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Differential Effects of Iron Deficiency on the Expression of CD80 and CD86 Co-Stimulatory Receptors in Mitogen-Treated and Untreated Murine Spleen Cells

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Abstract The interaction of CD28 and its ligands (CD80, CD86) on antigen presenting cells and that of TCR/CD3-MHC are required for T lymphocyte activation. To determine whether impaired lymphocyte proliferation associated with iron deficiency is due to reduced expression of these ligands, spleen cells obtained from eight to nine C57BL/6 mice/group of iron deficient (ID), iron replete (R), control (C), pair-fed (PF), and high iron (HI) mice were labeled with anti-CD80-fluorescein isothiocyante (FITC) and anti-CD86-FITC. Diets differed only in iron concentration: 5, 50, and 125 mg/kg for the ID, C, and HI, respectively. Mean levels of hemoglobin and liver iron stores of ID and R mice were less than 50% those of C mice ($P < 0.005$). In non-activated and concanavalin A-treated cultures, significant differences were observed among groups in the percentage of CD80+ cells: ID>R>C=PF=HI ($P < 0.05$). The same trend was observed for CD86+ cells ($P > 0.05$). Fluorescence intensity (FI) of either marker did not significantly change by iron status. In vitro iron chelation by deferoxamine (20, 200 μg/ml) for 1, 2, and 24 h increased FI of both markers on unactivated B and T cells ($P < 0.05$). However, it had no effect on FI of either marker of mitogen-treated cells presumably because the maximum levels are achieved by the mitogen. Lymphocyte proliferative responses to mitogens positively and significantly correlated with CD80 and CD86 FI ($r = 0.41-0.59$) but negatively correlated with the percentages of CD80+ cells ($r = -0.48$) ($P < 0.05$). Data suggest that impaired lymphocyte proliferation associated with iron deficiency is not due to reduced CD80 and CD86 expression. J. Cell. Biochem. 86: 571–582, 2002.

Key words: anemia; iron deficiency; CD80 receptor; CD86 receptor; co-stimulatory signal; lymphocyte proliferation; mice

T lymphocytes require two signals before they can be activated, secrete cytokines, and proliferate. The first signal, signal one, is delivered through the T cell receptor (TCR)/CD3 complex binding with the antigen-major histocompatibility complex on antigen-presenting cells (APC), and the second signal through several costimulatory molecules (intercellular adhesion molecule-1, LFA-3, vascular cell adhesion molecule-1, heat stable antigen, B7-1, and B7-2) on APC [June et al., 1994; Das et al., 1995]. Of all these co-stimulatory molecules, the B7

Abbreviations used: Anti-CD3, monoclonal antibody raised against CD3 (cluster of differentiation) molecule 3; anti-CD3-PE, anti-CD3 labeled with phycoerythrin (PE); anti-CD19-PE, monoclonal antibody raised against CD19 (B cell receptor) and tagged with PE; anti-CD80, monoclonal antibody raised against CD80 molecule; anti-CD80-FITC, anti-CD80 antibody conjugated with fluorescein isothiocyanate; anti-CD86, monoclonal antibody raised against CD86 molecule; anti-CD86-FITC, anti-CD86 antibody conjugated with fluorescence isothiocyanate; Con A, concanavalin A; C, control; DFO, deferoxamine B mesylate (also called desferrioxamine B mesylate); FCS, fetal calf serum; h, hour; HI, high iron; ID, iron deficient; min, minutes; PF, pair-fed.

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family has been shown to be required for TH1 (T helper 1) cell activation, cytokine secretion, and T cell clonal expansion.

B7-1 (CD80) and B7-2 (CD86) are expressed on several APC cells including B cells, monocytes, interferon-gamma activated monocytes, dendritic cells, eosinophils, natural killer cells, keratinocytes, and Langherans cells [Guinan et al., 1994]. At least in the mouse, these receptors are also expressed on activated lymphocytes [Hathcock et al., 1994; Das et al., 1995]. CD80 and CD86 have two counter receptors on T cells: CD28 and CTLA-4 [Freeman et al., 1993; June et al., 1994]. Although the interaction of CD28 and its ligands is required for optimal T cell activation and subsequent proliferation, the contribution of CD80 and CD86 molecules toward lymphocyte mitogenic response is not identical [Perrin et al., 1997]. In murine lymph node cells, it has been shown that the addition of both anti-B7-1 Fab and anti-B7-2 Fab to the culture medium completely suppressed the proliferative response to concanavalin (Con) A [Perrin et al., 1997]. In contrast, while anti-B7-2 Fab partially inhibited this response, anti-B7-1 Fab had no effect.

We and other investigators have previously observed impaired lymphocyte proliferation, cytokine secretion, and several other in vivo and in vitro immune responses in iron deficient laboratory animals and human subjects [reviewed by Brock, 1992; Kemp, 1993; Kuvibidila et al., 1993]. Impaired lymphocyte proliferation associated with iron deficiency is not limited to lymphocytes treated with T cell mitogens, but is also observed in antigen and lipopolysaccharide-treated cultures [Kuvibidila et al., 1983; Kemahli et al., 1988]. The mechanisms of impaired lymphocyte proliferation associated with iron deficiency are multifactorial. They include, though are unlikely limited to, reduced activity of iron-dependent enzymes, specifically ribonucleotide reductase, impaired protein kinase C activation, a defect in the hydrolysis of cell membrane phospholipids, and reduced secretion of interleukin-2 [Furukawa et al., 1992; Galan et al., 1992; Kuvibidila et al., 1992, 1998, 1999; Alcantara et al., 1994]. In a recent study, we observed that the expression of CD28 molecules on thymocytes was reduced by in vivo iron deficiency and in vitro iron chelation by deferoxamine [Kuvibidila and Porretta, 2001]. Not only was the percentage of CD28+ reduced, but so were CD28 relative levels measured by fluorescence intensity of anti-CD28-phycoerythrin. Considering the effects of iron deficiency on CD28 expression, we hypothesize that iron deficiency may also alter or reduce the expression of CD80 and CD86. Such an effect has not been previously investigated in either laboratory animals or humans. The aim of this study was twofold: (a) to determine whether iron regulates the expression (relative levels measured by fluorescence intensity) of either CD80, CD86, or both co-stimulatory molecules in mitogen-treated and untreated murine splenic lymphocytes and (b) whether professional APC such as B cells and non-professional APC (T) cells are equally affected by iron levels.

