recipients developed human adjuvant disease, as well as connective tissue diseases. Being those diseases apparently evoked by foreign material, this led the scientific community to investigate further this polymer and its clinical applications [1–5].

Normally the tissue reaction observed around a silicone breast prostheses is typical of a mild non-specific foreign-body reaction, but sometimes an excessive fibrotic response occurs around the silicone device, resulting in a thick fibrous layer which often causes discomfort to the patient. Silicone droplets released *in vivo* from damaged envelopes, or silicone bleeding from intact envelopes are often suggested to be the causative agents for undesired reactions around the implanted devices.

Many groups have started reviewing their clinical or experimental experience on silicone implants in order to detect any deleterious potential of such material.

This study has focused on the response of lymphocytes from patients carrying silicone implants upon re-exposure *in vitro* to silicone leachables. The hypothesis is that silicone has an antigenic potential: lymphocyte populations which had a long-term exposure *in vivo* to silicone should actively respond to a second antigenic challenge *in vitro* with silicone.

Lymphocyte surface antigens have been analysed by flow-cytometry, and also different functional assays have been performed on lymphocytes, including measurement of cytotoxic activity, proliferation and activation.

2. Materials and methods

A group of 22 recipients of silicone-gel-filled breast implants for reconstructive (73%) or augmentation (27%) purposes for more than one year was selected.

They were aged 35–64 years (mean: 49). All patients were found to have a fibrous capsule with different contracture degree which was scored based on the Baker scale. The average duration of implantation was 4 years and 10 months (range 1–10 years).

Ten healthy women (mean age 33 years and 7 months) with no silicone implants were selected as control population.

Silicone (PDMS) was extracted in cell culture medium, following the guidelines given by the Amer-

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ican Society for Testing and Materials for the extraction of polymers [6]. Briefly, 1 g silicone gel was placed in 5 ml cell culture medium (RPMI, see below) in a borosilicate glass container and kept at 37 °C for 5 days. The extract was aliquoted and kept at − 20 °C until used.

2.1. Cell isolation

Blood from patients or healthy subjects was collected with either EDTA for whole blood assays or sodium heparin for isolated lymphocyte assays.

Human lymphocytes from heparin-collected blood samples were separated by centrifugation over density gradient and cultured in RPMI 1640 to which was added 10% FCS, 1% antibiotic-antimicotic and glutamine 2 mmol.

Blood or cells were exposed to silicone extract for different time intervals, depending on the test to be performed.

2.2. Test procedures

EDTA-collected blood samples were treated with either saline or PDMS extract for 30 min and 48 h. The surface antigen expression, following binding to fluorescein (FITC)- or phycoeritrin (PE)-conjugated monoclonal antibodies, was examined by flow cytometry.

The isolated lymphocytes were used for measuring the cytotoxic activity of either untreated or 24-h PDMS-treated cells towards K562 target cells: the propidium iodide uptake method was used to determine this activity [7].

Finally, both PHA-stimulated (Ly + PHA) and resting lymphocytes (Ly) were exposed to silicone extracts for 3 and 7 days. Then, their proliferation was tested by the uptake of tritiated thymidine (3H-TdR) method and their state of activation was checked by the reduction of tetrazolium salt method (MTT test) [8].

2.3. Data presentation

Data were presented as the arithmetic mean, plus and minus the standard error or the standard deviation of the mean, of the indicated number of samples. Student's t test was applied to determine the statistical significance of the differences observed between the means of the groups, with p values $\leqslant 0.05$ considered significant.

3. Results

No significant differences have been detected in membrane antigen expression by patients' lymphocytes in comparison with healthy subjects, both at 30 min and 48 h endpoints. Moreover, the exposure of lymphocytes to PDMS-extract did not change this picture.

Membrane antigen expression has also been evaluated following separation of patients into two groups: patients with severe capsular contracture and patients with only minor fibrous thickening ($\geqslant 3$ and < 3, respectively, according to the Baker scale).

At 30 min no significant difference was evident between patients and normal subjects or between patients with severe contracture and patients with minor complications, even if patients with severe contracture have an increased number of CD3 + cells and CD57 + cells compared to patients with moderate fibrous development.

At 48 h patients with severe contracture showed a number of CD57 cells/mmc significantly higher per $p = 0.04$ than those of normal subjects (307 ± 150 and 160 ± 53, respectively) and those of < 3 patients (189 ± 102), even if the exposure to PDMS extract does not modify this proportion. It is to be noticed that CD57 is specific for cells having killer activity.

Employing the assay of cytotoxic activity to K562 target cells, significant differences were observed only in patients with high capsular contracture. Ly from patients rated < 3 in terms of fibrous contracture apparently have the same activity as Ly from healthy subjects. Patients rated $\geqslant 3$ showed an increased cytotoxic activity compared with both normal subjects and < 3-patients ($p < 0.005$). Exposure to silicone leaves the cytotoxic activity substantially unaltered in both groups (Fig. 1).

