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In Vivo and In Vitro Iron Deficiency Reduces Protein Kinase C Activity and Translocation in Murine Splenic and Purified T Cells

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Abstract We investigated the effects of iron deficiency anemia, iron repletion, and iron chelation by deferoxamine on protein kinase C (PKC) activity, an enzyme that plays a crucial role on T lymphocyte proliferation. The study involved 23 control (C), 18 pairfed (PF), and 24 iron deficient (ID) mice or ID mice that were repleted for 3 (n = 14), 7 (n = 17), or 14 (n = 14) days. The low iron (0.09 mmol iron/kg) and iron-supplemented (0.9 mmol iron/kg) diets were fed to mice for 53 days. Mean hemoglobin, hematocrit, and liver iron stores of ID mice were one third of those of C mice. Lymphocyte proliferation was reduced ($P < 0.05$) in spleen and purified T cells in ID but not PF mice. In concanavalin A, phytohemagglutinin, and anti-CD3 antibody-treated and untreated cells that were incubated in serum-free and serum-containing medium, PKC activity was significantly ($P < 0.05$) reduced in ID but not PF mice and returned to normal before correction of anemia. In mitogen-treated cells, while the ratios of membrane-bound to cytosol activity increased nearly seven-fold (from 0.4–0.63 in resting cells to 1.43–7.23) in spleen cells from C, PF, and repleted mice and 11-fold in T cells ($P < 0.005$), they remained below 1 in ID mice suggesting reduced translocation. In vitro iron chelation by deferoxamine for 120 min prior to cell activation reduced ($P < 0.05$) PKC activity by 46–60% in C and PF and 28–53% in ID mice. The data suggest that: 1) it is iron-deficiency but not anemia or differences in the proportion of immunocompetent T cells that reduced PKC activity in cells from ID mice; 2) reduced PKC translocation may play an important role on altered lymphocyte proliferation and associated functions in iron-deficient individuals. J. Cell. Biochem. 74:468–478, 1999.

Key words: iron deficiency; anemia; lymphocyte activation; protein kinase C; concanavalin A; phytohemagglutinin; mice; deferoxamine

Protein kinase C (PKC) plays a crucial role in signal transduction mechanisms associated with growth and differentiation. The enzyme is present in many tissues including lymphoid organs. In quiescent cells, more than 60% of PKC is present in the cytosol in inactive form and whereas only 40% or less is associated with the plasma membrane [Adamo et al., 1988]. In contrast, rapidly dividing cells have most of the PKC activity associated with the particulate fraction (membrane-bound) and less than 40% is in the cytosol. During T cell activation, PKC translocation from the cytosol to the plasma membrane is one of the early events that take place. It is believed that the translocation is a result of an increase in diacylglycerol originating from the hydrolysis of plasma membrane phospholipids (phosphatidyl inositol-4,5-bisphosphate) which facilitate the binding of PKC to cell membrane [Abbas et al., 1991]. PKC translocation is followed by phosphorylation of many proteins that have regulatory functions such as transferrin receptor and interleukin-2 receptor. In contrast to resting T cells, which do not express intracellular or plasma membrane

Abbreviations used: ANOVA, analysis of variance; BSA, bovine serum albumin; C, control; Con A, concanavalin A; d, day; DFO, deferoxamine; Hct, hematocrit; Hb, hemoglobin; h, hour; IP3, inositol-1,3,5-triphosphate; ID, iron-deficient; PF, pairfed; PIP2, phosphatidyl inositol-4,5-bisphosphate; PHA, phytohemagglutinin; PKC, protein kinase C; R, repleted.

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transferrin receptor, activated lymphocytes express transferrin receptor [Kemp, 1993]. It is uncertain whether phosphorylation of transferrin receptor is a prerequisite for its expression on the cell surface of activated T cells. However, it is known that an increase in transferrin receptor is required for iron uptake by activated cells.

