

Differences in iron requirements by concanavalin A-treated and anti-CD3-treated murine splenic lymphocytes

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Fe availability is critical for optimal lymphocyte proliferation; however, the minimum required levels are unknown. Such information is valuable when assessing *in vitro* immune responses in Fe-deficient subjects, because serum (Fe) added to the culture medium may replete lymphocytes. To address this issue, splenic lymphocytes obtained from seventeen 3-month-old C57BL/6 mice were incubated without and with 1 mg/l concanavalin A or 50 µg/l anti-CD3 antibody in media that contained between 0.113 and 9.74 µmol Fe/l. Fe was provided by either fetal calf serum (FCS, 0–100 ml/l), newborn calf serum (NBCS, 0–100 ml/l), or NBCS (10 ml/l) plus ferric ammonium citrate. As expected, the rate of DNA synthesis increased with Fe levels ($P < 0.01$). Maximum DNA synthesis was obtained with 2.26 µmol Fe/l (50 ml FCS/l) for concanavalin A and 0.895 µmol/l (20 ml FCS/l) for anti-CD3-treated cells. In serum-free media (0.113 µmol Fe/l), the proliferative responses to concanavalin A were below the background, while they rose 5.5-fold in anti-CD3-treated cells ($P < 0.05$). In apotransferrin-supplemented media (0.13 µmol Fe/l), the proliferative responses to concanavalin A and anti-CD3 antibody were 18.6 and 71 %, respectively, of that obtained with 4.66 µmol Fe/l (100 ml FCS/l). Interleukin 2 secretion also followed the same trend as lymphocyte proliferation. Since differences between both mitogens persisted after FCS was substituted with NBCS, we can rule out an effect on ribonucleotide reductase activity, or by other serum growth factors. We speculate an Fe effect at an early step of T-cell activation. Data suggest that the minimum Fe concentration required for lymphocyte proliferation varies with the mitogen.

Iron: Lymphocyte proliferation: Concanavalin A: Anti-C antibody: Interleukin 2

It has been established that Fe plays crucial roles on many cellular functions and it is also required for proliferation of all cells, specifically lymphocytes (Mainou-Fowler & Brock, 1985; Cazzola *et al.* 1990; Brock, 1992). Peripheral T cells from healthy individuals as well as leukaemic cell lines fail to proliferate when they are incubated in serum-free and transferrin-free media (Mainou-Fowler & Brock, 1985; Cazzola *et al.* 1990; Haq *et al.* 1995). In fact they undergo apoptosis (Haq *et al.* 1995). Antigen and mitogen-treated T lymphocytes show an increase in transferrin receptor molecules to ensure sufficient Fe uptake from culture media to satisfy the increased demand of proliferating cells (Cazzola *et al.* 1990). The role of Fe in T-lymphocyte proliferation is in part due to the fact that this trace element is a cofactor of ribonucleotide reductase, the enzyme that

reduces ribonucleosides to deoxyribonucleosides, which are later converted to deoxyribonucleotides. This enzyme has a rapid turnover and therefore Fe must be supplied continuously in order to sustain DNA synthesis and cell proliferation (Thelander *et al.* 1983; Furakawa *et al.* 1992).

In lymphocytes cultured *in vitro*, Fe is usually supplied in the form of fetal calf serum (FCS), bovine serum, newborn calf serum (NBCS), or human AB serum at a final concentration of 100 ml/l. However, the minimum concentrations of either Fe or serum required by murine splenic lymphocytes activated by different mitogens is unknown. Such information is valuable especially when studying the effects of Fe deficiency in human subjects and laboratory animals on *in vitro* lymphocyte proliferation and other functions. A high serum concentration may replete Fe-deficient cells

Abbreviations: BSA, bovine serum albumin; Con A, concanavalin A; FCS, fetal calf serum; IL, interleukin; NBCS, newborn calf serum.

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in vitro and thus mask the differences between Fe-normal and Fe-deficient cells. A very low concentration of serum will reduce Fe concentration in the culture medium and reduce the rate of DNA synthesis even of the Fe-normal cells, thus leading to negative results. In the present study, we determined whether there are differences in the requirement for Fe and/or serum by normal murine splenic lymphocytes that are activated by concanavalin A (Con A) and anti-CD3 antibody.

