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Mouse inflammatory response to stainless steel corrosion products

R. B. TRACANA, J. P. SOUSA
National Insitute for Biomedical Engineering (INEB), Praça Coronel Pacheco 1 4000 Porto, Portugal

G. S. CARVALHO
Department of Biology, Aveiro Univeristy, Portugal and National Insitute for Biomedical Engineering (INEB), Praça Coronel Pacheco 1 4000 Porto, Portugal

Corrosion occurs regularly following long-term implantation of stainless steel. Little is known about the inflammatory and immunological potential of stainless steel corrosion products. AISI 316L stainless steel was anodically dissolved in a physiologically solution, HBSS, through a chronoamperometric process by imposing an external constant current of 0.5 mA. The solution, containing 245 μg of Fe, 112 μg of Cr, 75 μg of Ni and 13 μg of Mo, was injected in the peritoneal cavity of male C57BL/6 mice. Five animals were used per survival period of time: 4, 16, 24, and 48 h, 1, 2, 4, 8 and 16 weeks. Three control mice per survival period of time were injected with HBSS. For each assay, peritoneal samples were analysed not only for the total number of cells but also for the percentages of macrophages, lymphocytes and polymorphonuclear (PMN) cells, which were estimated by differential counting on Wright-stained cytocentrifuge preparations. Our follow-up study showed that stainless steel corrosion products induced an acute inflammatory response for a period of one week as demonstrated by the influx of PMN cells and macrophages. In contrast neither a chronic inflammation nor an immune response was observed indicating that the stainless steel solution caused a minor tissue response.

1. Introduction

Stainless steel is commonly used in orthopaedic surgery. Corrosion is a main problem in the long-term application of such biomaterial due to the biological aggressive effects. Stainless steel corrosion products include iron, chromium, nickel and molybdenum ions which can accumulate in tissues surrounding the implant [1] or be transported to distant parts of the body [2–4]. Our previous *in vitro* studies have demonstrated that the exposure of human lymphocytes to the above metal ions causes alteration of the expression of lymphocyte surface antigens [5–7] and inhibits the immune response as assessed by lymphocyte proliferation [8–10]. Further *in vivo* studies have demonstrated that subcutaneous injection of stainless steel corrosion products cause not only toxic effects on mouse testicular seminiferous epithelium [11] but also alterations to spleen cellular populations [12].

Inflammation is a complex process developed in response to tissue injury, and is characterized by the accumulation of large quantities of extracellular fluid and recruitment of polymorphonuclear (PMN) cells and macrophages. The peritoneal cavity, being a clearly defined and distinct body space, has been used for studying the inflammatory response to biomaterials as it provides an excellent site for analysing the local cell populations [13–5]. In the present paper we describe a follow-up study of the alterations to mouse peritoneal cell populations, following intraperitoneal injection of stainless steel corrosion products, in order to evaluate the inflammatory response to such metals.

2. Materials and methods

2.1. AISI 316L stainless steel dissolution

Stainless steel, type AISI 316L, was anodically dissolved in HBSS (Hank's balanced salt solution, SIGMA H2513) through a chronoamperometric process by imposing an external constant current of 0.5 mA. This current value was applied to the system using a galvanostat/potentiostat model DGR 16 connected to an amperimeter to monitor the selected current. The electrochemical equipment was switched off after a certain period of time given by Faraday's law, assuming that iron was dissolved preferentially. This is a rough assumption, however, the resulting slurry composition was analysed by atomic absorption spectrophotometery (AAS). Due to the chlorine evolution occurred during the dissolution process there was a significant increase in pH, rising from around 7 to 4. The adjustment of pH was carried out by adding NaOH 0.1 M to the metallic slurry. The stainless steel solution was sterilized in a pressurized autoclave at 120 °C for 20 min and the AAS analysis