MATERIALS AND METHODS

Materials

Reagents were received from the following sources: Sigma (St. Louis, MO): concanavalin A (Con A), deferoxamine B mesylate (DFO, also called desferrioxamine mesylate), Drabkin's reagents, hemoglobin standard, iron assay kits; GIBCO (Grand Island, NY): RPMI-1640 with 25 mmol/L HEPES, penicillin/streptomycin, fetal calf serum (FCS); Harlan Teklad (Madison, WI): the iron deficient test diet and the deficient diet supplemented with iron; New England Nuclear (NEN) company (Boston MA): ^{3}H-thymidine (6.7 Ci/mmol); ICN (Costa Mesa, CA): Cytoscint; PharMingen (Costa Mesa, CA): anti-CD80 conjugated with fluorescein isothiocyanate (anti-CD80-FITC), anti-CD86-FITC antibodies, anti-CD3 conjugated with phycoerythrin (PE), anti-CD19-PE.

Experimental Design and Mice Feeding

C57BL/6 female mice (n = 41), 21–23 days of age were purchased from Charles River breeding laboratories (Wilmington, MA). Upon receipt, they were put on adjustment period for 7 days during which they received the control diet that contained 50 mg (0.895 mmol) of iron per kg diet in the form of ferrous sulfate and sterile deionized water. Following the adjustment period, mice were randomly assigned to the following dietary treatment groups: control (C, n = 8), iron deficient (ID, n = 16), pair-fed (PF, n = 8), and high iron (HI, n = 9). Iron deficiency was induced by feeding mice a diet that contained only 5 mg (0.0895 mmol) of iron per kilogram. The C and PF groups received the

same diet except that it was supplemented with 50 mg of iron per kilogram (0.895 mmol/kg). Moderate iron overload was induced by feeding a diet that contained 125 mg (2.238 mmol) iron per kilogram. Except for iron, the concentrations of protein, fat, carbohydrates, vitamins, and minerals of these diets were identical and have been previously reported [Kuvibidila et al., 1998]. While C, ID, HI mice had free access to their diets 24 h/day, PF mice received the control diet in amounts equal to the mean that ID mice had consumed during the preceding 24 h. Mice were housed in sterile microisolator system cages (product 109EI, Laboratory Products, Maywood, NJ) and they received sterile deionized water. The light/dark cycle was set for 12 h, and the room temperature at 22°C. The study was approved by the Institutional Committee for Animal Care and Use of Louisiana State University School of Medicine. The feeding period lasted 62 days.

Evaluation of Iron Status at the End of the Feeding Period

Three days prior to the experiment, eight ID mice with a hematocrit less than 0.20 were given the control diet (iron repletion protocol—R group). The repletion period was chosen based on our previous studies that showed improvement in lymphocyte proliferation and T cell activation prior to correction of indicators of iron-deficiency anemia [Kuvibidila et al., 1998]. At the time of killing, mice were anesthetized by ether inhalation for 30–60 s. After blood was drawn from the retro-orbital plexus, mice were killed by cervical dislocation. Hemoglobin and hematocrit were measured by the cyanmethemoglobin method and centrifugation, respectively [Rodak, 1992]. The iron concentrations of the liver and the diets were assayed as previously reported [Kuvibidila et al., 1998].

Preparation of Single Cell Suspension

Spleens were removed under sterile conditions and immediately placed in a preweighed sterile culture tube that contained 1-ml wash medium. The wash medium was supplemented with 10 g/L bovine serum albumin, 50 mg/L streptomycin, and 50,000 U/L penicillin and contained 0.113 μmol/L iron [Kuvibidila et al., 1998]. After the tubes were weighed, single-cell suspensions were prepared by grinding the lymphoid organs on a nylon mesh attached to a sterile 50-ml beaker secured with a rubber band. The nylon mesh was washed with 10-ml serum-free RPMI-1640 and cells were collected into the beaker, then transferred to a 15-ml conical centrifuge tube. Cells were washed at 400*g*, 4°C for 10 min. The supernatant was decanted and the pellets were resuspended in 1-ml ice-cold sterile deionized water to lyse red blood cells. Cells were further washed twice under the same conditions. The pellets were then resuspended in 2 ml of wash medium. Total and viable cells were counted under a light microscope after cells were diluted in a solution of trypan blue (4 g/L).

Estimation of the Percentage of CD80 + and CD86 + Cells

Three types of studies were conducted. In Study 1, spleen cells (2×10^6) were transferred to a 12 × 75 mm culture tube, followed by 500 ng of either anti-CD80-FITC or anti-CD86-FITC in a total volume of 250 μl of serum-free phosphate buffered saline (PBS). The final concentration of each antibody was 2 μg/ml. Tubes were incubated at 37°C for 30 min, protected from light. Since in a preliminary study, we observed that spleen cells incubated at 37°C showed brighter fluorescence intensity than those that were incubated at 4°C, we labeled cells at the former temperature. (However, when cells were labeled at 4°C, the observation made when cells were incubated at 37°C was corroborated—Study 3.) At the end of incubation period, cells were washed twice in PBS at 400*g*, 4°C for 10 min each. Each pellet was resuspended in 300 μl of 4% formaldehyde solution. Tubes were incubated at room temperature (22°C) on a shaker (Thermolyne Maxi-Mix III type 65800, Durbuque, IO) set at 200 rpm for 30 min. Fixed cells were analyzed within 24 h on a FACSCALIBUR flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA) using CELLQUEST Software. An electronic gate based on forward versus side scatter was constructed around lymphocyte population. Approximately 5,000-gated events were collected.

In Study 2, 2×10^6 viable spleen cells obtained from the five study groups were incubated in 12 × 75 mm culture tubes without and with 1.25 μg/ml Con A at 37°C, 5% CO_2, in a humidified atmosphere in NAPCO incubator (Model 5100, Portland, OR) for 14 h. The culture medium contained the following elements per liter or RPMI-1640: 10 ml FCS, 50 mg

streptomycin, 5×10^4 U, 1 mmol sodium pyruvate, 0.1 mmol nonessential amino acids, 50 μmol beta-mercaptoethanol, and 0.238 μmol iron. At the end of incubation period, cells were washed twice with serum-free PBS by centrifugation at 400*g*, room temperature for 10 min. After the second centrifugation, the supernatant was decanted. Anti-CD80-FITC and anti-CD86-FITC were added to parallel tubes; they were incubated and fixed under the same conditions as Study 1.

