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Effects of Iron Deficiency on the Secretion of Interleukin-10 by Mitogen-Activated and Non-Activated Murine Spleen Cells

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Abstract Interleukin (IL)-10 plays crucial regulatory roles in immune responses by inhibiting the secretion of several cytokines (IL-2, IL-12, interferon-gamma (IFN-γ)) and lymphocyte proliferation. Iron deficiency, a public health problem for children, alters these immune responses. To determine whether these changes are related to altered IL-10 secretion, we measured IL-10 in 24 and 48 h supernatant of spleen cell cultures from iron deficient (ID), control (C), pairfed (PF), and ID mice fed the control diet (iron repletion) for 3 (R3) and 14 (R14) days (d, $n = 12$/group). Mean levels of hemoglobin, hematocrit, and liver iron stores varied as follows: C ≈ PF ≈ R14 > R3 > ID ($P < 0.01$). Mean baseline IL-10 levels of ID mice tended to be higher than those of other groups ($P > 0.05$, ANOVA). Mean IL-10 levels secreted by concanavalin A (Con A) and antibody raised against cluster of differentiation molecule 3 (anti-CD3)-treated cells (±background) were lower in ID than in C (48 h) and iron replete mice ($P < 0.05$). Underfeeding also reduced IL-10 secretion by anti-CD3-treated cells (48 h, $P < 0.05$). Lymphocyte proliferative responses to anti-CD3 ± anti-CD28 antibodies were lower in ID than in C and PF mice, and they were corrected by iron repletion ($P < 0.05$). IL-10 levels negatively correlated with indicators of iron status ($r \leq -0.285$) and lymphocyte proliferation ($r \leq -0.379$ [$r \leq -0.743$ for ID mice]), but positively correlated with IFN-γ levels ($r \leq 0.47$; $P < 0.05$). Data suggest that iron deficiency has a generalized deleterious effect on cells that secrete both cytokines. Reduced IL-10 secretion by activated cells does not overcome the inhibition of lymphocyte proliferation due to other factors of T cell activation that are regulated by iron. J. Cell. Biochem. 90: 278–286, 2003.

Key words: iron deficiency; hemoglobin; macrophages; spleen; mice; cytokine; IL-10

Interleukin (IL)-10, a regulatory cytokine produced by T lymphocytes (TH0, TH1, TH2), B cells, macrophages, monocytes, and mast cells, plays crucial roles in several immune responses [Murray et al., 1997]. It down-regulates T cell activation and proliferation, secretion of IL-2, and several pro-inflammatory cytokines, and the expression of B7 (CD80, CD86) cell receptors on antigen presenting cells [Fiorentino et al., 1991; Taga and Tosato, 1992; Abbas et al., 1997]. IL-10 also inhibits macrophage activation and proliferation and by doing so regulates the secretion of IL-12 and indirectly that of interferon-gamma (IFN-γ) [D'Andrea et al., 1993]. IL-10 also inhibits the oxidative burst and nitric oxide secretion, factors that are essential for microbial killing [Monari et al., 1997; Murray et al., 1997].

Iron deficiency is a worldwide public health problem that affects primarily children and women of childbearing age [Looker et al., 1997]. This nutritional deficiency impairs several immune responses, including the respiratory burst required for nitric oxide production,

Abbreviations used: Anti-CD3, antibody raised against cluster of differentiation molecule 3; C, control; Con A, concanavalin A; ID, iron deficient; IFN-γ, interferon-gamma; IL-10, interleukin-10; PF, pairfed; R, iron replete; CPM, counts per minute.

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lymphocyte proliferation, and the secretion of several cytokines such IL-1, IL-2, and interferon-gamma (IFN-γ) [reviewed by Helyar and Sherman, 1987; Galan et al., 1992; Omar and Blakley, 1994; Walter et al., 1997; Kuvibidila and Baliga, 2002]. Recently, we observed that iron deficiency reduced the secretion of IL-12 and IFN-γ by activated murine spleen cells, but increased the expression of CD80 (B7.1) receptors [Kuvibidila and Porretta, 2002; Kuvibidila et al., 2003]. Iron chelation also increased the expression of CD80 as well as CD86 in murine spleen cells [Kuvibidila and Porretta, 2002]. The effect of iron deficiency on IL-10 secretion has not been previously studied. Considering the inhibitory effect of iron deficiency on the secretion of IL-12 and IFN-γ, and the down-regulatory action of IL-10 on the secretion of IFN-γ and IL-12, we hypothesized that iron deficiency may increase IL-10 secretion; and that there is a negative correlation between IL-10 levels and those of IFN-γ obtained from the same culture. We also hypothesized that increased IL-10, if any, may be a contributing factor for reduced lymphocyte proliferation usually associated with iron deficiency in humans and laboratory animals. The present study was undertaken to test our hypotheses.