Materials and methods

Materials

Reagents were received from the following sources: Sigma (St Louis, MO, USA) apotransferrin, Con A, β -mercaptoethanol, Drabkin's reagent, ferric ammonium citrate, haemoglobin standard, Fe assay kits, bovine serum albumin (BSA); GIBCO (Grand Island, NY, USA) RPMI-1640 with 25 mM-HEPES, FCS, NBCS, nonessential amino acids, sodium pyruvate, penicillin-streptomycin; Bio-Whittaker (Walkersville, MD, USA) L-glutamine; Harlan Teklad (Madison, WI, USA) the AIN-76 diet that contained 0.895 mmol Fe/kg (ferrous sulfate); NEN Dupont (Boston, MA, USA) [3 H]-thymidine (247.9 GBq/mmol); ICN (Costa Mesa, CA, USA) Cytosint; PharMingen (Costa Mesa, CA, USA) anti-CD3 antibody; R & D Systems (Minneapolis, MN, USA) interleukin (IL) 2 assay kits.

Evaluation of iron status at the end of the feeding period

Seventeen 6-week-old C57BL/6 female mice were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA). Two to three mice were housed per microisolator system cage, which was fitted with a filter. The cages were sterile. Mice had free access to their feeds and sterile deionized water for 2 months. The vivarium temperature was set at 22°C, with a 12 h light-dark cycle. At the time of killing, mice were anaesthetized by diethyl ether inhalation for 30–60 s. After blood was drawn from the retro-orbital plexus, they were killed by cervical dislocation. Haemoglobin and packed cell volume were measured by the cyanmethaemoglobin method and centrifugation respectively. The livers were removed, weighed and immediately frozen at -40°C until used for Fe assay. Liver homogenates were prepared as we reported previously (Kuvibidila *et al.* 1998). Fe concentration in each supernatant fraction was assayed by colorimetric method using a kit that was purchased from Sigma (catalogue no. 565). Briefly, 250 μ l liver Fe extract or Fe-free distilled deionized water was mixed with 2.5 ml Fe buffer reagent in a plastic cuvette. The background optical density was read (determined in a Beckman DU-64 spectrophotometer; Fullerton, CA, USA) set at a wavelength of 560 nm. After 50 μ l colour reagent (ferrozine) was added to each cuvette and mixed for 10 s by a vortex mixer, samples were incubated at 37°C in a water-bath for 10 min. Cuvettes were vortexed again for 10 s and the final optical density was determined at the same wavelength as for the initial reading. The Fe

concentrations were calculated by the following formula: Fe concentration (μ g/l) = ((sample final optical density - sample initial optical density)/(standard final optical density - standard initial optical density)) \times 5000, where 5000 μ g/l is the Fe concentration of the standard. All assays were done in duplicate.

Preparation of spleen cell suspension

After spleens were removed under sterile conditions, single-cell suspensions were prepared by standard techniques in serum-free RPMI-1640 wash medium that was supplemented with 10 g BSA/l, 50 mg streptomycin/l and 5×10^4 units penicillin/l. This medium contained 0.113 μ mol Fe/l. Cells were washed at 400 g at 4°C for 10 min. The supernatant fraction was decanted and the pellets were resuspended in 1 ml ice-cold sterile deionized water to lyse erythrocytes. Cells were further washed twice under the same conditions. The pellets were then resuspended in 2 ml wash medium. Total and viable cells were counted using a haemocytometer under a light microscope after cells were diluted in a solution of Trypan Blue (4 g/l).