In Study 3, the effect of iron chelation by deferoxamine (DFO) in vitro on the expression of CD80 and CD86 markers was examined in mitogen-treated and untreated spleen cells incubated in complete medium as described in Study 2. Viable spleen cells (2×10^6) were mixed with Con A (1.25 μg/ml), anti-CD3 (50 ng/ml) or no mitogen, and without or with, DFO (20 μg/ml or 200 μg/ml) in a total volume of 1 ml complete medium. Mitogen-treated and untreated cells were incubated at 37°C, 5% CO_2, in NAPCO incubator in a humidified atmosphere for either 1, 2, or 24 h. Cells were washed by centrifugation at room temperature, 400*g*, for 10 min prior to being labeled with either anti-CD80 or anti-CD86 antibodies. In order to identify the cell types that are affected by iron chelation, non-activated and Con A-treated spleen cells were also labeled with either anti-CD3-PE (4 μg/ml) (T cells) or anti-CD19-PE (5 μg/ml) (B cells) along with either anti-CD80-FITC or anti-CD86-FITC. In all experiments that involved time response, DFO dose response, and evaluation of CD3+ and CD19+ cells that expressed either CD80 or CD86, cells were labeled at 4°C (results of Figs. 5–7). All other incubation conditions were identical to those of Study 1 and 2.

Lymphocyte Proliferation

Two types of cultures were prepared: one with 20 μg/ml DFO and the other without DFO. Viable nucleated cells (1×10^6) obtained from normal control mice were mixed with optimal concentrations of either Con A (1.25 μg/ml), anti-CD3 antibody (50 ng/ml) or a mixture of anti-CD3 (50 ng/ml) and anti-CD28 (50 ng/ml). For background DNA synthesis, culture medium was added instead of the mitogen. The culture medium was identical to that used in Study 2 and 3 described above. However, when lymphocyte proliferation was evaluated in the five study groups, the culture medium contained 5-ml FCS/100 ml and no DFO.

Activated and non-activated cells (2×10^5) were transferred to 96-well plates in triplicate. The plates were incubated under the same conditions and for the same duration as macrocultures used for CD80 and CD86 staining. To estimate the rate of DNA synthesis in DFO-treated and untreated cells, 37 kBq (1 μCi) ^{3}H-thymidine were added to each well during the last 6 of the 24-h incubation period. In cultures that involved spleen cells from the five study groups, ^{3}H-thymidine was added during the last 24 of the 72-h incubation period. At the end of incubation period, cells were harvested onto filter strips (PHD Cell Harvester, Cambridge Technology, Watertown, MA). Filters from each well were transferred to a scintillation vial that contained 2-ml Cytoscint. The radioactivity incorporated into DNA was measured by counting each vial for 1 min in LKB liquid scintillation counter (Model 1219, Turku, Finland).

Calculations and Statistical Analysis

Descriptive statistics (mean ± SEM), analysis of variance (ANOVA), and Pearson's correlation coefficients were calculated by the use of Microstatistical program (Ecosoft, Inc., Indianapolis, IN) as described in the literature [Munro, 1993]. When ANOVA detected significant differences among study groups, Scheffé's test was used to determine which pairs of means were different. The level of significance was set at $P < 0.05$.

RESULTS

Indicators of Iron Status and the Weights of Lymphoid Organs

The mean concentration of indicators of iron status of ID mice were significantly lower than those of C and PF mice (Table I; $P < 0.005$). Iron repletion for 3 days improved but did not fully correct the decreased indicators. However, the differences between ID and R mice were significant for all three indicators of iron status ($P < 0.05$). Both the absolute and relative spleen weights were significantly increased by iron deficiency and were not corrected by iron repletion for 3 days ($P < 0.001$). Seven of the eight ID and all eight R mice had splenomegaly (relative spleen weight greater than 5.2 mg/g body weight, the highest observed in C mice). No significant differences were observed between the C and PF groups with any indicator of

TABLE I. Means of Indices of Iron Status, Weights of Body, and Lymphoid Organs

	Control	Pair-fed	Iron-deficient	Iron-repleted	High iron
Hemoglobin (g/L)	176.7 ± 3.64^{a}	174.8 ± 4.46^{a}	33.28 ± 3.3^{c}	57.05 ± 12.45^{b}	177.46 ± 3.32^{a}
Hematocrit	0.508 ± 0.007^{a}	0.514 ± 0.004^{a}	0.154 ± 0.0019^{c}	0.234 ± 0.0041^{b}	0.502 ± 0.0064^{a}
Liver iron (μmol/g liver)	1.05 ± 0.1^{b}	1.244 ± 0.09^{b}	0.359 ± 0.05^{d}	0.941 ± 0.20^{c}	1.676 ± 0.10^{a}
Weight (g)	20.55 ± 0.38	19.66 ± 0.47	17.49 ± 0.70	17.97 ± 0.87	20.91 ± 0.48
Spleen (mg)	89.88 ± 4.93^{c}	97.50 ± 6.20^{c}	261.25 ± 65.94^{b}	511.25 ± 97.5^{a}	118.89 ± 18.29^{c}
Spleen (mg/g body weight)	4.372 ± 0.22^{c}	4.943 ± 0.26^{c}	9.80 ± 3.47^{b}	28.31 ± 4.8^{a}	5.67 ± 0.89^{c}

Values are mean ± SEM; n = 8/group, 9 mice for the HI group. Within a row, values followed by different superscript letters are significantly different, $P < 0.05$; a > b > c > d.

iron status or the weights of body and spleen. Feeding mice the high iron diet had no effect on either hemoglobin or hematocrit, but it increased liver iron stores about 60% compared to C and PF groups ($P < 0.05$).

Percentage of CD80 + and CD86 + spleen cells. In freshly prepared spleen cells, iron deficiency was associated with a significant increase in the percentage of cells that expressed the CD80 receptor (Fig. 1A, $P < 0.05$) but not CD86 marker (Fig. 1B). Feeding ID mice, the control diet (R group) for 3 days did not reduce the percentage of CD80 + cells to the levels found in C mice. Neither underfeeding, nor feeding mice the high iron diet altered the percentage of CD80 + and CD86 + cells.

Incubation of spleen cells with Con A overnight resulted in a significant increase in the percentages of CD80 + (Fig. 1A) and CD86 + cells (Fig. 1B) in all study groups ($P < 0.05$). However, ID and R mice still showed significantly higher percentages of CD80 + spleen cells than the other study groups ($P < 0.05$). In contrast, iron deficiency and iron repletion had no significant effect on the percentage of CD86 + cells in Con A-treated cultures. As for freshly prepared cells, neither underfeeding nor feeding mice the high iron diet had any effect on the percentages of CD80 + and CD86 + cells of Con A-treated cells.

