JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 56–61

Stainless steel corrosion products cause alterations on mouse spleen cellular populations

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Stainless steel is a metallic biomaterial commonly used in orthopaedic surgery. In this study we looked at the effects of stainless steel corrosion products on spleen, in order to evaluate their potential immunotoxicological effects. For this purpose stainless steel, type AISI 316L, was electrochemically dissolved in a physiological salt solution. The final solution, containing 490 μg/ml Fe, 224 μg/ml Cr and 150 μg/ml Ni, was injected subcutaneously in mice. After several periods of time (4, 10 and 14 days) spleens were removed and analysed for: (a) their contents in Fe, Cr and Ni, (b) histological alterations, (c) enumeration of cellular populations. Results showed that stainless steel corrosion products accumulated in spleen, caused histological alterations, and induced changes in cellular populations. When compared to chromium and nickel, iron was the metal ion preferentially accumulated in spleen. The increase of the multinucleated giant cell population was accompanied by depletion of lymphocyte populations. This study indicates that stainless steel corrosion products can spread systemically and accumulate in spleen inducing histological and cellular alterations which may give rise to immunotoxicological consequences.

1. Introduction

Stainless steel, type AISI 316L, is widely used in orthopaedic surgery. Like all metallic implants, the corrosion and dissolution processes of its components are accelerated by the highly aggressive body fluids (reviewed in [1]). Several studies have shown that electrochemical reactions at the implant–tissue interface are the source of metal ion release into the tissue adjacent to the implants [2, 3]. Furthermore, chemical analyses of surrounding tissues have shown that metal ratios differ from those of the alloy, suggesting differences in transport of chemical species [4, 5]. Hildebrand and colleagues have reported that the metabolism and clearance of metal ions in patients after removal of orthopaedic implants is different [6]. They have shown that in contrast to cobalt, both nickel and chromium species form extracellular and intracellular precipitates, and that the biological transport of nickel is slower than that of cobalt but faster than that of chromium [6]. More recently, Merritt and Brown have demonstrated that nickel and molybdenum are rapidly and completely eliminated in the urine, most of the cobalt is excreted, and chromium is mostly accumulated in the kidney and spleen [7–9].

Toxicological research has recently selected the immune system as a target for toxic damage due to its complex nature. Immunotoxicology, or the study of adverse effects on the immune system resulting from exposure to chemicals or drugs, has been primarily involved with occupational and environmental situations (reviewed in [10]). Several studies have shown that most heavy metal ions are toxic for immune components at dosages below those resulting in general toxicity [11]. These minor dosages can cause effects at the level of regulatory immune cells, giving rise to dramatic consequences for the organism as a whole.

In the present study we dissolved stainless steel electrochemically and after injecting it into mice we looked at the effect on the predominant lymphoid organ for lymphocyte recirculation, the spleen. AISI 316L was electrochemically dissolved in a physiological salt solution, the resulting solution was injected subcutaneously in mice and their spleens were analysed for their metal contents, histological alterations, total cell numbers and lymphocyte populations.

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 H1641) 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. Thus, the electrochemical equipment was

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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 spectrophotometry.

2.2. Animals

Near-60-days-old male mice Charles River (29–40 g) were supplied from "Centro de Biologia" of "Instituto Gulbenkian de Ciência", Oeiras, Portugal. They were housed in groups of six per cage, and food and water were given *ad libitum*. A volume of 0.5 ml of SS-solution containing 245 μg/ml Fe, 112 μg/ml Cr and 75 μg/ml Ni was injected subcutaneously at days 0, 3, 6, 9 and 13, in a total of 20 mice. Control mice ($n = 10$) were equally injected but with HBSS only. Animals were sacrificed on days 0, 4, 10 or 14.

2.3. Atomic absorption spectrophotometry analysis

Mice spleens were analysed by atomic absorption spectrophotometry (AAS) in order to determine their contents of Fe, Cr and Ni. Spleens were removed from mice and immediately frozen at − 20 °C. Prior to AAS analysis, several steps were carried out to eliminate the water and organic matter present in the spleen matrix. First of all, the frozen spleens were allowed to thaw, placed inside teflon vessels and dried in a microwave oven model CEM MDS 2000. In order to optimize the drying period, several combinations of power, pressure and time were investigated using a four-stage drying program. The most suitable combination is listed in Table I.

