In vitro immuno-cytotoxicity of iron evaluated by DNA synthesis of human T lymphocytes stimulated via CD2 and CD3

G. S. CARVALHO
National Institute for Biomedical Engineering (INEB), Praca Coronel Pacheco, Porto, Portugal, and Department of Biology, University of Aveiro, Portugal
I. BRAVO*
National Institute for Biomedical Engineering (INEB), Porto, Portugal

In previous studies it was demonstrated that the *in vitro* exposure of human lymphocytes to iron, nickel or cobalt salts causes a significant reduction of lymphocytes expressing CD2 and CD3 surface antigens. Since both molecules are involved in T lymphocyte activation, these studies suggest that the above metals might affect T-cell activation and proliferation. Thus a method was developed for the stimulation of lymphocytes in which both CD2 and CD3 molecules were triggered simultaneously. For this purpose an anti-CD3 monoclonal antibody (mAb) was chemically bound to human erythrocytes (HE), forming HEαCD3 conjugates, which were used for lymphocyte stimulation. In this work the effects of iron on lymphocyte proliferation was studied, following stimulation via CD2 and CD3, in order to evaluate the immuno-cytotoxicity of iron. Increasing concentrations (5×10^{-3} μM–10^{2} μM) of iron citrate (Fe-citrate) showed that the higher concentration range (10 μM–10^{2} μM) caused moderate inhibitions of lymphocyte DNA synthesis (ranging between 18.3% and 78.6%). Furthermore the presence of monocytes in culture did not interfere in the inhibitory effect of Fe-citrate. Phenotypic characterisation of DNA-synthesizing cells in the presence of Fe-citrate showed that the $CD8^{+}$ (suppressor/cytotoxic) subset was the most reduced one. This study showed that iron inhibited T lymphocyte proliferation, particularly the suppressor/cytotoxic cells, suggesting that the presence of high levels of iron in *in vivo* situations can cause immunosuppression and, consequently, contribute to the onset of opportunistic infections and tumours.

1. Introduction
Stainless steels are widely used in orthopaedic applications as they combine good mechanical strength with low cost. Normally, they are passivated during manufacturing to improve the protective characteristics of the naturally formed oxide layer. When implanted in the body, host cells and tissue fluids interact with this passivation layer causing changes that lead to corrosion. The main disadvantage of stainless steels is this relatively low resistance to corrosion following implantation [1]. As iron is, at about 60%, the major alloying component in such alloys, high levels of iron could accumulate close to the implant as well as spread throughout the body.

It has been acknowledged that a material intended to interface with biological systems has to be evaluated with respect to its ability to perform with an appropriate host response in a specific application [2]. The host immune response may play an important role in biocompatibility as it is responsible for the maintenance of the physiological integrity of the body. Cellular interactions occurring at biomaterial/tissue interfaces have been recognized as important factors in determining the *in vivo* biocompatibility. Cell cultures, which constitute useful tools for studying in detail specific interactions at cellular and molecular level, can also be used as *in vitro* assays for cytocompatibility evaluation of biomaterials. Lymphocytes, being the effector cells of the immune response, should be considered as important targets for assessment of biomaterials cytocompatibility.

Lymphocyte surface antigens have been defined with specific monoclonal antibodies (mAb) leading to the identification and characterization of human lymphocyte subpopulations. In previous studies it was demonstrated that the *in vitro* exposure of human lymphocytes to iron, nickel or cobalt salts (but not to molybdenum salt) cause a significant reduction of lymphocytes expressing CD2 and CD3 surface antigens [3–5]. In contrast, none of the following antigens CD4, CD8, CD1, CD22, CD10 and HLA-DR were observed to be influenced by the exposure to metal salts [4, 5]. Since CD3 and CD2 are the T lymphocyte molecules involved in T-cell activation [6–9], previous

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studies suggested that the above metal salts might affect T-cell activation and proliferation. Thus it was decided to develop a method for the stimulation of lymphocytes in which both CD2 and CD3 molecules were triggered simultaneously. For this purpose human erythrocytes (which provide the ligand for CD2 [10–12]) were used as carriers for an anti-CD3 monoclonal antibody (which binds to CD3). The αCD3 mAb coupled to human erythrocytes (HE) formed HEαCD3 conjugates which were used for normal human peripheral blood lymphocyte stimulation. In the present study a method for human lymphocyte stimulation via CD2 and CD3 is described, and the inhibitory effects of increasing iron concentrations on DNA synthesis and proliferation of lymphocytes are analysed.