Because of differences in the absolute and relative spleen weights among the five study groups, the numbers of nucleated spleen cells, CD80 +, and CD86 + cells were also compared. Although the absolute (per spleen) and relative (per mg spleen) numbers of nucleated cells were lower in ID and R mice than in C, PF, and HI mice, only the mean relative cell number of the R group that was significantly different from the other four study groups (Table II, $P < 0.0005$). Iron deficiency slightly, but non-significantly increased the overall mean number of CD80 + cells/spleen; and also slightly, and non-significantly decreased mean number of CD80 + cells/mg spleen. While iron deficiency had no significant effect on the absolute number of CD86 + cells per spleen, it was associated with a 50% reduction in CD86 + cells per mg spleen ($P = 0.089$, ID vs. HI). In contrast to what one

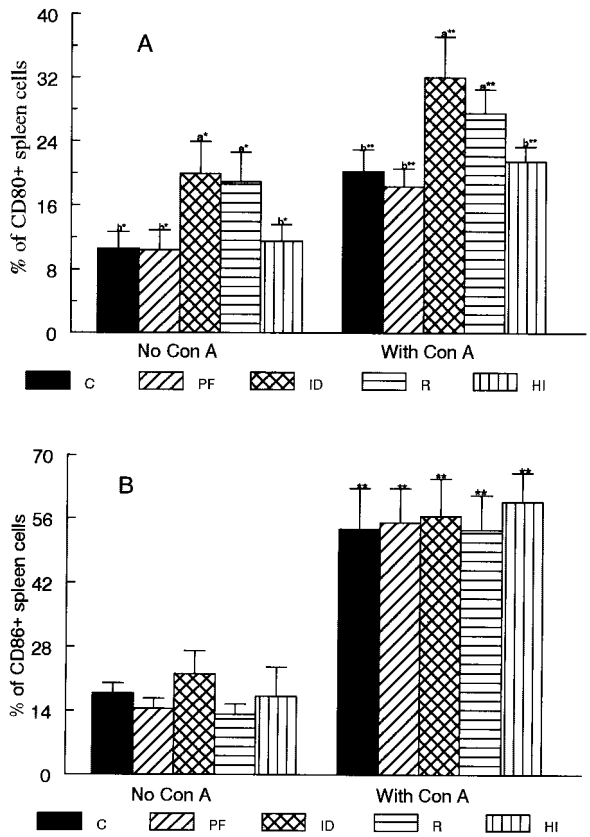

Fig. 1. Percentages of CD80 + (**A**) and CD86 + (**B**) spleen cells in mice in different dietary treatment groups. Abbreviations and sample sizes are: control (8 C), pair-fed (8 PF), iron-deficient (8 ID), and high iron (9 HI). Values are mean ± SEM. In untreated and Con A-treated cells, bars followed by different letters are significantly different: a* > b*, $P = 0.059$; a > b, $P < 0.05$; **$P < 0.01$, Con A-treated cells > untreated cells from the same dietary treatment group.

TABLE II. Number of Nucleated Cells, CD80+ and CD86+ Cells in the Spleens of Mice Different Levels of Iron

	Control	Pair-fed	Iron-deficient	Iron-replete	High iron	*P*
$N \times 10^6$ cells/spleen	45.1 ± 5.27	46.43 ± 2.60	36.55 ± 8.35	37.14 ± 7.90	58.82 ± 3.77	NS
$N \times 10^6$ cells/mg spleen	0.51 ± 0.07^a	0.50 ± 0.06^a	$0.294 \pm 0.17^{a,b}$	0.113 ± 0.04^b	0.556 ± 0.06^b	0.006
$N \times 10^6$ CD80+ cells/spleen	4.92 ± 1.41	4.86 ± 1.25	6.17 ± 1.08	6.30 ± 1.66	6.56 ± 1.15	NS
$N \times 10^6$ CD86+ cells/spleen	7.98 ± 1.39	6.70 ± 1.12	6.57 ± 0.96	4.84 ± 1.45	9.68 ± 1.22	0.089
CD80+ cells/mg spleen	0.058 ± 0.02	0.051 ± 0.012	0.048 ± 0.024	0.015 ± 0.004	0.063 ± 0.013	NS
CD86+ cells/mg spleen	0.093 ± 0.019^a	0.070 ± 0.011^a	0.047 ± 0.02^b	0.015 ± 0.065^b	0.098 ± 0.02^a	0.005

Values are mean ± SEM; n = 8/group, 9 mice for the HI group. Single cell suspensions were prepared and labeled with different antibodies within 2–3 h. NS, not significant. In any row, values followed by different letters are significantly different (a > b, $P < 0.05$).

would expect, iron repletion was associated with a 39% reduction in the absolute number of CD86+ cells (per spleen) and of about 84% of relative CD86+ cell number (per mg spleen). The reduction was significant for the relative but not absolute CD86+ cell numbers ($P < 0.005$). The decrease in the absolute and relative CD86+ cell numbers was very likely due to the fact that in the mouse, spleen can also be used for erythropoiesis when iron is limiting.

Fluorescence intensity of CD80+ and CD86+ spleen cells. Under the experimental conditions, neither iron deficiency, nor underfeeding or iron overload altered fluorescence intensity of CD80+ and CD86+ freshly prepared spleen cells (Fig. 2A,B). While incubation of spleen cells with Con A overnight did not alter fluorescence intensity of CD80+ cells in any study group, it significantly increased CD86 fluorescence intensity in all five groups ($P < 0.01$). However, no significant differences were observed among groups.

Effects of iron chelation by DFO on the percentages of CD80+ and CD86+ Cells. To further elucidate the possible role of iron on the expression of CD80 and CD86, spleen cells obtained from normal (iron sufficient) mice were incubated without and with 20 μg/ml DFO, 1.25 μg/ml Con A, or 50 ng/ml anti-CD3 for 24 h prior to labeling with anti-CD80-FITC and anti-CD86-FITC. While iron chelation resulted in a fourfold increase in the percentage of CD80+ cells of non-activated cultures ($P < 0.001$), it did not alter that of mitogen-treated cultures (Fig. 3A). Compared to non-activated cells, incubation of cells with either Con A or anti-CD3 without DFO resulted in a 7–8-fold increase in the percentage of CD80+ cells ($P < 0.001$). In contrast, incubation of cells with the same mitogens, but in the presence of DFO, resulted only in an 1.47- to 1.77-fold increase. Nevertheless, the increase was significant ($P < 0.001$).