To control the degree of spleen dryness, the samples were weighed after each drying stage. Only when the difference between consecutive stages was lower than 0.0001 g, were the samples assumed to be sufficiently dry.

Dry spleens were attacked with 5.0 ml of HNO_3 super pure purchased from Merck and placed inside the microwave oven to promote their digestion. The microwave parameters used in the four-stage digestion program are shown in Table II.

TABLE I Microwave parameters used to dry mice spleens

Stage	1	2	3	4
Power (%)	20	25	45	45
Pressure (psi)	50	50	50	50
Time (min)	15	30	10	10
Fan speed (%)	100	100	100	100

TABLE II Microwave parameters used for acid digestion of dried spleens

Stage	1	2	3	4
Power (%)	50	70	100	100
Pressure (psi)	30	40	70	80
Time (min)	5	5	5	10
Fan speed (%)	100	100	100	100

The resulting residues were dissolved in triply distilled water to a final volume of 25.0 ml. These solutions were then used to determine the metal ion levels by AAS. Iron ion analysis was carried out in a flame chamber Model 357 whereas chromium and nickel were analysed in a graphite furnace using a Perkin Elmer Model 4100ZL, which has higher sensitivity. The work programmes for the determination of the latter two elements are presented in Table III. The multiple addition method was the one chosen to perform the experimental AAS measurements.

2.4. Histological studies

For histological studies, mice spleens were fixed in Bouin's solution. After dehydratation in graded ethanol series and embedding in paraffin, sections were prepared in a micrototome Model Micron HM 320. Sections (4–6 μm thick) were counterstained with hematoxylin and eosin. Observations and photographs were obtained with a microscope model Olympus BH2-RFC equipped with an automatic photomicrographic system Model PM-10ADS.

2.5. Enumeration of spleen cells and immunocytochemistry

Cell suspensions from fresh spleens were prepared by teasing the organs apart in PBS (phosphate buffer saline). The connective tissue was retained over a fine wire mesh and the cell suspensions were collected in 10.0 ml of PBS for quantification of erythrocytes and leukocytes. After diluting the cell suspensions in methylene blue (1:2), the number of erythrocytes and leukocytes *per* spleen was estimated in a haemocytometer, using a microscope Olympus BH2.

Leukocytes were isolated from the total cell suspension by histopaque (d = 1.083, SIGMA) density gradient centrifugation at 300 g (SIGMA 3-12). The white layer containing leukocytes was collected and cells were washed three times by centrifugation in PBS. Leukocyte concentration was adjusted to 10^6 ml^{-1}, and 3.0 ml of each sample were equally distributed in three eppendorf tubes. Cells were centrifuged for 1 min at 10000 rpm in a microcentrifuge (Sanyo MSE, model Microcentaur). Tubes were kept on ice, and 50 μl of (a) diluted (1:100) monoclonal antibody to T lymphocytes (FITC-Thy1.2), (b) diluted (1:250) polyclonal antibody to B lymphocytes (FITC-polyvalent IgG, IgA and IgM) or (c) diluted (1:100) monoclonal

TABLE III Perkin Elmer programme used for Ni and Cr

Step	1	2	3	4	5
Nickel					
Temperature (°C)	110	130	1000	2250	2400
Time ramp (s)	4	6	12	0	1
Time hold (s)	16	28	20	7	2
Chromium					
Temperature (°C)	95	130	1500	2200	2400
Time ramp (s)	2	7	6	0	1
Time hold (s)	20	32	16	5	2

antibody to Thy1.1 antigen (FITC-Thy1.2) as a negative control (all from SIGMA) were added to each tube. After 30 min of incubation on ice, cells were washed by centrifugation to remove the unbound antibody. Cells were resuspended, mixed with one drop of glycerol-glycine mounting medium and placed on a microscope slide and covered with a coverslip. Cell preparations were examined under an incident light UV microscope Olympus BH2-RFC. For each microscope field, the number of fluorescently stained cells (under UV light) and the number of total lymphocytes (under visible light) were counted. At least 200 cells under visible light were counted and the percentage of fluorescing lymphocytes *per* preparation was calculated.