MATERIALS AND METHODS

Source of Reagents

Reagents were purchased from the following companies: Sigma (St. Louis, MO): concanavalin A (Con A), hemoglobin standard, Drabkin's reagents, iron assay kits; GIBCO (Grand Island, NY): RPMI-1640 with 25 mmol/L HEPES, penicillin/streptomycin, fetal calf serum; Harlan Teklad (Madison, WI): the iron deficient (ID) test diet and the deficient diet supplemented with iron; PharMingen (Costa Mesa, CA): antibody raised against cluster of differentiation molecule 3 (anti-CD3) antibody.

Experimental Design and Induction of Iron Deficiency in Mice

Iron deficiency was induced in C57BL/6 female mice (n = 60), as we previously reported [Kuvibidila et al., 1998]. Briefly, 3-week-old mice, purchased from Charles River (Wilmington, MA), were put on adjustment period for 7 days (d) during which they received the control diet that contained 50 mg iron/kg (ferrous sulfate) and sterile deionized water. Following the adjustment period, mice were randomly assigned to the following dietary treatment groups: C (n = 12), ID (n = 36), and pairfed (PF, n = 12). Iron deficiency was induced by feeding 24 mice the test diet that contained only 5 mg/kg. The C and PF groups received the same diet except that it was supplemented with 50-mg iron/kg. Except for the iron, the concentrations of protein, fat, carbohydrates, vitamins, and minerals of both diets were identical [Kuvibidila et al., 1998]. While C and ID mice had free access to their diets 24 h/d, PF mice received the control diet in amounts equal to the mean that ID had consumed during the preceding 24 h. The PF group was added to account for the effect of reduced food intake associated with severe iron deficiency anemia. Mice were housed in microisolator system cages (product 109EI, Laboratory Products, Maywood, NJ) and 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 Health Sciences Center. The feeding period lasted 51 d.

Evaluation of Iron Status at the End of the Feeding Period

Three and 14 d prior to sacrifice, 24 ID mice with hematocrit $\leq 25\%$ were given the control diet (repletion protocol, R3, R14 groups, 12/group). At the time of sacrifice, mice were anesthetized by ether inhalation for 30–60 s and weighed. After blood was drawn from the retro-orbital plexus, they were killed by cervical dislocation. The liver, thymus, and spleen were removed and weighed. 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 we previously reported [Kuvibidila et al., 1998].

Preparation of Single Cell Suspensions

Single cell suspensions were prepared from spleen in serum-free (wash) medium by standard techniques [Kuvibidila and Porretta, 2002]. The serum-free medium contained 25 mmol HEPES, 2 mmol L-glutamine, 10 g bovine serum albumin, 50 mg streptomycin, and 5×10^4 penicillin units, and RPMI-1640 (total volume, 1,000 ml). After cells were washed twice by centrifugation at 400*g* at 4°C for 10 min, and red

blood cells lysed by osmotic shock, each cell pellet was resuspended in 2 ml of wash medium. After cells were diluted with a solution of trypan blue (4 g/L), total and viable cells were counted under light microscope.

Preparation of Macro Cultures and Measurement of IL-10

Viable spleen cells, 2×10^6, were mixed with either 2.5 μg/ml Con A or 50 ng/ml anti-CD3 antibody. Culture medium was added to non-activated cells to a total volume of 1 ml. The culture medium contained 25 mmol HEPES, 50 ml fetal calf serum, 50 mg streptomycin, 5×10^4 U penicillin, 2 mmol L-glutamine, 1 mmol sodium pyruvate, 0.1 mmol non-essential amino acids, 50 μmol of β-mercaptoethanol, and RPMI-1640 in a total volume of 1,000 ml. Tubes were incubated at 37°C, 5% CO_2, in a humidified atmosphere (NAPCO, incubator, Portland, OR). After 24 and 48 h, tubes were centrifuged at 400*g*, at 4°C, for 10 min. Supernatants were collected, aliquoted in 400 μl per vial before freezing at −70°C. IL-10 was measured by enzyme immunoassay with kits purchased from R&D Systems (Minneapolis, MN). For cultures that were harvested after 24-h incubation period, IL-10 was measured in eight mice/group. Instructions provided by the vendor were carefully followed. Standards, controls, and test samples were assayed in duplicate.