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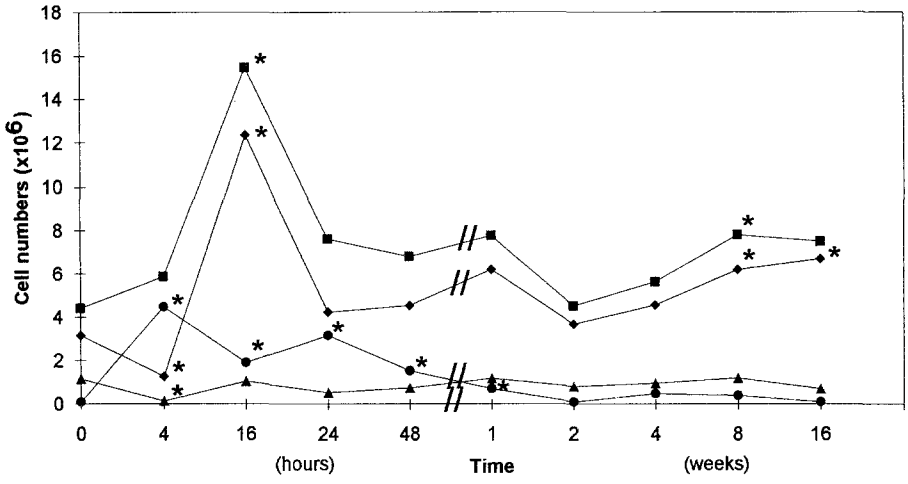

Figure 1 Total cell number and cell type distribution in mouse peritoneal cavity after injection of stainless steel corrosion products. *Significant differences ($p < 0.05$) between SS-treated and normal control (or time 0) groups. ■ Total cells; ◆ macrophages; ▲ lymphocytes; ● PMN cells.

showed that it contained 490 μg/ml of Fe, 224 μg/ml of Cr, 150 μg/ml of Ni and 26 μg/ml of Mo.

2.2. Intraperitoneal treatment of mice

Near-60-days-old male C57BL/6 mice (25–30 g) were purchased from "Instituto Gulbenkian de Ciência", Oeiras, Portugal. They were housed in groups of five per cage, and food and water were given *ad libitum*. Mice were injected intraperitoneally with 0.5 ml of SS-solution. At least three animals were used per survival period of time: 4, 16, 24, and 48 h, 1, 2, 4, 8 and 16 weeks. Three control mice per survival period of time were injected with the vehicle only, i.e. 0.5 ml of HBSS. Seven untreated mice were used as normal controls (untreated mice or time zero).

After the treatment time period the animals were killed by decapitation under deep ether anaesthesia. Peritoneal cells were collected following extensive washing of the peritoneal cavity with 4 ml of chilled PBS (phosphate-buffered saline SIGMA (D1408): 3–4 ml of peritoneal cell suspension was harvested per animal.

2.3. Differential cell counting of peritoneal cells

Three samples of each peritoneal cell suspension was used to count the number of cells per millilitre in an automatic Coulter counter. Other samples were taken to prepare smears of single-cell suspensions, by using a cytocentrifuge technique, in order to identify morphologically the peritoneal cell types. For this, cytocentrifuge (Heraeus sepatech, Labofuge Ae) was loaded with glass slides and filter paper strips, and 1 drop of 1% BSA (bovine serum albumin, SIGMA A9306) was added to each carrier block. According to the cell suspension concentration estimated as above, 8 to 20 drops of each cell suspension were added to the carrier blocks. Cells were centrifuged at 300 g for 10 min. Glass slides were removed from the carrier carefully to keep the slide and filter-paper strip together. After removing the strips without smearing the cell preparations, the cell smears were dried in the air and fixed for 2 min in 10% formalin in ethanol. After washing in water and dried in the air, the smears were stained by using the Wright method. Smears were examined under a microscope Olympus BH2. At least 200 cells were counted and the percentage of macrophages, lymphocytes and polymorphonuclear (PMN) cells were estimated.