2.6. Statistical analysis

Student's *t*-test was applied to determine the statistical significance of the differences observed between means of the two groups. P values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Metal contents in spleens

Metal contents in the stainless steel solution was 490 μg/ml Fe, 224 μg/ml Cr, 150 μg/ml Ni and 26.5 μg/ml Mo. This composition (55.0% Fe, 25.2% Cr, 16.8% Ni and 3.0% Mo) is similar to the one present in the original solid alloy AISI 316L (reviewed in [12]).

Accumulation of iron, chromium and nickel in mice spleens following several days of stainless steel treatment is shown in Table IV. In contrast to iron, both chromium and nickel showed higher levels at day 4 of treatment (13.81 $\mu g\,g^{-1}$ and 11.92 $\mu g\,g^{-1}$ of dry tissue, respectively). After 14 days of treatment, iron levels increased 2.8 times (1054 to 2932 $\mu g\,g^{-1}$ of dry tissue), chromium 8.0 times (1.72 to 13.72 $\mu g\,g^{-1}$ of dry tissue), and nickel 4.4 times (1.84 to 8.01 $\mu g\,g^{-1}$ of dry tissue) when compared to their respective controls (Table IV).

The accumulation of metal ions in spleens on day 14 (compared to day 0) was 1878 $\mu g\,g^{-1}$ Fe, 12.0 $\mu g\,g^{-1}$ chromium, and 6.2 $\mu g\,g^{-1}$ nickel (see Table IV). Therefore the proportions of accumulated metals in spleen were 99.04% Fe, 0.63% Cr, and 0.33% Ni (Fig. 1). Bearing in mind the composition of the stainless steel solution (55.0% Fe, 25.2% Cr and 16.8% Ni), our results indicate that iron, compared to chromium and nickel, was preferentially accumulated in spleen (Fig. 1).

3.2. Histological alterations

After 4 days of stainless steel administration, spleen sections showed an apparent depletion of lymphocytes from white pulp. Consequently reticular cells and fibres were evident (Fig. 2a). It was difficult to distinguish the peripheral region of the periarteriolar lymphocyte sheath (PALS) from the marginal zone or the central region. In the red pulp an accumulation of erythrocytes near the capsule was observed and some multinucleated giant cells (MNGC) could be found (Fig. 2b). After 10 days of treatment a general tissue discolouration, brownish, was observed. Compared to 4 days of treatment, after 10 days a higher accumulation of erythrocytes near the capsule was clear and MNGC appeared in higher numbers and showed a dense cytoplasm (Fig. 2c). After 14 days of stainless steel reatment, deposits of metals were found in the red pulp and no clear separation between white pulp and red pulp was observed due to the infiltration of erythrocytes in white pulp (Fig. 2d). The morphology of MNGC on day 14 was similar to the control.

Furthermore, morphometry of multinucleated giant cells (Fig. 3) showed that at days 4 and 10 these cells presented a higher ($P < 0.05$) diameter (119.0 $\pm$ 22.7 μm and 116.5 $\pm$ 38.6 μm, respectively) than the control (96.5 $\pm$ 10.1 μm). In contrast, no significant differences ($P > 0.05$) were observed between MNGC at day 14 (97 $\pm$ .2 μm) and the control (Fig. 3).

3.3. Enumeration of spleen cells

Countings of erythrocytes and total leukocytes *per* spleen showed no significant differences ($P > 0.05$) between mice treated for 14 days on stainless steel and respective controls (Fig. 3). In contrast, a significant decrease of isolated leukocytes ($P < 0.05$) was observed in spleens of treated mice ($18.8 \pm 1.4 \times 10^6$) when compared to the control ($24.4 \pm 1.5 \times 10^6$) (Fig. 4 and Table V).