Lymphocyte proliferation

Viable cells (1×10^6) were mixed with either 1 mg Con A/l or 50 μ g anti-CD3 antibody/l. These concentrations were the optimal levels based on mitogen dose responses that were performed in preliminary experiments. For background DNA synthesis, culture medium was added to cells instead of the mitogen. The culture media were composed of RPMI-1640, 50 mg streptomycin/l, 5×10^4 units penicillin/l, 2 mM-L-glutamine, 1 mM-sodium pyruvate, 0.1 mM-nonessential amino acids and 50 μ M- β -mercaptoethanol. FCS was added at the following concentrations: 0, 10, 20, 50 and 100 ml/l. The Fe concentrations of these culture media were 0.113, 0.450, 0.895, 2.260 and 4.660 μ mol/l respectively. In some experiments, 25 mg apotransferrin/l, 10, 20, 50 or 100 ml NBCS/l were added to the culture media instead of FCS. The Fe concentrations of the apotransferrin and NBCS-supplemented media were: 0.130, 0.201, 0.403, 0.806 and 1.925 μ mol/l. In another subgroup of mice (n 5), ferric ammonium citrate was added to the culture medium that contained 0.201 μ mol Fe/l in the form of NBCS (10 ml/l). The final concentrations of Fe were 2.100, 4.975, and 9.740 μ mol/l. Mitogen stimulated and unstimulated cells were incubated at 37°C, 5% CO₂ in a humidified NAPCO incubator (Model 5100; Portland, OR, USA) for 48 h before being pulsed with 37 kBq [3 H]thymidine per 2×10^5 cells. After 24 h, the cultures were harvested (PHD Cell Harvester; Cambridge Technology, Watertown, MA, USA). Each filter was transferred to a scintillation vial that contained 2 ml Cytosint (ICN). The radioactivity incorporated into DNA was measured by counting each sample for 1 min in an LKB liquid scintillation counter (Model 1219; Turku, Finland).

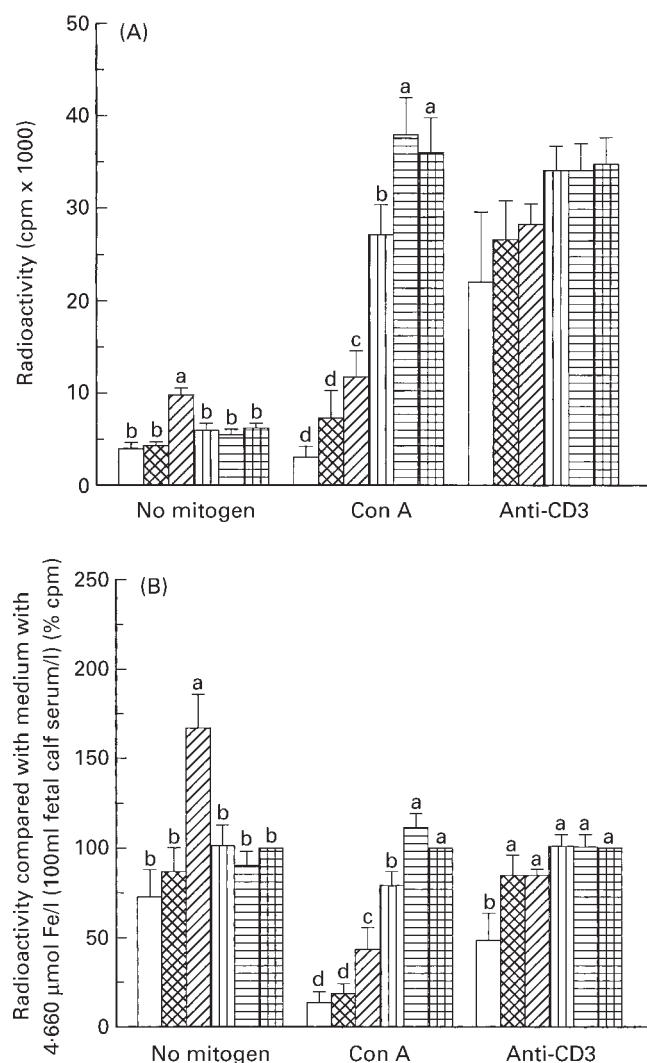


Fig. 1. Effects of iron and fetal calf serum concentrations on splenic lymphocyte proliferation. For details of procedures, see p. 68. Con A, concanavalin A, □, 0.113 µmol Fe/l; ▨, 0.130 µmol Fe/l; ▩, 0.450 µmol Fe/l; ▪, 0.895 µmol Fe/l; ▫, 2.260 µmol Fe/l; ▬, 4.660 µmol Fe/l. The corresponding fetal calf serum concentrations are: 0 ml/l (bovine serum albumin added to medium), 0 ml/l (apotransferrin added to medium), 10, 20, 50 and 100 ml fetal calf serum/l for 0.113, 0.130, 0.450, 0.895, 2.260 and 4.66 µmol Fe/l respectively. Values are means for seventeen mice with standard errors shown by vertical bars. ^{a,b,c,d}Mean values for the same mitogen treatment with unlike superscript letters were significantly different ($P < 0.05$). cpm, counts per minute.