Iron chelation for 24 h of non-activated cells also increased the percentage of CD86+ cells ($P < 0.001$) but had no significant effect on mitogen-treated cells (Fig. 3B). In basal (DFO-free) medium, Con A and anti-CD3 antibody

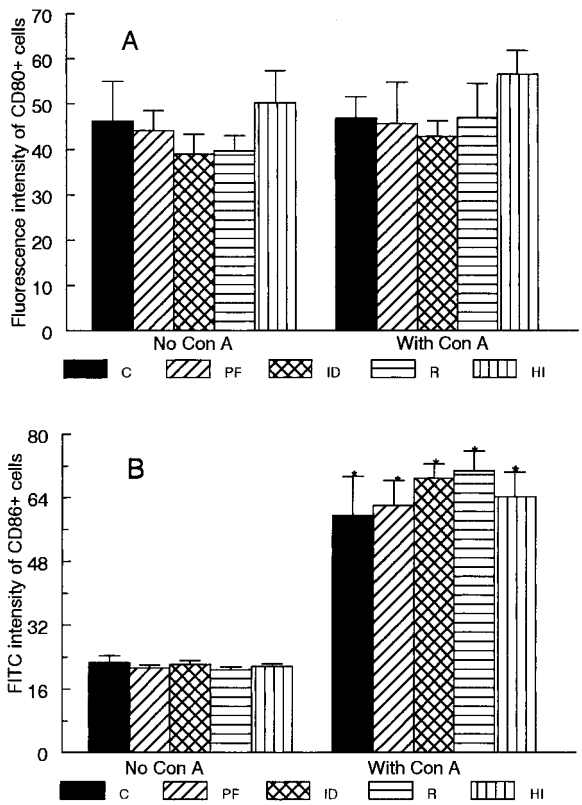

Fig. 2. Fluorescence intensity of CD80 (**A**) and CD86 (**B**) markers of positive spleen cells obtained from 8 control (C), 8 pair-fed (PF), 8 iron-deficient (ID), and 9 high iron (HI) mice. Values are mean ± SEM. In Con A-treated and untreated cultures, no significant differences were observed among groups. *$P < 0.01$, Con A-treated cells > untreated cells from the same dietary treatment group.

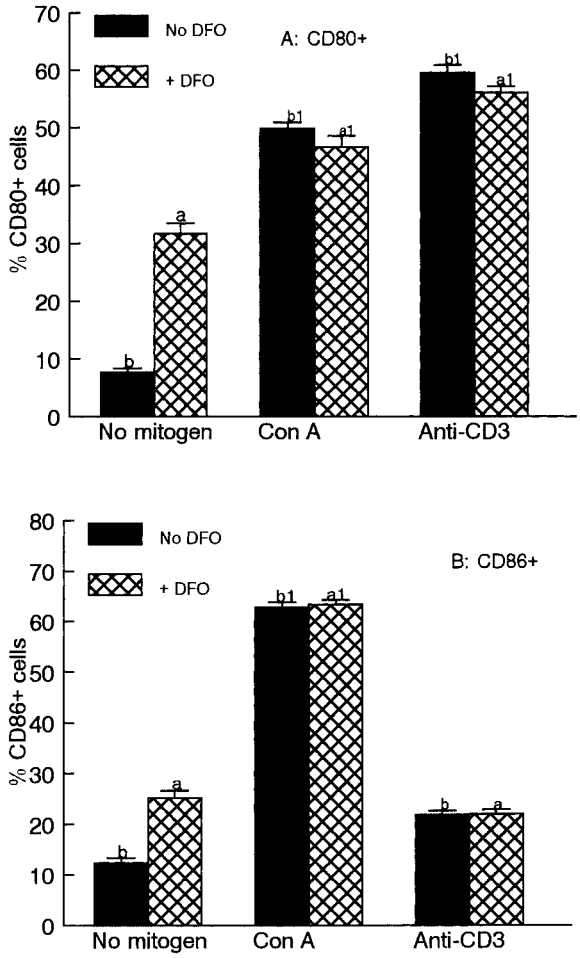

Fig. 3. Effect of iron chelation by deferoxamine (DFO) and mitogen-treatment for 24 h on the percentages of CD80+ (**A**) and CD86+ (**B**) spleen cells from five normal (iron-replete) mice. Values are mean ± SEM. In non-activated cultures, the percentages of CD80+ (A) and CD86+ (B) cells were increased by DFO (CD80+: a > b, $P < 0.001$; CD86+: a > b, $P < 0.05$). In the presence and absence of DFO, bars followed by different letters are significantly different (non-activated cells compared with Con A- and anti-CD3-treated cells; a1 > a; b1 > b; $P < 0.001$).

increased the percentage of CD86+ cells by 5-fold and 1.77-fold, respectively, when compared to non-activated cultures ($P < 0.001$). When DFO was included in the culture medium, Con A, but not anti-CD3 increased the percentage of CD86+ cells by 2.5-fold ($P < 0.001$). Iron chelation for 1 and 2 h did not affect the percentages of CD80+ and CD86+ cells (data not shown).

Effects of iron chelation by DFO on fluorescence intensity of CD80 and CD86 molecules. In non-activated spleen cells, iron chelation for 24 h was associated with a small, but significant increase in fluorescence intensity of CD80 (Fig. 4A) and CD86 receptors (Fig. 4B) ($P < 0.05$). In DFO-free medium, Con A, but not anti-CD3 also increased CD86 fluorescence intensity ($P < 0.001$). DFO increased the relative concentration of CD80 and CD86 markers to the same levels as Con A and anti-CD3 antibody. Although, there was a wide variation between animals, iron chelation for 1 and 2 h also tended to increase fluorescence intensity of CD80 receptor in whole spleen cells (Fig. 5), CD80 and CD86 markers in B (CD19+) cells (Fig. 6), and CD80 in T (CD3+) cells (Fig. 7). DFO treatment of cells for 24 h significantly increased fluorescence intensity of

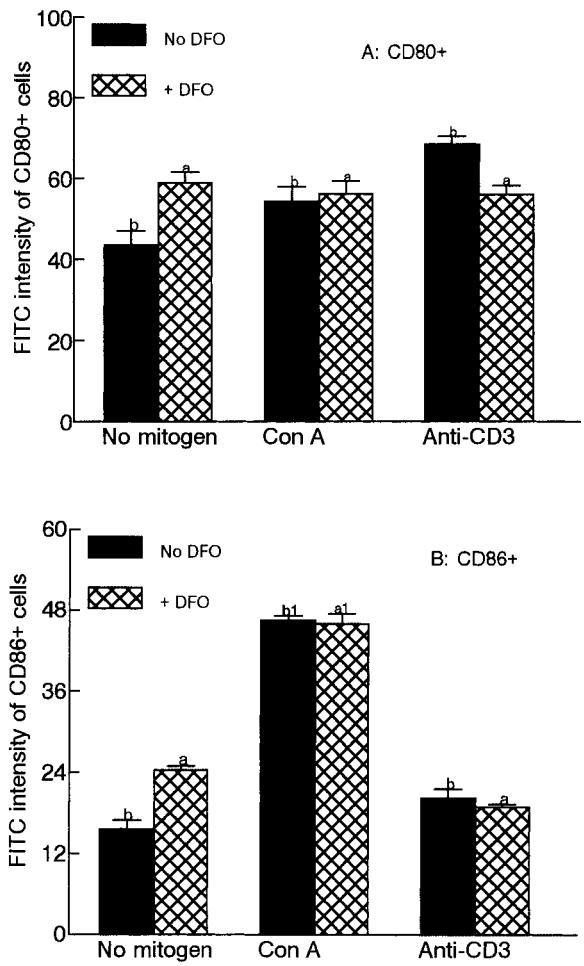

Fig. 4. CD80 (**A**) and CD86 (**B**) fluorescence intensity (FI) as a function of DFO treatment. Values are mean ± SEM, N = 5 normal mice. In non-activated cells, bars followed by different letters are significantly different (a > b; $P < 0.05$). For CD86 receptor, FI of Con A-treated cells is higher than that of non-activated cells incubated with and without DFO (a1 > a; b1 > b, $P < 0.001$).