Immunocytochemistry of isolated spleen leukocytes showed a reduction of both T cells (from $8.7 \pm 0.6 \times 10^6$ down to $4.6 \pm 0.7 \times 10^6$) and B cells (from $7.1 \pm 0.1 \times 10^6$ down to $3.7 \pm 0.8 \times 10^6$) after 14 days of stainless steel treatment (Table V and Fig. 5). The reduction of both T and B populations from a total of 15.8×10^6 ($8.7 \times 10^6 + 7.1 \times 10^6$) down to 8.3×10^6 (4.6

TABLE IV Concentrations of metal ions in dry weight mice spleens

Days of treatment	Fe ($\mu g\,g^{-1}$)			Cr ($\mu g\,g^{-1}$)			Ni ($\mu g\,g^{-1}$)		
0	1054	–	–	1.72	–	–	1.84	–	–
4	1471	(1.4)[a]	[417][b]	13.81	(8.0)	[12.1]	11.92	(6.5)	[10.1]
14	2932	(2.8)	[1878]	13.72	(8.0)	[12.0]	8.01	(4.4)	[6.2]

[a] Values in ( ) brackets represent the ratio between the values after treatment time and the control (day 0)
[b] Values in [ ] brackets represent the difference between the values after treatment time and the control (day 0)

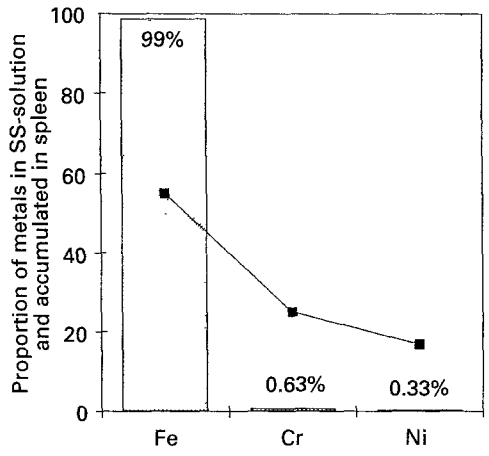

Figure 1 Proportions of iron, chromium and nickel present in stainless steel solution (—■—) and in spleens (columns) of mice treated with stainless steel for 14 days.

$\times 10^6 + 3.7 \times 10^6$) corresponds to an increase of the proportion of non-T and non-B cells, the so-called "null population", from 35.3% up to 55.9% (Fig. 5). Such negatively labelled cells may represent the macrophage/MNGC population.

4. Discussion

The immune system consists of an integrated mechanism in which several types of cells and molecules interact in order to ensure the survival of the body in a potentially hostile environment. This complex system makes the evaluation of immunological adverse effects of a compound very difficult. In fact, some immunotoxicants may (a) selectively target the immune system at doses below those that produce other evidence of toxic damage in other tissues, (b) cause immune dysfunction without histopathological changes, (c) and, conversely, cause histological changes with no signs of dysfunction (reviewed in [13]).

There is now a general consensus that the evaluation of immunological adverse effects of a compound requires a large panel of immunotoxicity tests. In our present work we addressed the question of immunotoxicological effects of metal ions released from stainless steel by looking at their immunopathological effects: (a) determination of their accumulation in spleen; (b) observation of spleen histological alterations; (c) enumeration of spleen total cell populations; and (d) estimation of changes in immune cell populations.

This study shows that mice spleens accumulate iron, chromium and nickel following injections of a solution containing stainless steel corrosion products. When compared to chromium and nickel, iron was the metal ion preferentially accumulated in spleen, probably due to iron sequester by iron-binding proteins and iron metabolism [14, 15]. Chromium was stored in spleen from day 4 to day 14, in contrast to nickel which was declining on day 14. These data are in agreement with Merritt and Brown reports demonstrating that chromium accumulates in spleen and is slowly eliminated in the urine, whereas nickel is rapidly eliminated [7–9].