2. Materials and methods

2.1. Coupling of α-CD3 mAb to erythrocytes (HEαCD3)

Rat monoclonal antibodies (mAb) reactive with monomorphic determinants of human CD3 (αCD3 mAb, named YTH-12.5) was kindly provided by Dr Herman Waldmann (Cambridge, UK). After ammonium sulphate precipitation of YTH-12.5 ascites fluid, the pellet was exhaustively dialysed against 0.09% NaCl and the protein concentration adjusted to 1 mg ml^{-1}. Anti-CD3 mAb was then coupled to papain treated human erythrocytes (HE), using $CrCl_3$. For the papain treatment the concentration of erythrocytes was adjusted to 20% (v/v) in phosphate buffer saline (PBS) and treated with 0.3 mg ml^{-1} of papain (EC 3.4.22.2, Sigma) for 20 min at 37 °C in the presence of L-cystein HCl (Sigma, Chemical Company, USA). After treatment cells were washed in 0.9% NaCl and kept at 4 °C. For coupling, 50 μl of packed papain treated human erythrocytes were added to an equal volume of 1 mg ml^{-1} of αCD3 mAb. While undergoing strong mixing, (using a vortex) 100 μl of 0.1% $CrCl_3$ (Sigma) in NaCl, were added dropwise. After 1 h of incubation at room temperature and under slow rotation, the αCD3-coupled HE (HEαCD3) were washed three times and resuspended in RPMI 1640 medium (GIBCO BRL, USA) to give an erythrocyte concentration of 1% (v/v).

2.2. Lymphocyte culture

Heparinized peripheral venous blood was obtained from healthy blood donors at the Blood Service of the Portuguese Oncology Institute (Northern Region). Peripheral blood mononuclear cells (PBM) were isolated by lymphoprep (Nycomed, Norway) density gradient centrifugation. For the isolation of peripheral blood lymphocytes (PBL), adherent cells were depleted by incubating PBM in RPMI 1640 medium in polystyrene culture dishes (Greiner, Germany) for 1 h at 37 °C in a 5% CO_2 atmosphere.

Cells (either PBM or PBL) were resuspended in culture medium to give a final concentration of 2×10^6 cells ml^{-1}. The culture medium was RPMI 1640 supplemented with 500 IU ml^{-1} penicillin (Atral, Portugal), 0.5 mg ml^{-1} streptomycin (Sigma), 24 μM HEPES buffer (Sigma) and 40% foetal calf serum (GIBCO) or, alternatively, 40% normal human serum, as indicated in the results section.

Preparation of $FeC_6H_5O_7$ (Fisher Scientific Co., USA) solution (Fe-citrate) in HBSS (GIBCO) has been described in detail previously [3]. $NaC_2H_3O_2$ (Sigma) solution (Na-citrate) was used as a negative control.

Several preliminary studies were carried out to analyse lymphocyte DNA synthesis (a) in relation to culture conditions such as the presence (PBM) or absence (PBL) of monocytes and the serum source (foetal calf serum, FCS, or normal human serum, NHS), and (b) to compare with both classical PHA mitogen and soluble αCD3 mAb stimulations.

Optimal conditions for lymphocyte stimulation via CD2 and CD3 were used in this paper. Thus, to 50 μl of the cell suspension at 2×10^6 cells/ml^{-1} were added 50 μl of 1% (v/v) CD2/CD3-stimulating agent (HEαCD3) and 100 μl of the appropriate Fe-citrate concentration (or Na-citrate, as a control). Cells were cultured in triplicates in round-bottom 96-well microtitre plates (Nunc, Denmark) at 37 °C in a 5% CO_2, humidified atmosphere.