Lymphocyte Proliferation

The methods for assessing lymphocyte proliferative responses to mitogen were previously described [Kuvibidila et al., 1998]. Briefly, 1×10^6 viable splenic lymphocytes were mixed with either culture medium (non-activated cultures), 2.5 μg/ml Con A, or 50 ng/ml anti-CD3 antibody ±50 ng/ml anti-CD28 antibody. The culture medium had identical reagents as that used for macro cultures. Cells, 2×10^5/200 μl, were transferred in triplicate to 96-well plates, and the plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere, in a NAPCO incubator for 48 h. Cultures were pulsed with 37 bq (1 μCi/well) for 24 h [Kuvibidila et al., 1998]. After cells were harvested onto filter strips, the radioactivity incorporated into DNA was estimated by counting the filters in a liquid scintillation counter (model LKB 1219, Turku, Finland) for 1 min.

Statistical analysis. Descriptive statistics (mean ± SEM), analysis of variance (ANOVA), paired *t*-test, and correlation coefficients were calculated by the use of a microstatistical prgram (Ecosoft, Inc., Indianapolis, IN) as described in the literature [Munro, 1993]. Since IL-10 levels were skewed, specifically for anti-CD3-treated cultures, two methods were used to compare groups: Mann–Whitney *U*-test and elimination of out-liers (values that fell outside the mean ± 2 SD range before performing ANOVA. (Mann–Whitney *U* is one of standard non-parametric tests that are used to compare groups when data are not normally distributed.) When ANOVA detected significant differences among study groups, Student's *t*-test was used to determine which pairs of means were different. The levels of significance was set at $P < 0.05$.

RESULTS

Indicators of Iron Status and the Weights of Body, Spleen, and Thymus

When mice were sacrificed, one C mouse was eliminated from all statistical analyses because it weighed only 11.34 g (compared with >15 g in the other 11 mice). Severe malnutrition was due to inability to ingest feed due to long teeth. The mean hematocrit and concentrations of hemoglobin and liver iron stores of ID mice were significantly lower than those of C and PF mice (Table I; $P < 0.01$). Iron repletion for 3 d improved, but did not correct the reduced levels of indicators of iron status. Although iron repletion for 14 d corrected both the hematocrit and hemoglobin concentrations, it did not fully correct liver iron stores. The mean body weights of ID and R3, mice were significantly lower than those of C and PF mice, and those of and R14 mice were also lower than those of C (Table I; $P < 0.05$). While both the absolute and relative thymus weights were significantly reduced by iron deficiency, those of spleen were significantly increased ($P < 0.01$). Iron repletion for 14 d, but not 3 d, nearly normalized the weights of both lymphoid organs.

IL-10 Levels in Non-Activated Cells

At each time-point (data not shown) or the pooled data for both time-points, while 100% samples from ID and R14 mice had detectable baseline IL-10 concentrations, only 73.7, 85, and 80% of those from C, PF, and R3 mice, respectively, did. When χ^2-test was computed without Yates'correction factor, the differences

TABLE I. Mean Indicators of Iron Status and Thymus Weight and Body Weight in Control (C), Pairfed (PF), Iron Deficient (ID), Iron Replete for 3 d (R3) or 14 d (R14)

	C	PF	ID	R3	R14
Hemoglobin (g/L)	169.5 ± 1.51	168.9 ± 2.88	37.6 ± 3.22*	66.87 ± 7.49**	157.5 ± 2.36
Hematocrit (%)	50.91 ± 0.32	51.4 ± 0.91	19.1 ± 1.29*	28.3 ± 2.86**	52.8 ± 0.92
Iron (μmol)/liver (g)	0.914 ± 0.06	1.012 ± 0.09	0.186 ± 0.012*	0.427 ± 0.59**	0.604 ± 0.06§
Weight (g)	18.5 ± 0.44	18.1 ± 0.5	15.6 ± 0.34*	16.9 ± 0.42**	17.3 ± 0.23‡
Spleen (mg)	69.36 ± 1.69	63.55 ± 3.34	192.8 ± 38.43*	351.6 ± 61.4***	89.25 ± 5.97
Spleen, body weight (ng/g)	3.76 ± 0.09	3.52 ± 0.15	12.23 ± 2.42*	20.69 ± 3.47***	5.21 ± 0.38§
Thymus (mg)	61.1 ± 2.54	68.3 ± 6.65	27.67 ± 3.3*	26.92 ± 2.82†	66.42 ± 3.27
Thymus, body weight (mg/g)	3.30 ± 0.1	3.78 ± 0.36	1.77 ± 0.21*	1.59 ± 0.17†	3.86 ± 0.20‡