We, as well as other investigators, have previously observed that iron deficiency in humans and laboratory animals reduced lymphocyte proliferation measured by ^{3}H-thymidine incorporation into DNA [Kuvibidila et al., 1989; Dallman, 1987]. In a preliminary study we also observed that PKC activity and translocation were altered by iron deficiency anemia in murine splenic lymphocytes [Kuvibidila et al., 1991]. PKC activity after repletion of iron deficient mice with dietary iron was not investigated nor was it studied in purified T cells. The present study was undertaken to determine whether: 1) full correction of iron deficiency anemia (hemoglobin and liver iron stores) is required to reverse the PKC defects; 2) the addition of iron in the form of serum to the culture medium for 2 h prior to cell activation would correct PKC activity and/or translocation in cells from iron deficient mice; 3) incubation of iron sufficient cells from control and pairfed mice in serum free medium with and without deferoxamine for 2 h prior to activation would alter PKC activity and distribution between the cytosol and membrane-bound fractions.

MATERIALS AND METHODS

Materials

All reagents were of high quality and were received from the following sources: Sigma (St. Louis, MO): concanavalin A, phytohemagglutinin, β-mercaptoethanol, hemoglobin standard, Drabkin's reagents, iron assay kits, deferoxamine; GIBCO (Grand Island, NY): RPMI-1640 with 25 mmol/L HEPES, fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, penicillin/streptomycin; Bio-Whitaker (Walkersville, MD): L-glutamine; Harlan Teklad (Madison, WI): the iron deficient test diet (TD 80396) and the deficient diet supplemented with iron; NEN Dupont (Boston, MA): ^{3}H-thymidine (6.7 Ci or 247.9 GBq/mmol), gamma-^{32}P-ATP, (specific activity 370 GBq/L), ICN (Costa Mesa, CA): cytoscint; R & D System: T cell enrichment columns.

Experimental Design and Mice Feeding

The study involved 110 female C57BL/6 mice, 21–23 days of age and were purchased from Charles River breeding laboratories (Wilmington, MA). After 5 days of adjustment period, during which the animals received the control diet that contained 0.90 mmol/kg (ferrous sulfate), a blood sample (approximately 150 μL) was drawn from the retro-orbital plexus. Mice were weighed prior to drawing the blood sample. Hemoglobin and hematocrit were measured by the cyanmethemoglobin method and centrifugation, respectively. The mean (± SEM) weight (g), hemoglobin (g/L), and hematocrit levels for the 110 mice were 14.14 ± 0.22, 139.7 ± 1.3, and 0.455 ± 0.003, respectively.

The 110 mice were then randomly assigned to one of the three groups: the control (C, n = 23), the iron deficient (ID, n = 69), and the pairfed (PF, n = 18). Iron deficiency was induced by feeding mice the iron deficient test diet that contained 0.09 mmol iron/kg. The C and PF groups received the iron supplemented diet that contained 0.90 mmol iron/kg diet. The compositions of the deficient and the control diets were identical with regard to protein, energy, vitamins, and minerals and they have been previously described [Kuvibidila et al., 1998]. Iron deficiency was induced as we previously reported [Kuvibidila et al., 1991]. While C and ID had free access to their diets 24 h per days, PF mice received the control diet in amounts equal to what the ID mice had consumed during the preceding 24 h. To monitor the development of anemia, hemoglobin, and hematocrit were measured in all mice on the low iron diet once every 2 weeks. When the hemoglobin (of ID mice) decreased to less than 70 g/L (45–56 days of feeding), 45 mice were given the control diet (repletion protocol) for either 3 (n = 14), 7 (n = 17), or 14 (n = 14) days. These groups are referred to as R3, R7, and R14 respectively throughout the text. The average feeding period for the iron deficient, iron sufficient, and repleted mice was 53 days (range 40–63 days).