2.3. Assessment of lymphocyte DNA synthesis

Incorporation of ^{3}[H]-thymidine in cellular DNA was used for assessment of lymphocyte proliferation. For this purpose, cultures were pulsed with 0.2 μCi of ^{3}[H]-thymidine (Amersham, Radiochemical Centre, UK) for the last 4 h of a 72-h total culture time. In some experiments different culture times were performed, as shown in the results section. The ^{3}[H]-thymidine incorporation was measured by liquid scintillation counting after harvesting by a semi-automatic multiple harvester (Skatron, Liebyen, Norway). The results are expressed as mean counts per min (cpm) of triplicate cultures. Percentage alteration of ^{3}[H]-thymidine incorporation was calculated according to the formula:

$$\%\ \text{alteration} = \frac{\text{cpm in Fe-citrate containing cultures} \times 100}{\text{cpm in Fe-citrate free cultures}} - 100$$

Student's *t*-test was applied to determine the statistical significance of the differences observed between groups: *p* values $\leqslant 0.05$ were considered significant.

2.4. Cell viability and phenotype of proliferating cells

For the determination of cell counts, cell viability and phenotype of proliferating cells, PBL were cultured with HEαCD3 and iron citrate in volumes ten times

higher than before, i.e., 0.5 ml of PBL at 2×10^6 cells ml^{-1}, 0.5 ml of 1% (v/v) HEαCD3 stimulating agent, and 1.0 ml of the appropriate Fe-citrate concentration. At days 0, 2, 5 and 7, cells were recovered from culture plates, washed and analysed for cell viability. For this purpose cultured cells were mixed with equal volume of 0.2% (w/v) Trypan Blue (Sigma) in PBS (phosphate buffer saline) and immediately read in a haemocytometer chamber. Trypan blue cannot penetrate the membrane of live cells, therefore only dead cells become stained.

Autoradiography was used for identification of DNA-synthesizing cells in 72-h cultured cells. For this purpose, 0.1 μCi of ^{3}[H]-thymidine was added to the culture 1 h before removing the cells from the culture plates. Cells were washed twice with HBSS and resuspended in RPMI containing 10% FCS. Cell smears were prepared onto clean glass slides by cytocentrifugation and stored at $-20\,°C$. Phenotypic characterization of cultured lymphocytes was performed by an immunocytochemical method, alkaline phosphatase anti-alkaline phosphatase (APAAP), according to the procedure described by DAKOPATTS (Denmark). Briefly, before staining, slides were allowed to reach room temperature, fixed with dehydrated cold acetone for 15 min and then washed with TBS for 3–10 min. 50 μl of diluted mAb to CD2 (M720) 1:100, to CD3 (M756) 1:50, to CD4 (M716) 1:10 or to CD8 (M707) 1:100 (all from DAKOPATTS) were added to the areas to be stained. After 30 min incubation, the smears were washed in TBS and 50 μl of diluted (1:30) rabbit anti-mouse immunoglobulins (Z259 from DAKOPATTS) were added. After washing with TBS, 50 μl of soluble conjugates of calf intestinal alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (D651 from DAKOPATTS) were added. Both steps using Z259 and D651 were repeated to enhance the reaction. After washing with TBS, cell smears were incubated with 100 μl of Fast-Red TR chromogenic substrate for alkaline phosphatase for 20 min. The reaction was terminated by rinsing with TBS. Smears were counter-stained for 3–5 min with haematoxylin. For autoradiography, smears were exposed to Ilford KS for 10 days at 4 °C in the dark, and developed with D-19 Developer and F-10 Fixer (Eastman Kodak Co., Rochester, USA). At least 200 lymphocytes were counted per slide.