Measurement of interleukin 2

Macrocultures that contained 1×10^6 viable spleen cells/ml were incubated without or with 1 mg Con A/l or 50 µg anti-CD3/l for 48 h in culture media that contained 0.130–4.660 µmol Fe/l supplemented in the form of FCS. Cultures were incubated in 12×75 culture tubes at 37 °C, 5% CO₂ in a humidified atmosphere. At the end of the incubation period, tubes were centrifuged at 4 °C, 670 g for 10 min. Supernatant fractions were divided into subsamples and frozen at –40 °C until IL-2 was assayed by enzyme immunoassay with kits that were purchased from R & D Systems. Instructions provided by the

vendor were carefully followed. A standard curve was included in each experiment and the assay was performed in duplicate. The minimum detection level was 15.6 pg/ml.

Calculations and statistical analysis

Descriptive statistics (mean values with their standard errors) and ANOVA were calculated by the use of Microstatistical program (Ecosoft Inc., Indianapolis, IN, USA) and as described in the literature (Munro, 1993). When ANOVA showed that there were significant differences between culture media, Scheffé's test was used to compare means of two media at a time. The level of significance was set at $P < 0.05$.

Results

The mean concentrations of haemoglobin (146.6 (SEM 4.822) g/l), liver Fe stores (1.03 (SEM 0.082) µmol/g liver), packed cell volume (49.6 (SEM 0.49) %), and body weight (20.0 (SEM 0.359) g) were within the range found in normal mice in our laboratory (Kuvibidila *et al.* 1998). As one would expect, the rate of DNA synthesis of splenic lymphocytes increased with Fe or FCS concentrations (Fig. 1(A) and (B)). [³H]Thymidine incorporation into DNA of non-activated cells was not different between cultures that were incubated in BSA- and apotransferrin-supplemented media. However, the addition of Fe in the form of FCS resulted in a significant increase in the rate of background DNA synthesis, which reached a peak at 0.450 µmol/l (10 ml FCS/l), but decreased at higher concentrations ($P < 0.05$).

In mitogen-treated cells, the rate of DNA synthesis significantly increased with Fe and FCS concentrations (Fig. 1(A) and (B), $P < 0.05$). However, the pattern of proliferative responses to Con A-treated cells was different from that of anti-CD3 treated cells. The maximum rate of DNA synthesis was obtained at 2.260 µmol Fe/l (50 ml FCS/l) and 0.895 µmol Fe/l (20 ml FCS/l) for Con A-treated and anti-CD3 treated spleen cells respectively. In serum-free BSA-supplemented medium that contained 0.113 µmol Fe/l, [³H]thymidine incorporation into DNA of Con A-treated cells was reduced by 24% compared with the background ($P > 0.05$), and it was only 13.7% of that obtained with 4.660 µmol Fe/l (100 ml FCS/l) ($P < 0.05$; Fig. 1(B)). When apotransferrin was added to the BSA-supplemented medium thus increasing the Fe levels to 0.13 µmol/l, the proliferative response to Con A rose to 18.6% of that obtained with 4.660 µmol Fe/l (100 ml FCS/l). The slight increase in the rate of DNA synthesis was very likely to be due to improved absorption of residual Fe by apotransferrin. In anti-CD3 treated cells, the absence of serum and apotransferrin did not inhibit DNA synthesis compared with the background. In fact, the proliferative response rose by 5.5-fold ($P < 0.001$) and it was 48.6% of the response obtained with 4.660 µmol Fe/l or 100 ml FCS/l (Fig. 1(B)). Apotransferrin significantly increased the proliferative responses to anti-CD3 ($P < 0.05$) to the point that they were 71% of the maximum. When the medium contained 0.895 µmol Fe/l, [³H]thymidine uptake by anti-CD3 treated cells was