Values are mean ± SEM, N = 12, but 11 C.
*$P < 0.01$, significantly different from all other groups.
**$P < 0.05$, R3 < C, PF, and R14; R3 > ID.
***$P < 0.01$, R3 greater than the other groups.
†$P < 0.01$, R3 < C, PF, and R14.
‡$P < 0.05$, compared to C.
§$P < 0.05$, compared to C and PF.

among groups in the proportion of mice with detectable IL-10 levels were significant ($\chi^2 = 10.49$, df = 4; $P < 0.05$).

Although the differences among groups were not statistically significant at $P < 0.05$ (ANOVA), the mean baseline levels of IL-10 of ID mice was 3× and 1.6× (time 1 and time 2, respectively) of those of C mice (Fig. 1). Underfeeding in PF mice also non-significantly increased mean baseline levels at the 24 h incubation period compared with C mice (69.8%). At the 48 h incubation period, the mean of ID mice was higher than that of PF at $P = 0.1$ (Student's t-test). Iron repletion, especially in cultures incubated for 48 h reduced mean IL-10 to normal levels. Within each group, baseline IL-10 levels increased by 2.9 to nearly sevenfold at 48 h when compared to 24 h time-point due to cell proliferation (paired t-test, $P < 0.05$).

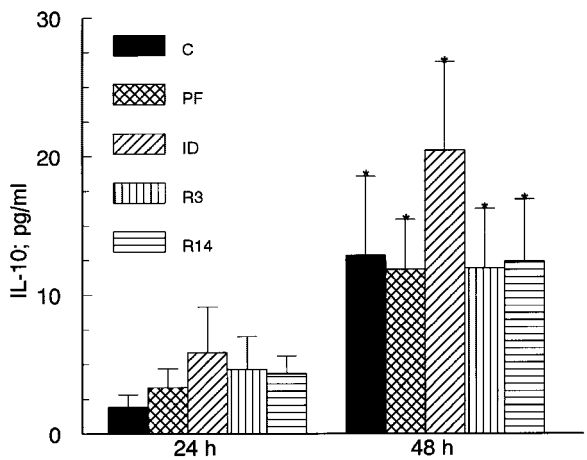

Fig. 1. Mean ± SEM baseline IL-10 levels in spleen cells from control (C), pairfed (PF), iron deficient (ID), ID mice replete with iron for 3 d (R3) or 14 d (R14). N = 7–8 at 24 h, and 11–12 at 48 h time-point. At 48 h time-point, PF < ID, $P = 0.1$, Student's t-test. For each iron treatment group, mean IL-10 levels at 48 h were higher than those of the 24 h (*$P < 0.05$, paired t-test).

IL-10 in Con A-Treated Cells

When cells were activated by Con A for 24 and 48 h, neither iron deficiency, nor underfeeding significantly altered the mean IL-10 levels compared to the C group (Fig. 2A, ANOVA). However, the mean IL-10 levels of ID, PF, and C groups were significantly lower than those of the R3 and R14 groups ($P < 0.05$). When results were expressed as relative concentrations (levels obtained with Con A minus baseline), we observed significant differences among groups in the mean IL-10 levels at both time-points (Fig. 2B; $P < 0.05$). Iron deficiency reduced the mean relative IL-10 levels compared to the other groups except R14 at 48 h incubation period ($P < 0.05$). The mean relative levels of IL-10 of the R3 (24 and 48 h) and R14 (24 h) groups were also significantly higher than those of the C and PF groups ($P < 0.05$). In each iron treatment group, IL-10 secretion was significantly higher at 48 h than at 24 h time-point ($P < 0.05$). The increase is atributed to cell proliferation.