Histological observations showed strong accumulation of erythrocytes and multimucleated giant cells (MNGC) in the red pulp. Bearing in mind that 75% of the volume of spleen is made up of red pulp [16], these results suggest that the deposition of metals in spleen

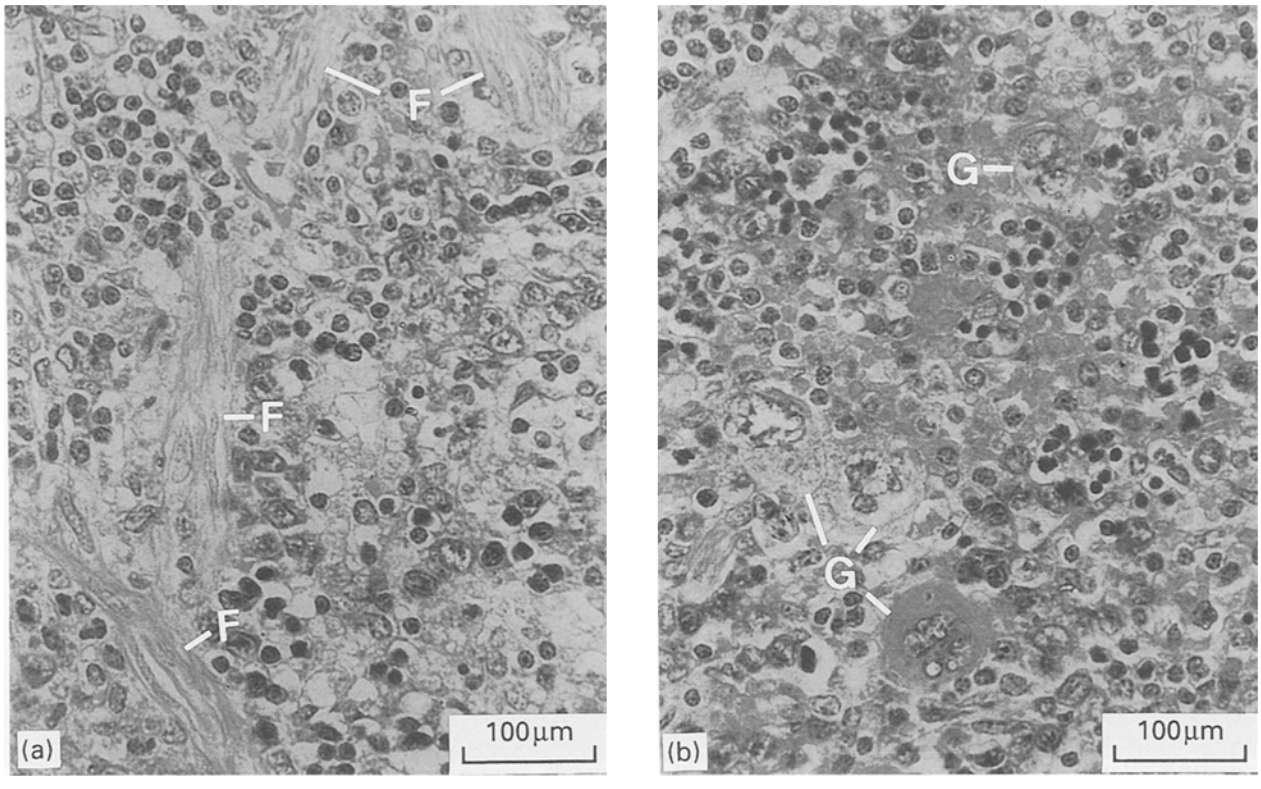

Figure 2 Spleen histological alterations observed after 4 days (a,b), 10 days (c) and 14 days (d). C: capsule, E: erythrocytes; F: reticular fibers; G: multinucleated giant cell; M: deposit of metals.