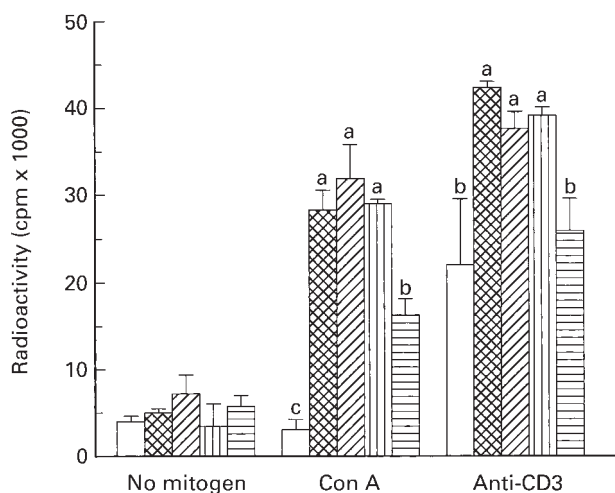


Fig. 2. Effects of iron concentrations added to the culture medium in the form of newborn calf serum on the rate of DNA synthesis in concanavalin A (Con A) and anti-CD3 treated cells. For details of procedures, see p. 68. □, 0-113 μM -Fe; ▨, 0-130 μmol Fe/l; ▩, 0-450 μmol Fe/l; ▪, 0-895 μmol Fe/l; ▫, 2-260 μmol Fe/l; ▬, 4-66 μmol Fe/l. The corresponding newborn calf serum concentrations for different iron levels were: 0, 10, 20, 50 and 100 ml newborn calf serum/l for 0-113, 0-201, 0-403, 0-806, 1-925 μmol Fe/l respectively. Values are means for five mice with standard errors shown by vertical bars. ^{a,b,c}Mean values for both Con A and anti-CD3 with unlike superscript letters were significantly different ($P < 0.05$). cpm, counts per minute.

101% of the maximum obtained with 4-660 μmol /l whereas it was less than 80% for Con A-treated cells (Fig. 1(B)).

When FCS was replaced by NBCS, the responses to Con A and anti-CD3 followed the same trend as those of cultures that were incubated in FCS-supplemented medium (Fig. 2). For example, there was a 13% increase in Con A proliferative responses of spleen cells when 20 ml NBCS/l (0-403 μmol Fe/l) was added to the culture media instead of 10 ml NBCS/l (0-201 μmol Fe/l). In anti-CD3-treated cultures, the proliferative responses did not increase, in fact they decreased by 11% ($P > 0.05$). The addition of different concentrations of ferric ammonium citrate to the culture medium that was supplemented with 10 ml NBCS/l resulted in 36–40% increase of the proliferative responses to Con A, whereas it resulted in a 4–20% decrease in the responses to anti-CD3 (Fig. 3, $P < 0.05$).

Similar to the results of cell proliferation, IL-2 secretion was also affected by the concentration of Fe or FCS in mitogen-treated and untreated cultures (Fig. 4). In non-activated cells, IL-2 secretion was very low in apotransferrin-supplemented medium, rose upon addition of Fe in the form of FCS to culture media and reached a peak at 2-260 μmol Fe/l (50 ml FCS/l). In Con A-treated cells, there was a steady rise in IL-2 secretion with the increase in Fe and FCS levels from less than 50 pg/ml in apotransferrin-supplemented medium to about 200 pg/ml in 4-660 μmol Fe/l (100 ml FCS/l)-containing medium ($P < 0.05$). In anti-CD3 activated spleen cells, the addition of 0-450 μmol Fe/l (10 ml FCS/l) to the culture medium slightly increased IL-2 secretion when compared with apotransferrin-supplemented medium. However, at

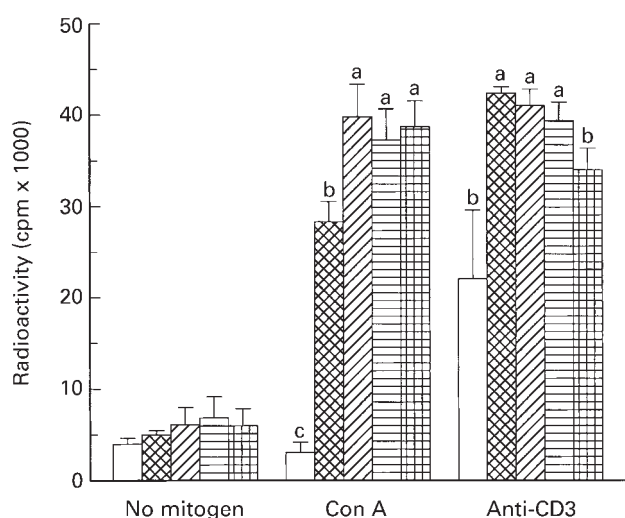


Fig. 3. Effects of iron concentrations added to the culture medium that contained newborn calf serum (10 ml/l) on the rate of DNA synthesis in mitogen treated and untreated spleen cells. For details of procedures, see p. 68, Con A, concanavalin A, □, 0-113 μmol Fe/l; ▨, 0-201 μmol Fe/l; ▩, 2-10 μmol Fe/l; ▪, 4-975 μmol Fe/l; ▫, 9-74 μmol Fe/l. Values are means for five mice with standard errors shown by vertical bars. ^{a,b}Mean values for each mitogen with unlike superscript letters were significantly different ($P < 0.05$). cpm, counts per minute.