IL-10 Secretion in Anti-CD3-Treated Cultures

When spleen cells were activated by anti-CD3 antibody for 24 h, neither iron deficiency, nor underfeeding reduced the rate of secretion of IL-10 (Fig. 3A). However, iron repletion for 3 d and for 14 d raised IL-10 means levels by 85 and 188% above normal ($P < 0.05$, C vs. R14, and $P < 0.06$, ID and PF vs. R14, Student's t-test). When cells were incubated for 48 h, both iron deficiency and underfeeding reduced the levels

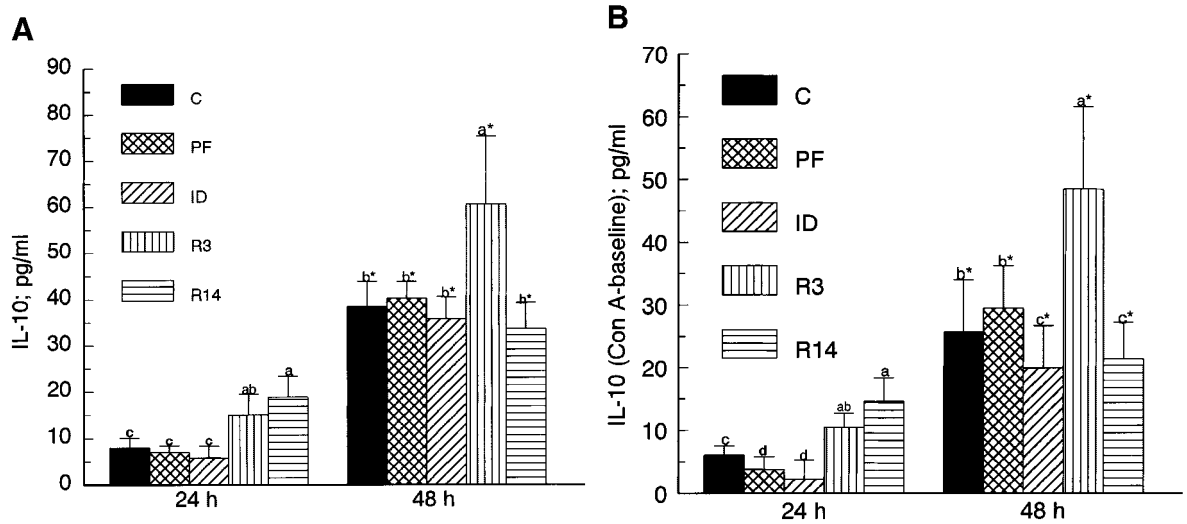

Fig. 2. Total (**A**) and relative (**B**) IL-10 levels secreted by concanavalin A (Con A)-treated spleen cells from C, PF, ID, and iron replete mice for 3 d (R3) or 14 d (R14). Values are mean ± SEM. N = 7–8 at 24 h, and 11–12 at 48 h time-point. For each incubation period, bars followed by different letters are significantly different ($a > b \geq c > d$, $P < 0.05$; $b > c$ at $P < 0.09$; Student's *t*-test and/or Mann–Whitney *U*-test). *$P < 0.05$ compared to IL-10 levels obtained at 24 h in the same dietary treatment group (paired *t*-test).

of IL-10 (Fig. 3A; $P < 0.05$). Iron repletion for 3 d corrected the reduced levels. Mean relative levels of IL-10 of anti-CD3-treated cultures (24 h time-point) were also significantly higher in the R14 and R3 groups than in the C, PF, and ID groups ($P < 0.05$). Similar to Con A-treated and non-treated cultures, IL-10 secretion significantly increased in all groups with incubation period with anti-CD3 antibody ($P < 0.05$).

Lymphocyte Proliferation

Iron deficiency was associated with reduced lymphocyte proliferative responses to anti-CD3 with and without anti-CD28 antibody; and iron repletion restored them to normal levels (Fig. 4, $P < 0.05$). Although the differences did not statistical significance at $P < 0.05$, iron deficiency also tended to decrease lymphocyte