2.4. Statistics

A two-tailed Student's t test was used to establish whether the difference between groups was significant: p values < 0.05 were considered significant.

3. Results

In normal controls, i.e. untreated mice, the peritoneal cavity contained a total of $4.4 \pm 1.2 \times 10^6$ cells. This peritoneal cell population was composed of $3.1 \pm 0.9 \times 10^6$ macrophages, $1.2 \pm 0.4 \times 10^6$ lymphocytes and $0.1 \pm 0.1 \times 10^6$ polymorphonuclear (PMN) cells, corresponding to a proportion of 71.1 ± 3.6%, 26.8 ± 5.6% and 2.1 ± 2.1%, respectively (Table I).

The SS-treatment of mice caused a significant ($p < 0.05$) increase in the total peritoneal cell numbers, from $4.4 \pm 1.2 \times 10^6$ cells at time 0 (or normal controls) up to $15.4 \pm 6.2 \times 10^6$ cells at 16 h and $7.8 \pm 3.1 \times 10^6$ cells at 8 weeks of treatment (Fig. 1). However only the peak at day 16 was significantly different ($p < 0.05$) when compared to its respective vehicle-control group (Table II. "Total cells" column; and Fig. 2).

TABLE I Cell number and cell distribution in normal mouse peritoneal cavity

Cell type	Cell number ($\times 10^6$)	Distribution (%)
Macrophages	3.1 ± 0.9	71.1 ± 3.6
Lymphocytes	1.2 ± 0.4	26.8 ± 5.6
PMN cells	0.1 ± 0.1	2.1 ± 2.1
Total cells	4.4 ± 1.2	100.0

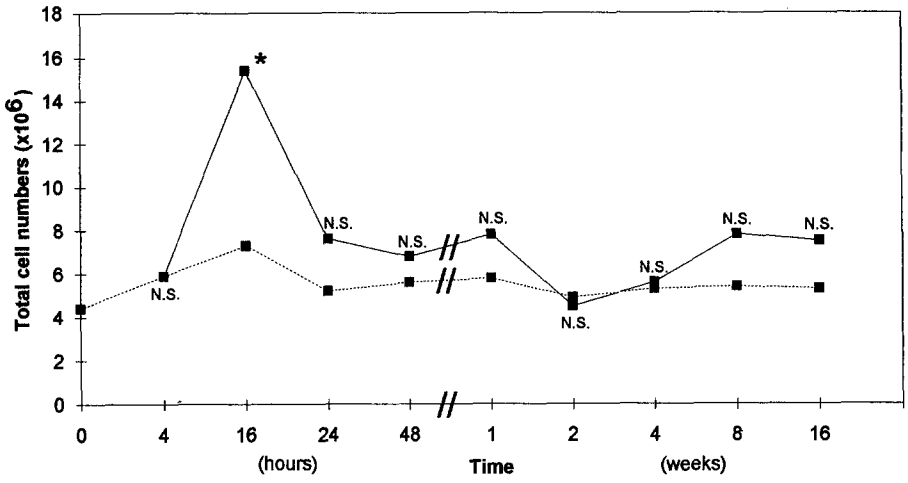

Figure 2 Total cell numbers in mouse peritoneal cavity after injection of stainless steel corrosion products or the vehicle. *Significant differences ($p < 0.05$) between SS-treated (—▮—) and their respective vehicle-control time groups (··▮··); N.S. no significant differences ($p < 0.05$) between SS-treated and their respective vehicle-control time groups.