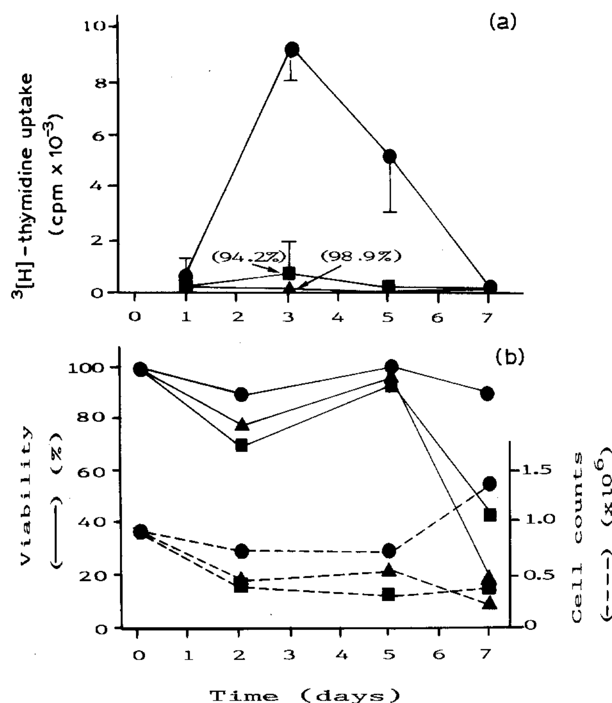

Figure 1 Time course study of Fe-citrate effects on DNA synthesis and proliferation of lymphocytes stimulated via CD2 and CD3. For the determination of DNA synthesis (a) 50 μl of PBL at 2×10^6 cells ml^{-1} were added to 50 μl of 1.0% (v/v) HEαCD3 and to 100 μl of 0.0 mM (●—●) 2.5 mM(■—■) or 10.0 mM (▲—▲) Fe-citrate. Cultures were pulsed with ^{3}[H]-thymidine for the last 4 h of the indicated culture time. Results are expressed as mean cpm ± SD values of triplicate cultures. Numerical figures in brackets express percentage inhibition of DNA synthesis. To estimate cell counts and cell viability by trypan blue exclusion test (b) larger cultures using 500 μl of PBL, 500 μl of HEαCD3, and 1000 μl of the appropriate Fe-citrate concentration were prepared in triplicate: standard deviations of results were below 5% (viability —; cell count - - -).

3. Results

Preliminary experiments using Na-citrate in parallel with Fe-citrate showed that only the latter was able to inhibit lymphocyte DNA synthesis, indicating that the citrate anion does not interfere in the DNA synthesis process.

Alterations in lymphocyte DNA synthesis as well as in cell numbers and cell viability caused by the presence of Fe-citrate were analysed in a time course study of CD2/CD3-stimulated lymphocytes (Fig. 1). Inhibition of lymphocyte DNA synthesis (assessed by ^{3}[H]-thymidine incorporation into cellular DNA) was observed when cells were incubated in the presence of Fe-citrate. In fact, at day 3 of cell culture, inhibitions of 94.2% and 98.9% were found when Fe-citrate was used at the final concentration of 1.25 mM or 5.0 mM, respectively (Fig. 1a). Cell counts and cell viability (determined by trypan blue dye-exclusion test) showed no significant variations on days 2 and 5 (Fig. 1b). However at day 7 the number of cells in the control culture, but not in Fe-citrate cultures, nearly doubled: an increase of 1.86 (Fig. 1b). Furthermore at day 7 the cell viability of Fe-citrate cultured lymphocytes was strongly reduced, down to 42.1% and 18.2% in 1.25 mM and 5.0 mM Fe-citrate, respectively, as compared to 89.1% in control cultures. Not only data from Fig. 1 but also preliminary control experiments (data not presented) performed in the absence of Fe-citrate, showed consistently maximum DNA synthesis at day 3 of cell culture. Thus in the following experiments the cells were cultured for a period of 3 days.