0-895 μmol Fe/l, IL-2 levels decreased. In fact at 2-260 and 4-660 μmol Fe/l, IL-2 secretion decreased below the baseline levels that were obtained in apotransferrin-supplemented media, although the reduction was not significant.

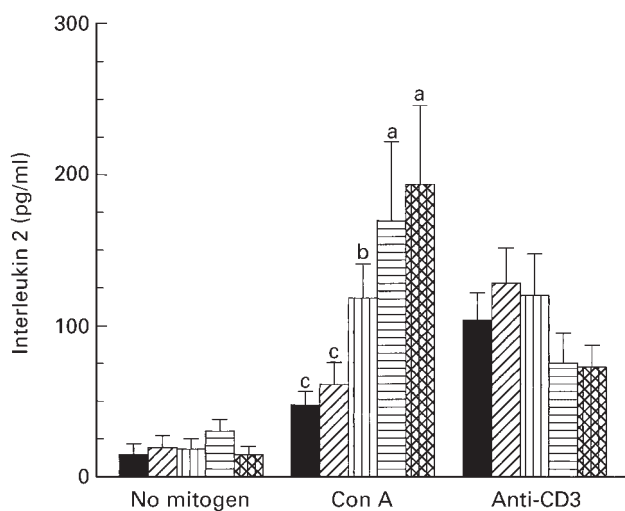


Fig. 4. Effects of iron concentrations added to culture medium in the form of fetal calf serum on interleukin 2 secretion by activated and non-activated murine spleen cells. For details of procedures, see p. 68. Con A, concanavalin A. ■, 0-130 μmol Fe/l; ▨, 0-450 μmol Fe/l; ▩, 0-895 μmol Fe/l; ▪, 2-260 μmol Fe/l; ▫, 4-660 μmol Fe/l. Values are means for twelve mice with standard errors shown by vertical bars. The corresponding fetal calf serum concentrations for listed iron levels are: 0 (25 mg apotransferrin/l), 10, 20, 50, 100 ml fetal calf serum/l for 0-130, 0-450, 0-895, 2-260, 4-660 μmol Fe/l respectively. ^{a,b,c}Mean values with unlike superscript letters for Con A were significantly different ($P < 0.05$). No significant differences were observed between the five iron concentrations in anti-CD3 treated cultures.

Discussion

The importance of Fe-transferrin on proliferation and functions of activated human as well as laboratory animal lymphocytes has been established (Brock, 1981; Lederman *et al.* 1984; Golding & Young, 1995). Most, though not all, clinical and animal studies have shown that Fe deficiency reduces *in vitro* lymphocyte proliferative responses to mitogens and antigens (for review see Brock, 1992; Thibault *et al.* 1993; Omar & Blakley, 1994; Kuvibidila *et al.* 1998). The lack of reduction in lymphocyte proliferation in the few studies is puzzling. Although the severity of Fe deficiency, differences in methods, and unknown confounding variables in the control group are thought to be the cause of the negative results, our present results may provide one other explanation. It seems that the type of stimulant used may be a key factor that determines the extent of impairment of lymphocyte proliferation and likely other immune responses during Fe deficiency.

The present study suggests that there are differences in the sensitivity to Fe and/or FCS concentrations of normal murine spleen cells incubated with either Con A or anti-CD3 antibody. Con A is a non-specific T-cell mitogen that binds to T cells as well as non-T cells through glucosyl and mannosyl receptors (Nagase *et al.* 1998). In contrast, anti-CD3 antibody is specific and binds only to the T-cell receptor (T-cell receptor-CD3 complex) (Abbas *et al.* 1997a). One of the main functions of Fe is to maintain the activity of ribonucleotide reductase, the enzyme that is responsible for the biosynthesis of deoxyribonucleotides (Hoffbrand *et al.* 1976; Thelander *et al.* 1983). This is a rate-limiting step in the pathways that lead to mitosis. It was surprising to find that the absence of serum and apo-transferrin inhibited the rate of DNA synthesis by 24% in Con A-treated cells, whereas that had no negative effect in anti-CD3 treated cells. Our results on anti-CD3 treated cells are similar to those of Golding & Young (1995), who observed that normal human peripheral blood lymphocytes incubated in serum-free medium in the presence of phytohaemagglutinin could proliferate to a certain degree. However, the addition of either human serum, FCS or Fe-transferrin was required for optimal cell proliferation. Unfortunately, these authors did not provide results from non-activated cells and they only studied one mitogen.