TABLE II Cell type distribution in mouse peritoneal cavity following SS-solution or HBSS injections

		Cell type ($\times 10^6$)			
	n	Macrophages	Lymphocytes	PMN cells	Total cells
Untreated	7	3.7 ± 0.9	1.2 ± 0.4	0.1 ± 0.1	4.4 ± 1.2
4 h HBSS	3	4.9 ± 2.5	0.2 ± 0.2	0.9 ± 0.9	5.9 ± 3.5
4 h SS-treated	4	1.3 ± 0.4[a]	0.1 ± 0.2	4.5 ± 1.4[a]	5.9 ± 1.2
16 h HBSS	3	7.0 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	7.3 ± 0.1
16 h SS-treated	5	12.3 ± 4.5[a]	1.0 ± 1.0	1.9 ± 1.3[a]	15.4 ± 6.2[a]
24 h HBSS	3	4.7 ± 0.6	0.3 ± 0.4	0.1 ± 0.1	5.2 ± 2.5
24 h SS-treated	3	4.2 ± 1.6	0.5 ± 0.6	3.1 ± 1.8[a]	7.6 ± 2.5
48 h HBSS	3	4.8 ± 2.2	0.9 ± 0.6	0.1 ± 0.0	5.6 ± 3.3
48 h SS-treated	5	4.5 ± 2.0	0.7 ± 0.5	1.5 ± 1.1[a]	6.8 ± 2.4
1 w HBSS	3	5.8 ± 0.6	0.4 ± 0.3	0.1 ± 0.1	5.8 ± 2.6
1 w SS-treated	4	6.2 ± 1.3	1.2 ± 0.6	0.7 ± 0.4[a]	7.8 ± 1.6
2 w HBBS	2	4.5 ± 0.5	0.2 ± 0.3	0.1 ± 0.1	4.9 ± 2.3
2 w SS-treated	3	3.7 ± 0.1	0.8 ± 0.2	0.1 ± 0.0	4.5 ± 0.3
4 w HBSS	2	4.6 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	5.3 ± 2.5
4 w SS-treated	3	4.6 ± 0.2	1.0 ± 0.4	0.5 ± 0.3	5.6 ± 0.5
8 w HBSS	3	5.0 ± 0.3	0.4 ± 0.2	0.1 ± 0.1	5.4 ± 2.3
8 w SS-treated	3	6.2 ± 2.7	1.2 ± 0.6	0.4 ± 0.3	7.8 ± 3.1
16 w HBSS	3	4.9 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	5.3 ± 2.3
16 w SS-treated	4	6.7 ± 4.5	0.7 ± 0.6	0.1 ± 0.1	7.5 ± 5.0

Values represent the means ± SEM of *n* independent experiments conunted in triplicate.
[a] Significant differences ($p < 0.05$) between SS-treated and HBSS (vehicle control) groups.

This increase in total peritoneal cells was mostly due to the expansion of the macrophage population (Fig. 1). When compared to normal controls (or time 0), changes of macrophages were statistically significant ($p < 0.05$) after 4 and 16 h as well as after 8 and 16 weeks of SS-treatment (Fig. 1). However, then comparing the macrophage populations of SS-treated mice with the respective populations of HBSS-injected mice (vehicle-control groups) significant ($p < 0.05$) increases were observed after 4 and 16 h of SS-treatment only (Table II, "Macrophages" column; and Fig. 3). These results indicate that the macrophages accumulation in the peritoneal cavity, as a result of SS injection, occurred in the period up to 24 h of SS-treatment.

The lymphocyte population of the peritoneal cavity of SS-treated mice was maintained close to the control throughout the 16 weeks of the present study, except at 4 h of SS-injection, when a reduction in cell numbers was observed, from $1.2 \pm 0.4 \times 10^6$ at time 0 (or normal control) down to $0.1 \pm 0.2 \times 10^6$ (Fig. 1). This reduction was statistically significant ({$p < 0.05$) when compared to the normal control (Fig. 1), however it was not significant ($p < 0.05$) when compared to its respective vehicle-control group (Table II, "Lymphocyte" column; and Fig. 4). These results indicate that the peritoneal lymphocyte population was not stimulated by the injection of SS-solution.