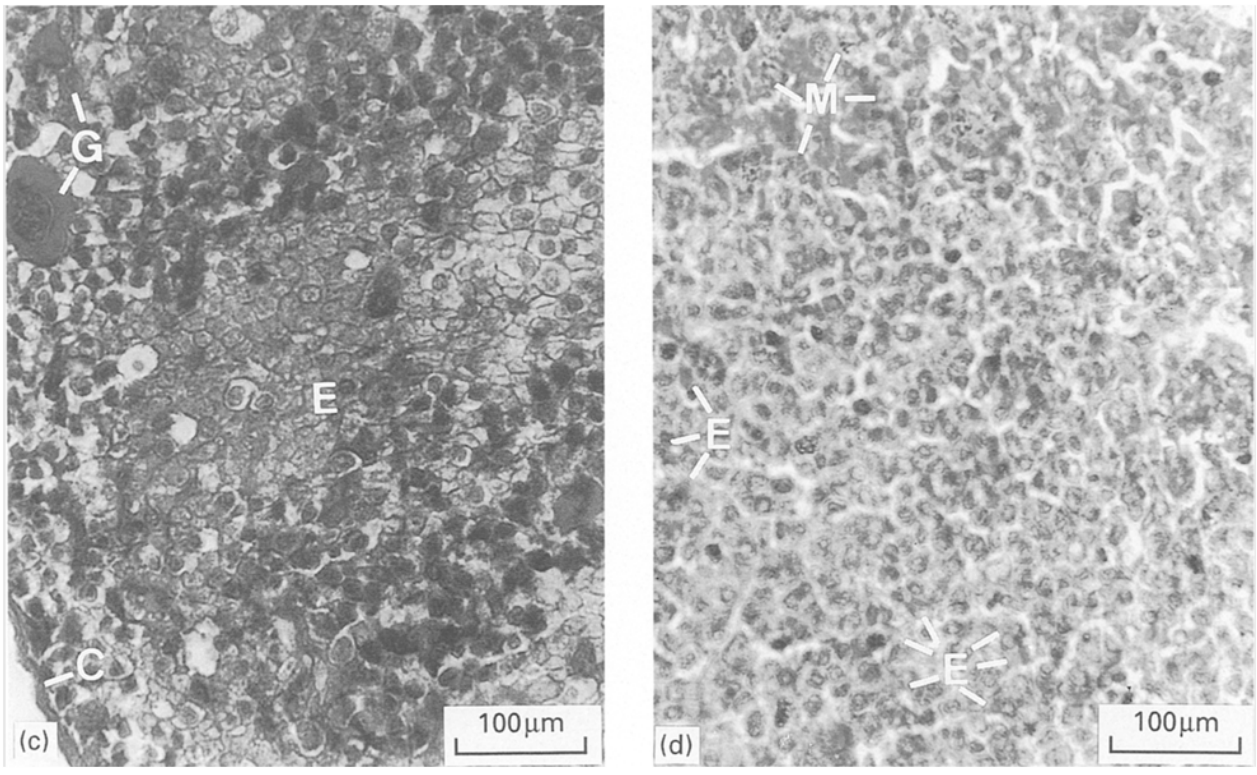

Figure 2 (Contd)

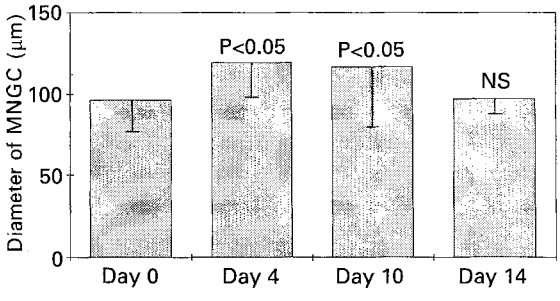

Figure 3 Morphometry of multinucleated giant cells (MNGC) in spleens of mice treated with stainless steel solution for different periods of time. Results are expresses as mean ± SD. $P < 0.05$: Means compared to the control (day 0) were significantly different. NS: Means compared to the control (day 0) were not significantly different.