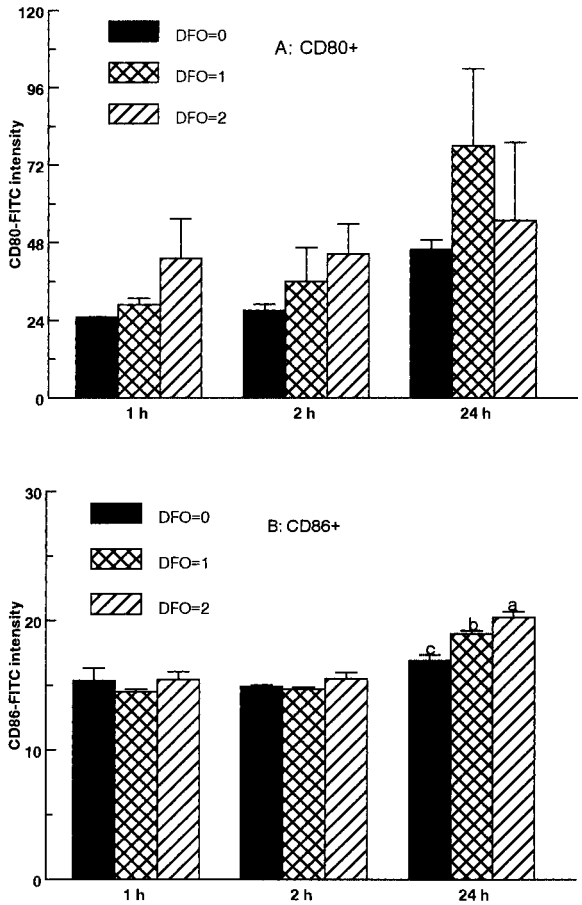

Fig. 5. Effects of incubation period and DFO concentrations on the expression of CD80 and CD86 receptors in spleen cells. Values are mean ± SEM; three mice/data point. Although $P > 0.05$, CD80 fluorescence intensity tended to increase within 60 min of DFO-treatment.

CD86 in CD19 + cells ($P < 0.05$; Fig. 6), and non-significantly in CD3 + cells. Iron chelation for 24 h also tended to increase CD80 relative levels in B and T cells, though $P > 0.05$.

Lymphocyte proliferation. In spite of the high expression of CD86 marker by Con A-treated spleen cells incubated without DFO (but in 1% FCS supplemented medium), ^{3}H-thymidine incorporation into DNA rose only 2.25-fold (over the baseline) compared to 12.55 for anti-CD3-treated cells that expressed less CD86 (Table III). In DFO-treated cells, ^{3}H-thymidine incorporation into DNA of Con A- and anti-CD3-treated cells was abolished presumably because of impaired activity of ribonucleotide reductase but not due to lack of expression of CD80 and CD86 [Furukawa et al., 1992]. Although there was a large overlap between groups, iron deficiency was associated with a 26–36% reduction in lymphocyte proliferative responses to Con A, anti-CD3 antibody, and the combination of anti-CD3 and anti-CD28 antibodies (Table IV). The differences between groups was marginally significant for anti-CD3/anti-CD28 antibody-treated cells ($P = 0.077$; ANOVA). The lack of significant reduction in the rate of DNA synthesis was in part due to the fact that the culture medium contained only 5% instead of 10% FCS that we have used in our previous studies [Kuvibidila et al., 1998]. When Student's t-test was used to compare groups, lymphocyte proliferative responses were lower in ID mice than of those of PF mice (anti-CD3, $P = 0.066$; anti-CD3/anti-CD28, $P = 0.0077$, Con A, $P = 0.087$), HI mice (anti-CD3/anti-CD28, $P = 0.039$), and C mice (anti-CD3/anti-CD28, $P = 0.068$). Moderate iron overload did not affect lymphocyte proliferation when compared to C and PF mice.

Hemoglobin and hematocrit, but not liver iron stores, positively and significantly correlated with lymphocyte proliferative responses to Con A and anti-CD3 (Table V; $P < 0.05$). While the percentages of CD80 + spleen cells negatively and significantly correlated with both hemoglobin and hematocrit levels ($P < 0.05$), and non-significantly with liver iron stores, those of CD86 + cells did not. Interestingly, lymphocyte proliferative responses to mitogens positively and significantly correlated with CD80 and CD86 fluorescence intensity, but negatively correlated with the percentages of CD80 + cells ($P < 0.05$). The negative correlation between proliferative responses and the percentage of CD80 + cells may imply that high number of CD80 + cells are inhibitory to mitogenic response.

DISCUSSION

The requirement of CD80 and CD86 for T cell activation in the mouse and the human has been established [Hathcock et al., 1994; June et al., 1994; Perrin et al., 1997]. It has been shown that blockade of B7 significantly reduced Con A induced murine spleen cell proliferation and induction of mRNA for interleukin-2 [Perrin et al., 1997]. In addition to B cells, CD80 and CD86 are expressed on a variety of cells including activated T cells [June et al., 1994; Das et al., 1995].

In the present study, we determined the influence of iron deficiency on the expression of CD80 and CD86 in non-activated and mitogen-treated spleen cells. We also studied the