In contrast to the lymphocyte population, a significant ($p < 0.05$) accumulation of polymorphonuclear

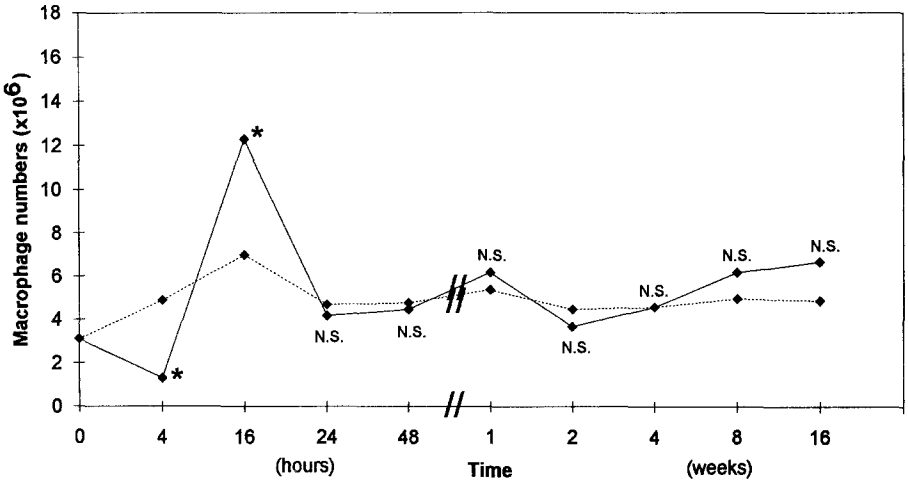

Figure 3 Macrophage numbers in mouse peritoneal cavity after injection of stainless steel corrosion products or the vehicle. *Significant differences ($p < 0.05$) between SS-treated (—◆—) and their respective vehicle-control time groups (·· ◆ ··); N.S. no significant differences ($p < 0.05$) between SS-treated and their respective vehicle-control time groups.

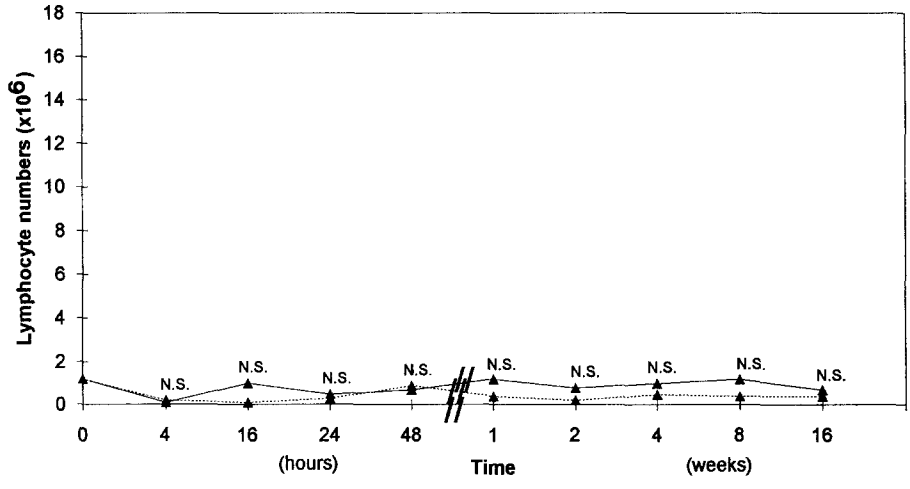

Figure 4 Lymphocyte numbers in mouse peritoneal cavity after injection of stainless steel corrosion products or the vehicle. *Significant differences ($p < 0.05$) between SS-treated (—▲—) and their respective vehicle-control time groups (··· ▲ ···); N.S. no significant differences ($p < 0.05$) between SS-treated and their respective vehicle-control time groups.