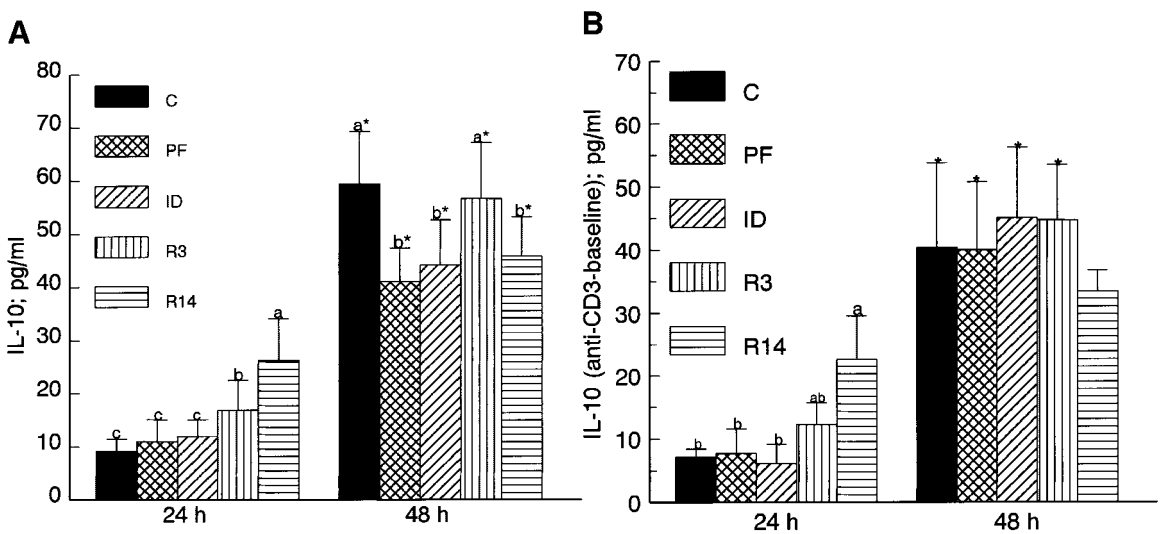

Fig. 3. Total (**A**) and relative (**B**) IL-10 levels secreted by antibody raised against cluster of differentiation molecule 3 (anti-CD3)-treated spleen cells from C, PF, ID, and iron replete for 3 d (R3) or 14 d (R14). Values are mean ± SEM. N = 7–8 at 24 h and 11–12 at 48 h time–point. For each incubation period, bars followed by different letters are significantly different ($a > b > c$; $P < 0.05$, Student's *t*-test and/or Mann–Whitney *U*-test). *$P < 0.05$ compared to IL-10 levels obtained at 24 h in the same dietary treatment group (paired *t*-test).

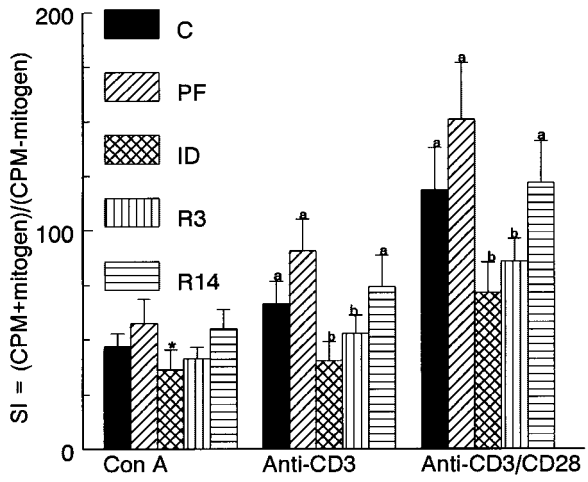

Fig. 4. Rate of DNA synthesis expressed as stimulation indexes (counts per minute (cpm) + mitogen/baseline cpm) by dietary treatment group. Abbreviations are: C, control; PF, pairfed; ID, iron deficient; R3, iron replete for 3 d; and R14, iron replete for 14 d. Values are mean ± SEM. N = 7–8 at 24 h and 11–12 at 48 h. With each mitogen, bars followed by different superscript letters are significantly different (a > b, $P < 0.05$, ANOVA followed by Student's test). *$P < 0.1$ compared to PF and R14.

proliferative responses to Con A ($P < 0.1$, ID compared to PF and R14). The smaller decrease in lymphocyte proliferation of ID Con A-treated cells was very likely due to the low serum concentration that we added to the culture medium [5% in the current study compared with 10% in previous studies; Kuvibidila et al., 1998, 1999]. Underfeeding had no significant effect on the rate of DNA synthesis.

Correlation coefficients. Baseline IL-10 levels negatively and significantly correlated with liver iron stores ($r = -0.285$, $P < 0.05$) and non-significantly correlated with hemoglobin and hematocrit. IL-10 levels secreted by Con A-treated cells negatively and significantly correlated with hemoglobin ($r = -0.229$, $P < 0.05$), and non-significantly with hematocrit ($r = -0.215$), and liver iron stores ($r = -0.202$). In contrast, there was no significant correlation between IL-10 levels secreted by anti-CD3-treated cells and indicators of iron status (data not shown). Body and thymus weights also negatively, but non-significantly, correlated with IL-10 levels (data not shown).