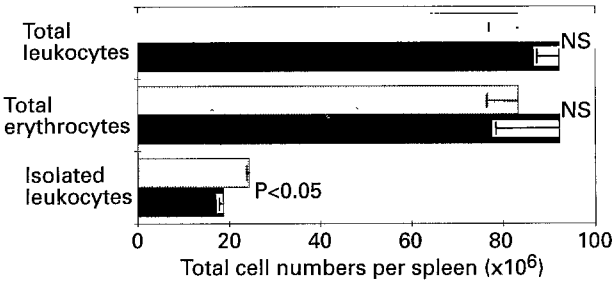

Figure 4 Enumeration of total leukocytes, total erythrocytes and gradient density isolated leukocytes in mice treated for 14 days (■) and controls (⊞).

leads to an accumulation of blood in spleen. Moreover, blood cells binding metal ions [17] may accumulate in red pulp and the expansion of MNGC may be associated to the intense activity of phagocytic cells in ingesting altered red blood cells [16]. This intense destruction of red blood cells may also have contributed to the increase of iron levels found in spleen.

TABLE V Spleen lymphocyte populations of untreated and stainless-steel-treated mice

Cells	Control ($\times 10^6$)	(%)	SS-treated mice ($\times 10^6$)	(%)
Leukocytes	24.4 ± 1.5		18.8 ± 1.4	
T cells	8.7 ± 0.6	(35.7 ± 2.6)	4.6 ± 0.7[a]	(24.6 ± 0.7)[b]
B cells	7.1 ± 0 1	(29.0 ± 0.6)	3.7 ± 0.8	(19.5 ± 4.3)

[a] Total cell numbers were determined at day 14 of SS treatment
[b] Number in parentheses represents percentage distribution

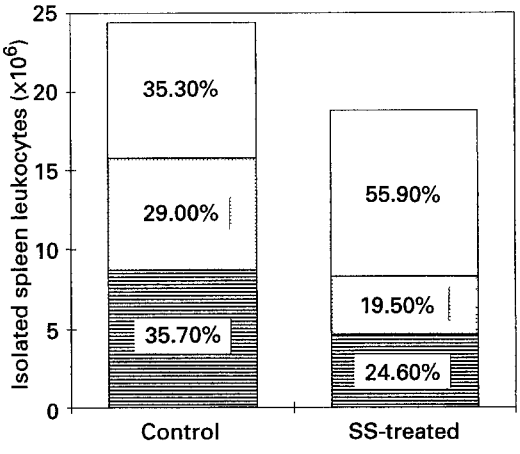

Figure 5 Immunocytochemical identification of lymphocyte populations in spleens of mice treated for 14 days and controls (□ null cells; ⊞ B lymphocytes; ■ T lymphocytes).

Histological observations of spleen suggested that metals accumulated in spleen caused a depletion of lymphocytes in white pulp. Such reduction of lymphocytes was confirmed by estimating the number of

isolated leukocytes and by immunocytochemical identification of lymphocytes. This lymphocyte depletion may be due to some interference of metal ions with molecules involved in lymphocyte adhesion to spleen endothelium. Adhesion molecules similar to homing receptors and addressins (well defined for lymph nodes, Peyer's patches, tonsils and inflammatory reactions) have not so far been identified in spleen (reviewed in [18]). Histological observations further showed that lymphocyte depletion was accompanied by erythrocyte infiltration in the white pulp, reinforcing putative alterations in spleen microenvironment and cellular distribution.

As the cell population isolated by density gradient centrifugation is mainly composed of lymphocytes and monocytes/macrophages [19], the increase of the null population (or non-T/non-B cells) must be due to an expansion of the monocytes/macrophage population. In addition to monocytes and macrophages, their derived MNGC (which were observed in high numbers in histological sections) may also integrate the null population. Further immunocytochemical labelling for monocytes/macrophages will clarify whether these non-T and non-B cells might be cells of the monocytes/macrophage lineage.

In conclusion, our results indicate that stainless steel corrosion products accumulate in spleen, cause histological alterations, and induce changes in cellular populations, in particular, lymphocyte depletion. In spleen, as in other lymphoid organs, the line between pathological and impressive but essential physiological reactions is vague [17], which makes difficult the interpretation of pathological alterations. Therefore further functional studies are now being developed in our laboratory in order to better clarify the immunotoxicological effects of metal ions released from metallic biomaterials.

Acknowledgements

This work was financially supported by project JNICT PBIC/C/SAU/1594/92.

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Received 25 October 1993
and accepted 31 March 1994