In our present study, differences in the sensitivity of murine spleen cells to Con A and anti-CD3 antibody were unlikely to be due to a growth factor (other than Fe) in the serum since when ferric ammonium citrate was added to medium supplemented with 10 ml NBCS/l, the differences between both mitogens persisted. The differential sensitivity of Con A *v.* anti-CD3 treated cells may also not be at the level of ribonucleotide reductase, but probably at an early step in T-cell activation pathways, such as mitogen binding to specific receptors, hydrolysis of cell membrane phospholipids, activation of protein kinase C and other protein kinases, or cytokine secretion. In support of this speculation is, first, the pattern of IL-2 secretion in Con A and anti-CD3 treated cells (Fig. 4). In apo-transferrin-supplemented media, anti-CD3 treated cells secreted

a mean of 103.0 pg IL-2/ml compared with 47.3 pg/ml for Con A-treated cells. The addition of 0.450 and 0.895 $\mu\text{mol Fe/l}$ (10 and 20 ml FCS/l respectively) only slightly increased IL-2 secreted by anti-CD3 treated cells, whereas it resulted in a 4-fold increase in Con A-treated cells. Second, in one experiment, we observed that the binding of Con A conjugated to fluorescein isothiocyanate was significantly decreased by approximately 90% when 100 ml FCS/l was added to the incubation medium (SR Kuvibidila and LE Leiva, unpublished results). We therefore speculate that in serum-free media and low serum levels, spleen cells bind more Con A, whereas the opposite is observed when the medium is supplemented with 100 ml FCS/l. Since high levels of Con A are toxic to lymphoid as well as non-lymphoid cells, we speculate that many Con A-treated cells, incubated in low-serum medium, die (Nagase *et al.* 1988). Third, the activity and mRNA levels of protein kinase C (Phillips *et al.* 1987; Alcantara *et al.* 1994; Kuvibidila *et al.* 1999) and certain cyclin (D type)-dependent kinases (Gao & Richardson, 2001) are significantly reduced by Fe chelation. These protein kinases are involved in signal transduction in the early phase of T-lymphocyte activation, prior to DNA synthesis and mitosis. However, it is unknown whether anti-CD3 antibody activates these kinases more efficiently than Con A.

IL-2 secretion, however, may not be the only cytokine involved in lymphocyte proliferation affected by different levels of iron in the culture medium. Scaccabarozzi *et al.* (2000) have recently shown that Fe chelation by desferrioxamine significantly reduced the secretion of tumour necrosis factor α by activated human monocytic THP-1 cells. Tumour necrosis factor α modulates the translocation of nuclear factor- κB from the cytoplasm to the nucleus, where it binds to specific DNA sequences and initiates the expression of several cytokine inducible genes including IL-2 and IL-2 receptor (Abbas *et al.* 1997b,c). This cascade of events leads to lymphocyte proliferation. No information is available on the role of Fe on the translocation of nuclear factor- κB . Whatever the mechanism involved in the differential Fe requirements by anti-CD3 and Con A-treated spleen cells for proliferation, our results provide one of the clues that may explain the normal lymphocyte proliferation occasionally reported in Fe-deficient human subjects and laboratory animals (Gross *et al.* 1975; Galan *et al.* 1992). Our results imply that an Fe-deficient individual may have either impaired or (nearly) normal lymphocyte proliferative responses depending on whether the stimulant primarily interacts with the T-cell receptor-CD3 complex or the glucosyl-mannosyl receptors (similar to those used by Con A).

In summary, our present results suggest that the minimum levels of Fe required by murine splenic lymphocytes and perhaps human lymphocytes for proliferation vary with the type of mitogen. We are unaware of a similar previous observation made by other investigators. Further experiments are required to determine which early events in the T-cell activation pathways, other than IL-2, are less affected by Fe levels in anti-CD3 treated spleen cells compared with Con A-activated lymphocytes.

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