When comparing data obtained from aesthetically augmented patients with reconstructed patients, the first group appeared to have a number of CD3 + CD4 + cells/mmc significantly higher than both the second group of patients (654 ± 109 versus 448 ± 110, $p = 0.002$) and the normal subject population (654 ± 109 versus 563 ± 107, $p = 0.05$). When PDMS-extract is added to the culture the difference between augmented and reconstructed patients is not found.

A similar finding has been noticed when patients are grouped depending on the presence of one or two devices. An increase in CD3 + CD4 + cells/mmc is observed in patients with bilateral implantations at both 30′ (582 ± 152 versus 455 ± 117, $p = 0.04$) and 48 h time intervals (975 ± 214 versus 794 ± 205, $p = 0.04$). Once again, the addition of PDMS extract makes the difference between the two patient groups disappear.

Functional assay of lymphocytes was also accomplished by 3H-TdR test and MTT test. After 3 days of exposure to PDMS extract, resting lymphocytes (Ly) of normal subjects do not incorporate more than

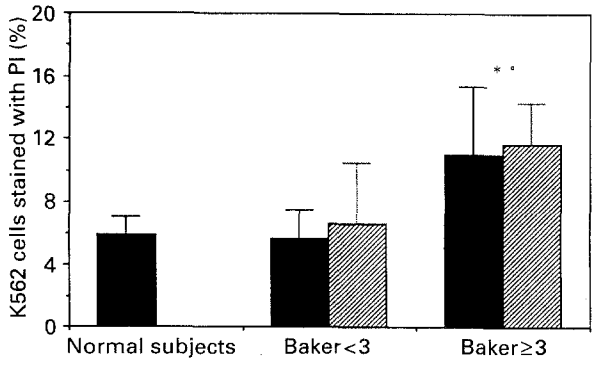

Figure 1 Cytotoxic activity of lymphocytes to K562 cells after 24 h culture. Symbols show the arithmetic mean ± sd (*: $p < 0.001$; °: $p < 0.05$): ■ Ly + medium; □ Ly + PDMS.

unexposed Ly (109 ± 26 versus 159 ± 21 dpm), but PDMS-treated Ly from patients showed a significantly increased incorporation of thymidine compared with untreated (237 ± 17 versus 179 ± 13 dpm; $p < 0.05$), i.e. an increased proliferation.

The same results were found when Ly + PHA were tested. Moreover, if the patients were grouped as 'augmented' and 'reconstructed' depending on the reason for the implantation, only the Ly + PHA from 'augmented patients' showed a consistently increased proliferation compared with unexposed Ly + PHA ($p < 0.05$); the results for 'reconstructed patients' were not significantly different from those for healthy subjects (Fig. 2).

Using the MTT test the same trend was observed. Following 3 days of exposure to PDMS, resting Ly from patients were more activated than those from unexposed Ly (207 ± 14 versus 144 ± 12 O.D. × 10^3, $p = 0.004$), whereas resting Ly from healthy subjects were no different from unexposed Ly (data not shown).

When the silicone–lymphocytes contact was prolonged to 7 days, no significant difference was found between exposed and unexposed Ly from patients by MTT test (data not shown). Once again, however, Ly from 'augmented patients' showed significantly increased activation ($p < 0.05$) compared with unexposed Ly, unlike Ly from 'reconstructed patients' which showed no significant activation when compared to unexposed Ly (Fig. 3).

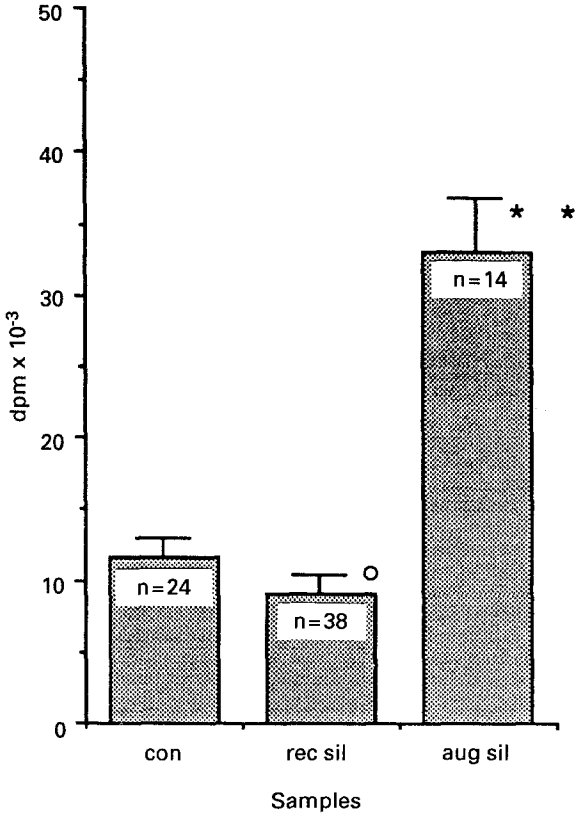

Figure 2 Media comparison in 3H-TdR assay. Values are expressed as arithmetic mean ± standard error of the indicated number of samples: con = Ly + control extract; rec-sil = Ly of reconstructed patients + silicone extract; aug-sil = Ly of augmented patients + silicone extract (O not significant versus control value; ** $p = 0.001$ versus control value).