The effects of various concentrations of Fe-citrate (5×10^{-3} μM–10^2 μM) on DNA synthesis and cell viability of either CD2/CD3-stimulated or unstimulated PBL are presented in Fig. 2. The addition of Fe-citrate to unstimulated cells did not cause effects on both DNA synthesis and cell viability. In contrast, high concentrations (10 μM–10^2 μM) of Fe-citrate in CD2/CD3-stimulated cultures caused a moderate inhibition of lymphocyte DNA synthesis, but no effect

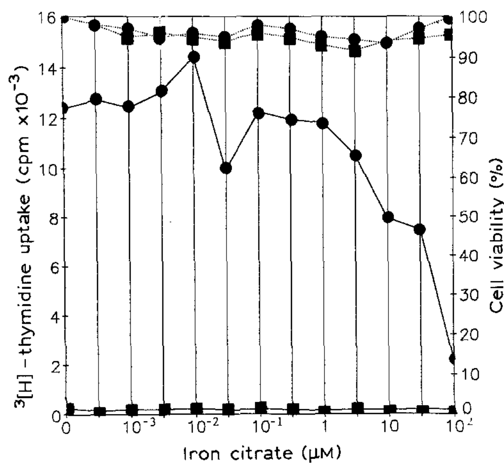

Figure 2 Effects of Fe-citrate on DNA synthesis and cell viability of either unstimulated or CD2/CD3-stimulated human lymphocytes. Results are expressed as mean of triplicate cultures and standard deviations were below 15%. Uptake: ■—□ no stimulation, ●—● CD2/CD3 stimulation; viability: ■----□ no stimulation, ●----● CD2/CD3 stimulation.

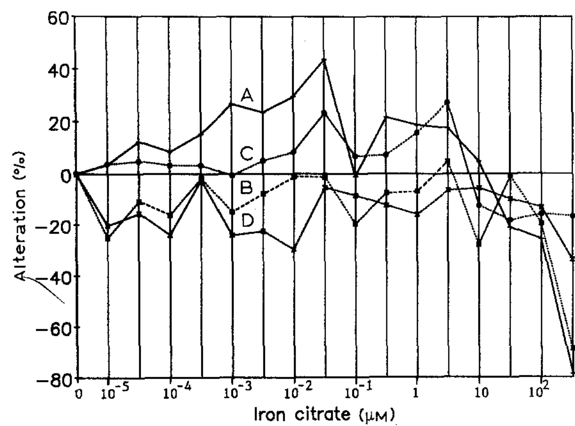

Figure 3 Effects of Fe-citrate on DNA synthesis of CD2/CD3-stimulated human lymphocytes cultured in the presence of monocytes. Results are expressed as percentage alteration compared to controls (Fe-citrate free cultures) of respective experiments (see Section 2).

on cell viability (Fig. 2). These results indicate that iron by itself is not able to induce lymphocyte DNA synthesis of unstimulated cells, and that it inhibits DNA synthesis of CD2/CD3-stimulated cells but does not cause cell death.

In order to better evaluate the effects of Fe-citrate on the immune respose—in which monocytes play an important role—Fe-citrate was added to cultures containing not only lymphocytes but also monocytes. As shown in Fig. 3 no significant alterations on lymphocyte DNA synthesis were observed with concentrations up to 10 μM Fe-citrate: a moderate increase (experiments A and C) as well as a moderate decrease (experiments B and D). However, over a Fe-citrate concentration of 10 μM a general decrease of lymphocyte DNA synthesis was found, reaching inhibition values of 18.3% (experiment B), 33.7% (experiment C), 68.7% (experiment D) and 78.6% (experiment A). The presence of monocytes in culture does not seem to interfere with the inhibitory effect of Fe-citrate upon lymphocyte DNA synthesis, since no significant differences ($p > 0.05$) were observed between PBL and PBM CD2/CD3-stimulated cultures (Fig. 4).