During the first 24 h incubation period, the rate of DNA synthesis negatively and in some instances significantly correlated with the levels of IL-10 (Table II, $P < 0.05$). The negative correlation persisted when the analysis involved only ID mice (Table II, $P < 0.05$). Surprisingly, at 48 h incubation period, the rate of DNA synthesis positively, and in many instances, significantly correlated with IL-10 levels (Table II, $P < 0.05$, overall study population as well as the subgroup of ID mice).

Since IL-10 down-regulates the secretion of IFN-γ, correlation coefficients between both cytokines were also calculated (Table III). (Results of IFN-γ were reported elsewhere along with those of IL-12 [Kuvibidila et al., 2003].) At both time-points, in contrast to what we expected, IL-10 levels positively, and in some

TABLE II. Correlation Coefficients Between IL-10 Levels and the Rate of DNA Synthesis in the Overall Study Population and in ID Mice

	No mitogen (R)	Con A (R)	Anti-CD3 (R)	Anti-CD3/ anti-CD28 (R)
IL-10, 24 h				
All mice				
No mitogen	−0.261	−0.148	−0.248	−0.265
Con A	−0.189	−0.167	−0.379*	−0.326*
Anti-CD3	−0.015	−0.165	−0.306*	−0.257
ID mice				
No mitogen	−0.476	0.034	−0.127	−0.003
Con A	−0.58	−0.564	−0.743*	−0.58
Anti-CD3	−0.433	−0.43	−0.595	−0.432
IL-10, 48 h				
All mice				
No mitogen	0.36*	0.282*	0.279*	−0.221*
Con A	0.286*	0.521*	0.407*	0.435*
Anti-CD3	0.50*	−0.069	0.278*	0.236*
ID mice				
No mitogen	0.687*	0.619*	0.709*	0.685*
Con A	0.127	0.560*	0.392	0.347
Anti-CD3	0.748*	0.394	0.582*	0.496

*$P < 0.05$.

TABLE III. Correlation Coefficients Between Levels of IL-10 and Those of IFN-γ Secreted in Untreated and Mitogen-Treated Cells

	IFN-γ, no mitogen (R)	IFN-γ, Con A (R)	IFN-γ, anti-CD3 (R)
IL-10, 24 h			
No mitogen	0.264	0.277	0.003
Con A	0.305*	0.24	0.212
Anti-CD3	0.459*	0.184	0.317*
IL-10, 48 h			
No mitogen	0.47*	−0.003	−0.091
Con A	−0.364*	0.122	0.137
Anti-CD3	−0.151	0.21	0.268*

*$P < 0.05$.

cases, significantly correlated with those of IFN-γ in the same culture ($P < 0.05$). The only exception is that baseline levels of IFN-γ negatively and significantly correlated with IL-10 levels in Con A-treated cultures ($P < 0.05$). Positive correlations were very likely due to some feedback mechanism such as IFN-γ activating macrophages leading to increased secretion of IL-10.

DISCUSSION

In the current study, we tested three hypotheses. The first is that iron deficiency increases the secretion of IL-10 by spleen cells. The second is that there is a negative correlation between the levels of IL-10 and those of IFN-γ in the same culture. In other words, reduced secretion of IFN-γ that was previously reported is related to increased IL-10 secretion [Omar and Blakley, 1994; Kuvibidila et al., 2003]. The third is that increased IL-10 secretion is a contributing factor to reduced lymphocyte proliferative responses usually associated with iron deficiency. At least for non-activated cells, our first hypothesis was accepted since iron deficiency tended to increase the mean IL-10 levels at both 24 and 48 h incubation periods.

Contrary to our hypothesis, iron deficiency did not increase IL-10 secretion by activated murine spleen cells. In fact, it decreased either the overall or the relative levels in mitogen-treated cells (Figs. 2 and 3). In addition, when compared to iron replete mice, iron deficiency decreases the overall IL-10 levels secreted by Con A as well as anti-CD3-treated cells. Although underfeeding is a confounding variable, we believe that iron deficiency is the most important factor in altered IL-10 secretion for two reasons: (A) the higher relative IL-10 levels of PF compared to ID mice shown in Figure 2B (48 h); (B) the higher correlation coefficients observed between IL-10 levels and indicators of iron status as compared with those with body and thymus weights. The reason for the opposite effect of iron deficiency in non-activated versus activated cells is not clear. However, we speculate two possible mechanisms. The first is a feedback effect of other cytokines on lymphocytes and monocytes/macrophages: IFN-γ secreted by stimulated T and natural killer cells activate macrophages leading to increased IL-10 secretion. The second is that not all cells that produce IL-10 are equally affected by iron deficiency. As stated in the introduction, IL-10 is secreted by several cell types including T lymphocytes (TH0, TH1, and TH2), B cells, macrophages, and mast cells [D'Andrea et al., 1993]. It is very likely that the sensitivity (changes in intracellular iron levels as well as the percentage of cell subsets in the spleen) of these various cell types toward iron deficiency is not identical. Assuming that the proportion of TH2 lymphocytes (the major producers of IL-10) is reduced in spleen of ID mice compared to C (or iron replete) mice, then, the lower IL-10 levels in mitogen-treated cultures should not be surprising. In the current study, we did not assess the contribution of various spleen cell populations on IL-10 secretion in untreated and mitogen-treated cultures. However, experiments are planned to assess the importance of this factor.