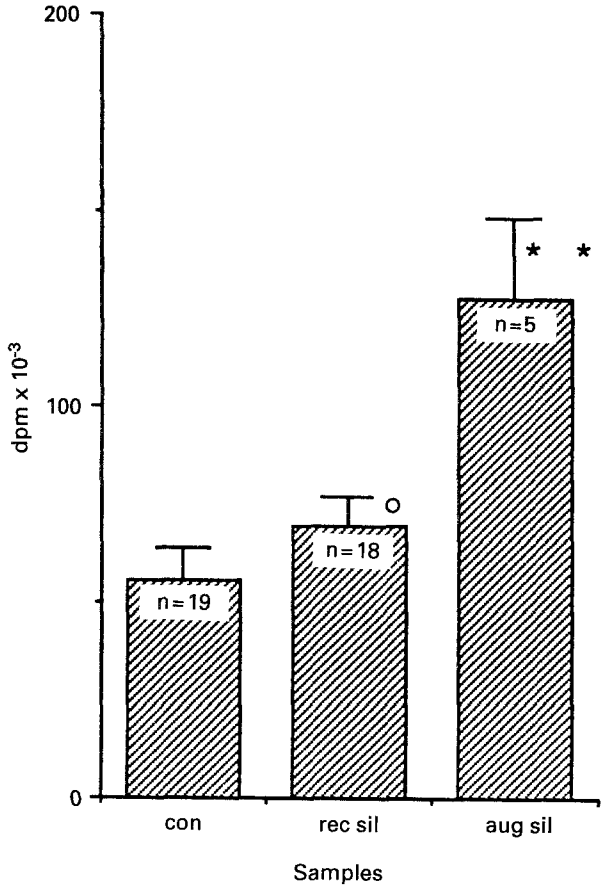

Figure 3 Media comparison in silicone MTT assay. Values are expressed as arithmetic mean ± standard error of the indicated number of samples: con = Ly + control extract; rec-sil = Ly of reconstructed patients + silicone extract; aug-sil = Ly of augmented patients + silicone extract (O not significant versus control value; ** $p = 0.0009$ versus control value).

4. Discussion

A number of reports have appeared in the literature describing the development of autoimmune connective tissue diseases or "human adjuvant disease" following the implantation of silicone in humans. This has led to the hypothesis that silicone could act in some way as an "immunogenic" moiety.

The spreading of PDMS particles from the implanted device has been demonstrated [9]. The particles apparently behave as haptens and could interact with the immune system of the host upon binding to protein carriers.

Because the role of lymphocytes is of fundamental importance in the complex mechanisms involved in the immune response, the study of the performance of such cells provides a useful tool in the understanding of biomaterial/immune system interactions.

Recently, Brantley *et al.* [10] have studied the response to mitogens and the lymphocyte subpopulation proportion in rats receiving silicone experimental implantation: no change in such parameters was detected, so it was concluded that no sensitizing activity was exerted by the silicone implants.

These results agree with our finding that silicone *per se* is not able to change the surface antigen expression and the proliferation of normal lymphocytes.

On the contrary, resting Ly from patients with silicone devices react to re-exposure to silicone by increasing their proliferation (3H-TdR uptake test at 3 days) and entering a state of activation (MTT test at 3 days). This would support our hypothesis that silicone might behave as an antigen able to evoke the response of a sensitized population upon re-exposure to it.

This would agree with some authors who reported on the strongly positive response of previously silicone-sensitized animals when intradermally injected with silicone: it was concluded that silicone-protein complexes could be immunogenic [11].

It is to be noticed also that the patients carrying a consistent amount of silicone, i.e. 'augmented patients' or patients with bilateral implantation, have a significantly higher number of CD3 + CD4 + cells: these are the cells actively involved in the specific immune response. In addition, the proliferation of both resting Ly and Ly + PHA from these patients is also increased and their state of activation is still detectable at 7 days.

It can also be speculated that a relationship exists between the immune system response and the development of a periprosthetic capsule: this aspect could perhaps be inspected by assaying the cytokine production.

As far as the degree of fibrous capsule contracture is regarded, we found that patients scoring $\geqslant 3$ according to the Baker scale, have a significant increase of CD57 + cells: this antigen is specific of natural killer cells and CD8 + cytotoxic/suppressor cells.

The presence of a high number of these cells is confirmed by the assessment of the cytotoxic activity against K562 target cells: we found a significant increase of this activity in patients with contracture rated $\geqslant 3$ compared to patients rated < 3, who showed an activity not different from that of normal subjects.

In conclusion, these results, although still preliminary being referred to a limited population of silicone implanted patients, suggest the possibility of an interaction between silicone and the immune system which cannot be disregarded when taking into account silicone-related complications. Further experimental and clinical investigations are warranted.

Acknowledgments

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