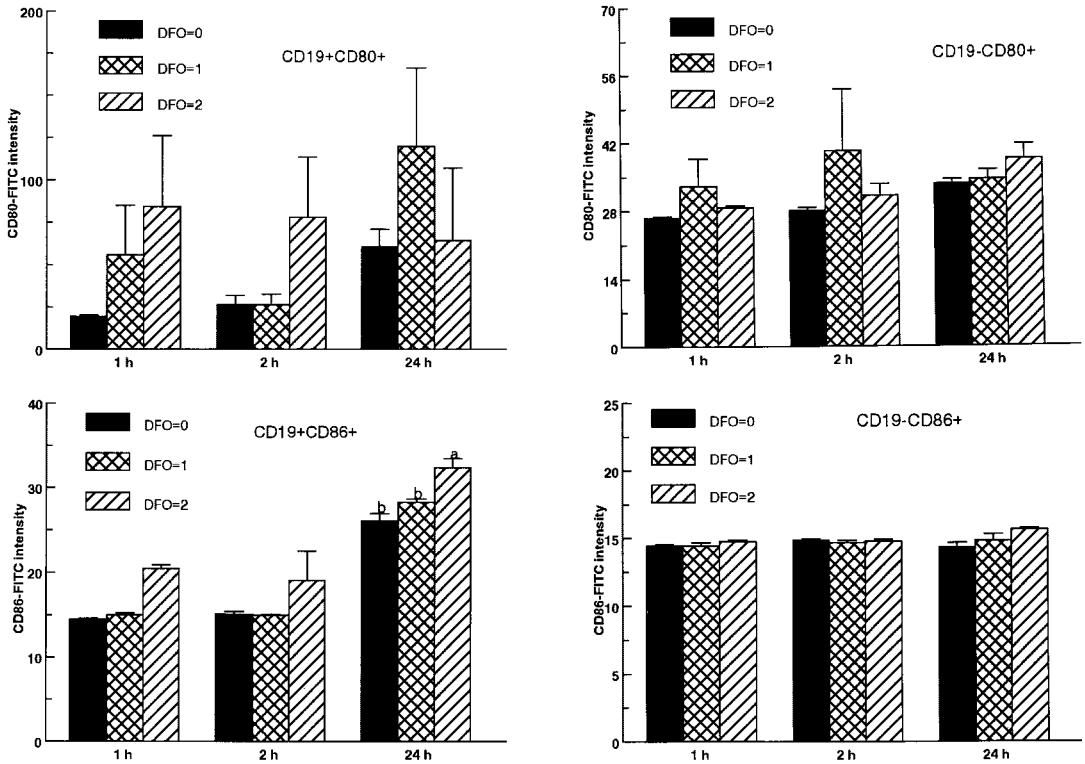

Fig. 6. Effects of incubation period and DFO concentrations on the expression of CD80 and CD86 receptors in B (CD19 +) cells. Values are mean ± SEM; three mice/data point. Although $P > 0.05$, CD80 fluorescence intensity tended to increase within 60 min of DFO treatment.

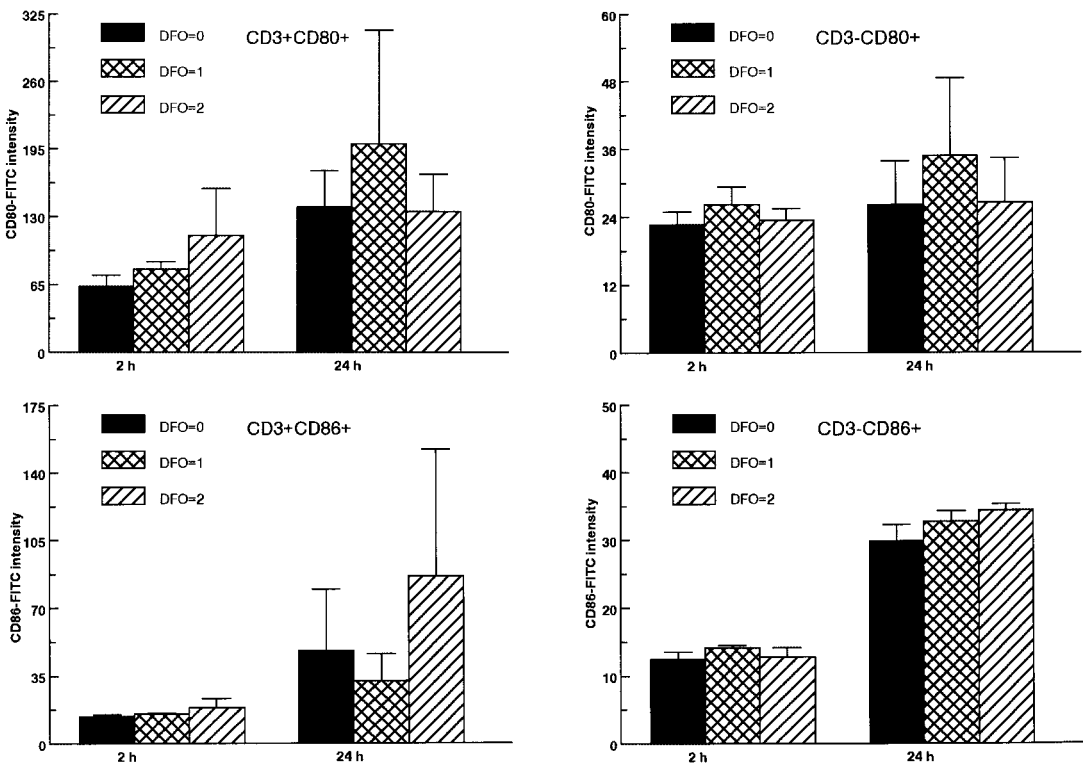

Fig. 7. Effects of incubation period and DFO concentrations on the expression of CD80 and CD86 receptors in T (CD3 +) cells. Values are mean ± SEM; three mice/data point. Although $P > 0.05$, CD80 fluorescence intensity tended to increase within 60 min of DFO treatment.

TABLE III. ^{3}H-Thymidine Incorporation Into DNA of Activated and Non-Activated Spleen Cells Incubated With and Without 20 μg/ml Deferoxamine (DFO)

	No DFO	With DFO	*P*
No mitogen	37 ± 13^c	36 ± 9.9^b	NS
Con A	84 ± 32^b	41 ± 7.7^b	0.05
Anti-CD3 antibody	467 ± 47^a	70 ± 9.7^a	0.05

Values are mean ± SEM; n = 5 normal mice; the unit is Bq (1 Bq = 29 cpm or 1 cpm = 0.034 Bq). NS, not significant. Data are based on 6 h pulsing of cells that were incubated with and without mitogens and/or DFO for 24 h. In each column, values followed by different statistical signs are significantly different; a > b > c, $P < 0.005$.

direct effect of iron chelation on the expression of these markers. Our results show that iron may have a significant role on the expression of these markers. In Con A-treated and untreated cultures, in vivo iron deficiency, but not moderate iron overload or underfeeding increased the percentage of CD80 + spleen cells but not that of CD86 + cells. In contrast, in vivo iron deficiency did not significantly increase fluorescence intensity of either marker.

Our strongest evidence that iron may modulate the expression of these markers, however, comes from the in vitro iron chelation experiments. In non-activated cells, iron chelation resulted in a significant increase in the percentages of cells that expressed CD80 and CD86, as well as fluorescence intensity of these markers. The results on increased percentage of CD80 + cells following iron chelation are consistent with those that we obtained from in vivo iron-deficiency experiments. The lack of significant increase in fluorescence intensity of either marker in spleen cells from ID and/or R mice suggest that in vivo iron deficiency is less efficient in depleting intracellular iron than DFO.