The identification of lymphocytes able to synthesize DNA in the presence of Fe-citrate was performed by autoradiography of ^{3}[H]-thymidine incorporated in cellular DNA combined with phenotypic characterization of proliferating lymphocytes by immunocytochemistry (APAAP method). It was observed that of the DNA-synthesizing cells in the presence of Fe-citrate (1.2 μM, 20 μM or 300 μM) the percentage of CD8 positive cells was the most reduced, when compared to CD2$^+$ or CD3$^+$ cells (Fig. 5). In contrast to CD8$^+$ cells, CD4$^+$ cells showed a tendency to increase in proportion in Fe-citrate cultured lymphocytes (Fig. 5c), and ratios of CD4/CD8 were shown to increase in parallel with increases of Fe-citrate concentrations in cell culture (Table I). These results indicate that Fe-citrate preferentially inhibits the proliferation of CD8$^+$ (T suppressor/cytotoxic) cells.

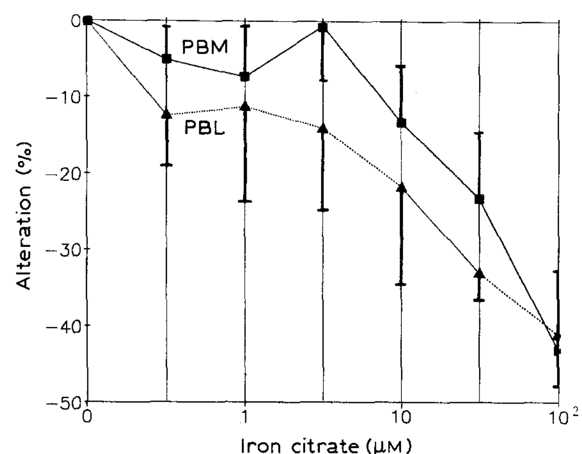

Figure 4 Effects of Fe-citrate on DNA synthesis of CD2/CD3-stimulated human lymphocytes cultured in the absence (PBL) or in the presence (PBM) of monocytes. Percentage alteration compared to controls (Fe-citrate free cultures) of respective experiments was determined as described in Section 2. Results are expressed as mean ± SD of three separate experiments.

TABLE I CD4$^+$/CD8$^+$ ratios of DNA-synthesizing lymphocytes following stimulation via CD2 and CD3 and cultured in the presence of Fe-citrate

Fe-citrate (μM)	Experiment A	Experiment B	Experiment C
0.0	0.87	0.72	0.65
1.2	1.47	1.22	0.81
20.0	1.80	1.28	0.91
300.0	2.02	2.38	1.05

4. Discussion

Iron plays an important role in functional activities of the body. Most of the total body iron is present as haemoglobin in erythrocytes, or stored as ferritin in different cell types [reviewed in 13]. Lymphocyte proliferation, which is a main feature of immune responses, requires the presence of a seric iron-containing protein known as transferrin [reviewed in 14]. Transferrin receptors (TfRs) are induced on T