With regard to our second hypothesis, we observed positive, instead of negative correlation between the levels of IL-10 and those of IFN-γ. It, therefore, appears that iron deficiency has a generalized negative effect on T and non-T cells that secrete these cytokines. An alternative explanation is that reduced secretion of IFN-γ by T lymphocytes and NK cells limits macrophage activation, and hence IL-10 secretion.

With regard to our third hypothesis, since lymphocyte proliferative responses to mitogens are usually reduced by iron deficiency, we expected not only higher IL-10 levels in the supernatant of ID mice, but also negative correlation between IL-10 levels and the rate of DNA synthesis. However, this is not what we observed. Iron deficiency reduced the rate of DNA synthesis expressed as either stimulation indices (ratio of counts per minute (cpm) in mitogen-treated over untreated cells) or

absolute cpm (data not reported) and IL-10 secretion. Although our hypothesis is not fully supported, it appears that IL-10 secretion had some negative effect on lymphocyte proliferation during the first 24 h of incubation period as shown by the negative correlation coefficients reported in Table III. The positive correlation between IL-10 levels (in the 48 h cultures) and spleen cell proliferation was very likely due to some confounding factors, such as IL-2 secretion and expression of CD80 and CD86 receptors. Increased secretion of IL-2 during the first 24 h of cell activation and expression of CD80 and CD86 on antigen presenting cells after 24 h, are required for lymphocyte proliferation [June et al., 1994; Abbas et al., 1997].

What is the implication of increased IL-10 baseline levels, but reduced levels in mitogen-activated spleen cells? Assuming that baseline IL-10 levels are also increased in blood levels of ID mice, our data may imply reduced baseline blood levels of proinflammatory cytokines such as tumor necrosis factor-α, IFN-γ, and IL-1 [Arai et al., 2000]. Elevated blood levels of IL-10 may be detrimental to the host defense system against pathogens because of reduced secretion of proinflammatory cytokines, the respiratory burst, and nitric oxide secretion, biological processes that are required for microbial killing [Murray et al., 1997; Clevemons et al., 2000]. Reduced respiratory burst has been reported in neutrophils from ID laboratory animals, and it may be related to increased baseline IL-10 levels [Murakawa et al., 1987]. Reduced levels of IL-10 in activated cells should, in theory, increase IFN-γ secretion [Kawachi et al., 2000]. However, as we recently reported, the levels of IFN-γ as well as those of IL-12, are reduced in ID mice [Omar and Blakley, 1994; Kuvibidila et al., 2003]. The bottom line is that neither reduced IL-10 levels nor reduced IFN-γ levels are beneficial to the host defense system related to pathogen killing [Clevemons et al., 2000].

In summary, our results suggest that, (A) iron deficiency tended to increase baseline IL-10 levels secreted in vitro; however it decreases or limits the increase associated with spleen cell activation by mitogens; (B) although underfeeding is a confounding factor, iron deficiency is the most important factor in the altered rate of secretion of IL-10; (C) reduced IL-10 secretion by activated spleen cells does not overcome the inhibitory effect of iron deficiency on lymphocyte proliferation very likely due to other factors in the T cell activation pathways that are regulated by iron. Some of these factors are the hydrolysis of cell membrane phospholipids, protein kinase C activation, IL-2 secretion, expression of co-stimulatory molecules, and of course, the activity of the iron-dependent enzyme ribonucleotide reductase [Furukawa et al., 1992; Galan et al., 1992; Kuvibidila et al., 1992, 1998, 1999, 2003; Alcantara et al., 1994; Kuvibidila and Porretta, 2003]. The mechanisms by which iron deficiency reduces IL-10 secretion by activated cells is not known. However, they could be related either to gene transcription, mRNA translation, or protein half life (degradation). Experiments are planned to elucidate these mechanisms.

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