Spleen cell activation by either Con A or anti-CD3 antibody increased the relative concentration of CD80 and CD86 as well as the percentages of CD80 + and CD86 + cells. However, the addition of DFO to the culture medium did not further increase either the levels or the percentages of positive cells beyond those obtained with mitogens alone. Our results imply that the maximum percentage of cells that express CD80 are obtained with the mitogen; consequently the presence of DFO does not and can not further increase it. In addition, the maximum expression of CD80 on each cell is achieved by iron chelation and the presence of a mitogen can not increase it any further.

The present data do not allow us to determine which professional and non-professional APC whose expression of CD80 and CD86 molecules are affected by in vivo iron deficiency. However, based on results obtained from in vitro iron chelation, iron regulates the expression of both markers in B and T cells, and starting as early as 1–2 h of incubation of cells with DFO. It is also possible that iron depletion has a similar effect on other APC.

We have previously reported that the percentages of B cells (assessed by immunoglobulin binding) and T cells (assessed by anti-Thy1.2, anti-L3T4, and anti-Ly2 antibodies) were significantly decreased in ID mice [Kuvibidila et al., 1990]. We also observed decreased lymphocyte proliferation of activated spleen cells and it was not corrected by T cell enrichment or the addition of serum to the culture medium; however, it was corrected by in vivo iron repletion [Kuvibidila et al., 1998, 1999]. In the present study, although the differences among groups were small, the proliferative responses of spleen cells obtained from ID mice were also lower than those of C and PF mice. The high

TABLE IV. ^{3}H-Thymidine Incorporation Into DNA of Activated and Non-Activated Spleen Cells Obtained From Mice Fed Different Levels of Iron

	Control	Pair-fed	Iron-deficient	Iron-replete	High iron
No mitogen	56.6 ± 13.03^b	200.2 ± 17.4^a	166 ± 16.61^a	156.5 ± 9.48^a	175.1 ± 19.63^a
Con A	3592 ± 960	4072 ± 881	2665 ± 432	3651 ± 741	3232 ± 578
Anti-CD3	$5514 \pm 1351^{a,b}$	5834 ± 1290^a	3542 ± 632^b	$4730 \pm 980^{a,b}$	$5153 \pm 1156^{a,b}$
Anti-CD3/anti-CD28	8958 ± 1440^a	9877 ± 135^a	6030 ± 348^b	$6698 \pm 1006^{a,b}$	7749 ± 643^a

Values are mean ± SEM; n = 8/group, 9 mice for the HI group. The rate of DNA synthesis was studied during the last 24 of a 72-h incubation period. Units: Bq (1 cpm = 0.034 Bq or 1 Bq = 29 cpm). For non-activated cells, $P < 0.001$, and for anti-CD3/anti-CD28-treated cells, $P = 0.077$, ANOVA. In any row, values followed by unlike letters are different by Student's *t*-test. PF > ID: $P < 0.008$ for anti-CD3/anti-CD28-treated cells, $P = 0.066$ for anti-CD3-treated cells, $P = 0.087$ for Con A-treated cells. C > ID, $P = 0.068$ for anti-CD3/anti-CD28-treated cells; HI > ID, $P < 0.05$, anti-CD3/anti-CD28-treated cells.

TABLE V. Correlation Matrix Between Indicators of Iron Satus and Expression of CD80 and CD86 Markers, and Lymphocyte Proliferative Responses to Mitogens

	Hb	Hct	% CD80+	% CD86+	CD80 FITC	CD86 FITC	Con A	Anti-CD3	Anti-CD3/ CD28
	r	r	r	r	r	r	r	r	r
Hb	1								
Hct	0.97*	1							
% CD80+Φ	−0.5*	−0.53*	1						
% CD86+Φ	−0.04	−0.07	0.62*	1					
CD80 FITCδ	0.27	0.29	−0.54*	−0.32*	1				
CD86 FITCδ	0.08	0.16	−0.41*	−0.24	0.52*	1			
Con A	0.22	0.26	−0.37*	−0.2	0.5*	0.52*	1		
Anti-CD3	0.30*	0.33*	−0.45*	−0.21	0.42*	0.41*	0.82*	1	
Anti-CD3/CD28	0.45*	0.47*	−0.48*	−0.13	0.56*	0.59*	0.93*	0.82*	1

Hb, hemoglobin; Hct, hematocrit; Φ, % of CD80+ or CD86+ cells non-activated cultures; δ, fluorescence intensity of non-activated cells. r values followed by asterisks (*) are significantly different from zero.

positive correlation coefficients between relative concentrations of CD80 and CD86 markers of freshly prepared spleen cells and the proliferative responses at 72 h, suggest that impaired blastogenesis associated with iron deficiency is not due to lack of expression of these markers. Instead, it is due to other mechanisms in the lymphocyte activation and proliferation pathways [reduced hydrolysis of cell membrane phospholipids, protein kinase C activation, interleukin-2 secretion, etc; Galan et al., 1992; Kuvibidila et al., 1992, 1998, 1999; Alcantara et al., 1994]. The strong negative correlation between lymphocyte proliferative responses to mitogens and percentage of CD80+ cells suggest that too many CD80+ cells are inhibitory, and therefore may also contribute to reduced lymphocyte proliferation during iron deficiency. Whether the increased CD80 and CD86 expression associated with iron deficiency and/or iron chelation is an attempt for APC to increase the affinity for CD28 requires further investigation. Further experiments are also required to delineate the mechanisms (altered degration, migration from the cytosol to the cell membrane de novo synthesis) by which iron deprivation (chelation) increases the expression of these markers). Experiments are also planned to determine the mechanisms by which high percentage of CD80+ cells associated with in vivo and in vitro iron deficiency may inhibit spleen cell proliferation.

In summary, our data suggest the following: (a) In vivo iron deficiency increases the percentage of spleen cells that express CD80 marker but it does not alter its relative concentration; (b) in vivo iron deficiency does not increase the percentage of cells that express CD86 marker or its relative concentration; (c) in vitro iron chelation increases the relative concentrations of CD80 and CD86 markers and the percentages of non-activated spleen cells that express these markers; but the effects of iron chelation and mitogen on the expression of these markers are not synergistic; (d) iron chelation increased the expression of CD80 and CD86 on B and T cells within 1–2 h; and (e) lymphocyte proliferative responses to mitogens positively correlate with CD80 and CD86 relative levels, but inversely correlate with the percentage of CD80+ cells.

In conclusion, reduced proliferative responses of splenic lymphocytes usually associated with iron deficiency can not be attributed to lack of expression of CD80 and CD86 but to the many other defects previously reported in the literature, and also perhaps to the abnormally high number of CD80+ cells. The regulation of expression of co-stimulatory markers by iron may have a significant health implication on autoimmune diseases in individuals (women) who are chronically iron deficient.

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