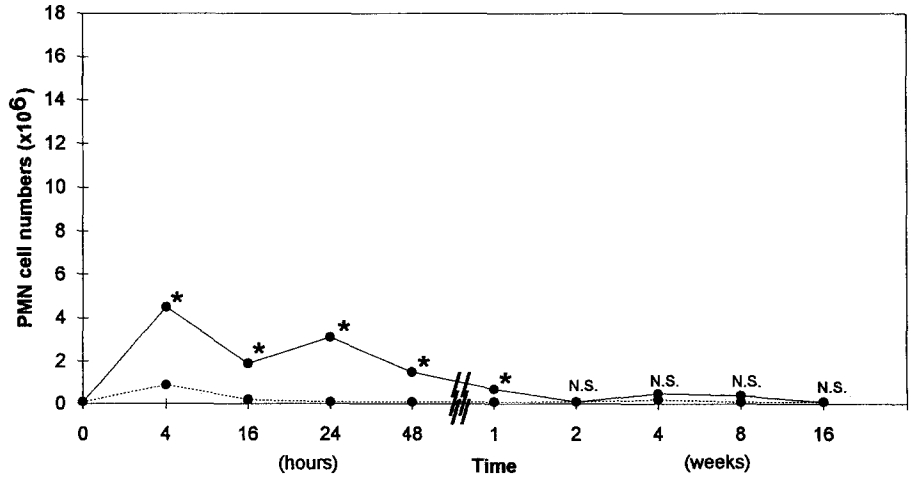

Figure 5 Polymorphonuclear cell numbers in mouse peritoneal cavity after injection of stainless steel corrosion products or the vehicle. differences ($p < 0.05$) between SS-treated (—●—) and their respective vehicle-control time groups (··· ● ···); N.S. no significant differences ($p < 0.05$) between SS-treated and their respective vehicle-control time groups.

(PMN) cells was observed for the period 1 week after SS-injection (Fig. 1). The changes in PMN cells after SS-injection was significantly different ($p < 0.05$) when compared not only with the normal control (or time 0) but also with their respective vehicle-control (or HBSS-treated) groups (Table II, "PMN cells" column; and Fig. 5). These results indicate that SS-injection caused an accumulation of PMN cells in the peritoneal cavity for a period of 1 week.

4. Discussion

In the present paper we used the mouse peritoneal cavity as an *in vivo* model to study the inflammatory response to stainless steel corrosion products because it is a well separated body space, thus an excellent site to study the alterations in cell populations associated to material injection.

The proportions of resident peritoneal cell populations found in untreated C57BL/6 mice were similar to those previously described by Plasman and Vray [16] for BALB/c mice: $69.4 \pm 4.5\%$ macrophages, $29.3 \pm 5.7\%$ lymphocytes and $0.9 \pm 0.3\%$ polymorphonuclear cells.

Our follow-up study showed that the injection of stainless steel in the mouse peritoneal cavity caused an inflammation for a period of 1 week. It revealed the characteristics of an acute inflammation as demonstrated by the influx of PMN cells and macrophages. In contrast, for the total 4 months analysed, no chronic inflammation was observed since the characteristic appearance of multinucleated giant cells, fibroblast-like cells, fibrin- or collagen-like fibres and granulomas did not occur. The absence of chronic inflammation was expected since we administered a solution, not a solid material, which was most probably spread rapidly through the body. In fact, chronic inflammatory reaction has been described following intraperitoneal implantation of solid materials, such as calcium phosphates [17], carbon fibre reinforced epoxy composites [18], and polymers [13, 15].

Of interest was the fact that the inflammatory response was not accompanied by an immune response, as lymphocytes were not stimulated by the presence of metals. This *in vivo* results confirm out previous *in vitro* studies demonstrating that metal ions are not able to stimulate the lymphocyte proliferation [10]; on the contrary, they inhibit the immune function [8–10]. This is in accordance with others [15] who have indicated that the inflammatory reaction developed as a result of peritoneal injection of polylactides is not followed by a proliferation of lymphocytes.

In this study we have demonstrated that stainless steel solution caused a minor tissue response as it induced an acute inflammatory response for a short period of one week. It remains, however, to be clarified whether the injection of stainless steel particles would induce the onset of a chronic inflammation.

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