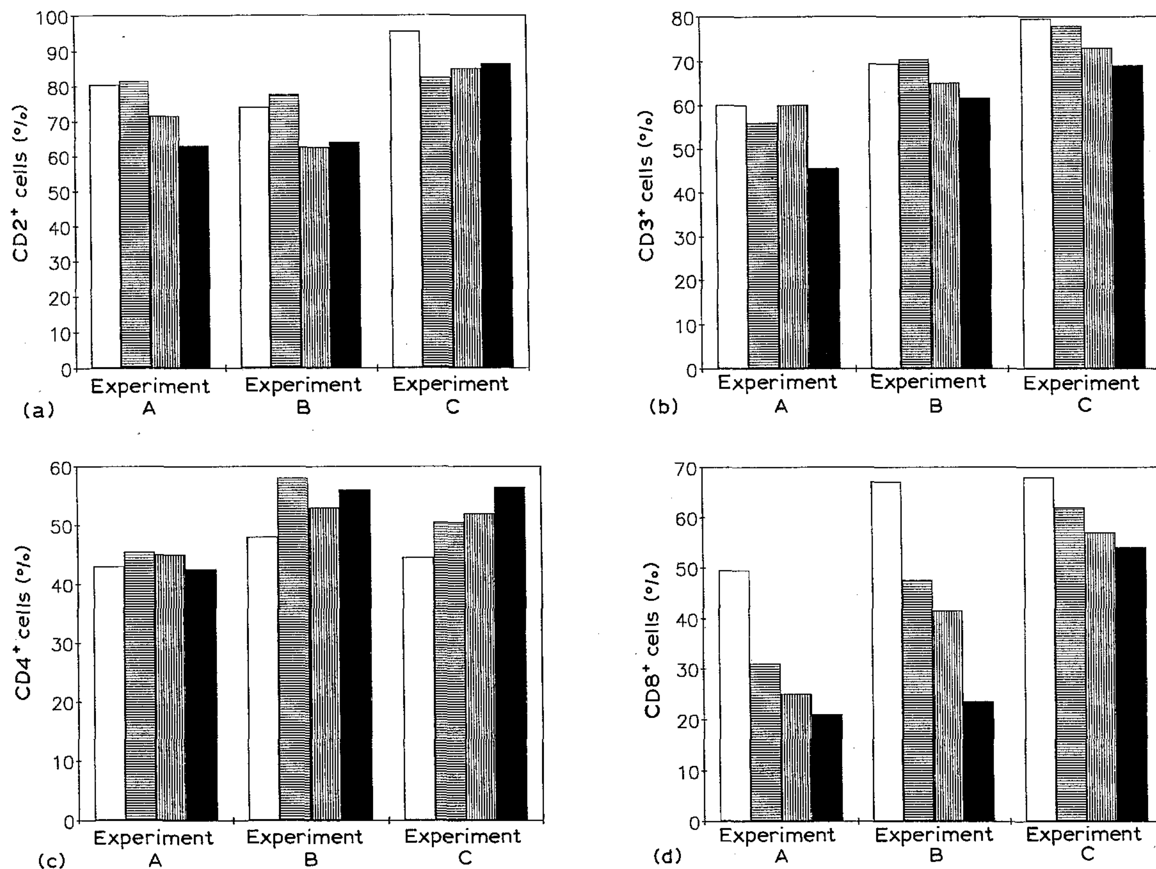

Figure 5 Phenotypic characterization of lymphocytes able to synthesize DNA in the presence of Fe-citrate, following CD2/CD3-stimulation. DNA-synthesizing lymphocytes were identified by autoradiography of ^{3}[H]-thymidine pulsed cell cultures, and the percentage of labelled cells positive for CD2, CD3, CD4 and CD8 was determined by the APAAP method described in Section 2 (□0.0 μM; ▤ 1.2 μM, ▥ 20 μM; ■ 300 μM).

lymphocyte surfaces following mitogenic stimulation, after which lymphocytes enter the S-phase of cell cycle [15]. It has been indicated that TfRs are significantly inhibited by supplementation of iron salts [16]. Similarly, in the present study we observed that iron causes inhibition of DNA synthesis and proliferation of lymphocytes stimulated via CD2 and CD3. This inhibition of lymphocyte DNA synthesis caused by iron might be due to a decrease of TfRs [16], thus impeding the initiation of the cell cycle [15] and, consequently, inhibiting lymphocyte proliferation. This inhibition of lymphocyte DNA synthesis is not a reversible process since the removal of iron from the culture medium (after 30 min or 24 h of exposure to lymphocytes) did not alter the inhibition of lymphocyte DNA synthesis (data not shown).

Cells of the monocyte/macrophage lineage have a main role in iron metabolism as well as in immune responses [17, 18], and our study showed that the presence of monocytes in culture did not alter the inhibitory effects of iron on lymphocyte DNA synthesis. Furthermore the suppressor/cytotoxic lymphocyte subpopulation was the one most affected by the presence of iron in the culture, indicating that immune functions related to foreign cell killing may be hampered. A similar *in vivo* situation, in which lymphocyte proliferation is inhibited and cytotoxic T cells are depressed by the presence of iron, can contribute to uncontrolled bacterial growth, leading to infections as well as to the onset of tumours. Such situations may occur in tissues surrounding an iron-base alloy and in other tissues where iron can accumulate.

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