All mice were housed in sterile cages and they received deionized sterile water. The room temperature was set at 25°C, and the light/dark cycle for 12 h. The study was approved by the Institutional Review Board for the Care and Use of Laboratory animals of Louisiana State University Medical School.

Evaluation of Iron Status at the End of the Feeding Period

At the time of sacrifice, mice were anesthetized by ether inhalation for 30–60 sec. After as much as possible was drawn from the retro-orbital plexus, mice were killed by cervical dislocation. In each experiment, between one and three mice were sacrificed per dietary treatment group, including repleted groups. Hemoglobin and hematocrit were measured by the cyanmethemoglobin method and centrifugation, respectively. The livers were removed, weighed, and immediately frozen at −40°C until used for iron assay. For liver iron contents, each liver was homogenized in 2 mL deionized water at 4°C. Iron in the homogenates was extracted twice with 3 mol/L nitric acid at 90°C for 3 h as we previously reported [Kuvibidila et al., 1998]. After cooling to room temperature, the samples were centrifuged at 29,000*g* (Sorvall, Newton, CT) for 10 min at 4°C. Iron content in each supernatant was assayed by colorimetry using a kit purchased from Sigma. Preliminary experiments which involved six livers obtained from iron sufficient mice, treated with nitric acid four times, showed that up to 97% of the iron was extracted in the first two treatments. The iron concentration of the test diet and the iron supplemented (control) diet was determined as we previously reported [Kuvibidila et al., 1998].

Preparation of Spleen Cell Suspension and Measurement of Protein Kinase C Activity

Spleens were removed under sterile conditions. Single-cell suspensions were prepared by standard techniques in serum-free RPMI-1640 medium that was supplemented with 1% bovine serum albumin (BSA), 50 mg/L streptomycin, and 50,000 units penicillin/L [Kuvibidila et al., 1991]. In a pilot study, T cells were isolated by negative selection from spleen cell suspensions on T cell enrichment columns as described in the literature [Binz and Wigzell, 1975]. While B cells were removed by the binding of F(ab) surface immunoglobulin to glass beads coated with anti-immunoglobulins, monocytes were removed through the binding of Fc fraction. The resulting T cells were used for PKC assay and lymphocyte proliferation.

For PKC measurement, 1×10^6 viable spleen or purified T cells were resuspended in 1 mL of either serum-free RPMI-1640 medium supplemented with 250 mg/L apotransferrin, 500 µg/L insulin, 1% bovine serum albumin, 50 mg/L streptomycin, 50,000 units/L penicillin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 50 µmol/L β-mercaptoethanol or complete medium. The complete (culture) medium differed from the serum-free medium only by having 10% fetal calf serum instead of apotransferrin, BSA, and insulin. The iron content of the culture medium and serum-free RPMI-1640 was 4.5 µmol/L, and 1.23 µmol/L, respectively. Cells were incubated without deferoxamine, (Study 1), or with 250 mg/L deferoxamine, (Study 2) at 37°C, 5% CO_2, in a humidified incubator for 2 h. At the end of this period, either 2.5 mg/L concanavalin A (Con A), 2.5 mg/L phytohemagglutinin (PHA), or 25 µL of serum-free or culture medium per 1 mL of cell suspension was added to the cells. In the second study that involved purified T cells, 20 µg anti-CD3 antibody/L were added instead of Con A or PHA. Cells were mixed and further incubated under the same conditions for 30 min. Cells were washed twice at 4°C, 670*g*, for 10 min in serum-free RPMI-1640. After the second wash, the pellet was resuspended in 200 µL of digitonin buffer (500 mg/L in RPMI-1640). Cells were mixed and incubated on ice for 5 min. After centrifugation at 4°C, 670*g*, for 10 min, the supernatant was carefully removed with an automatic pipet and was transferred to a pre-labeled cryovial (cytosol-associated PKC). To collect the membrane-bound PKC activity, the pellet was resuspended in 200 µL of digitonin buffer containing 1% Triton X-100. The tubes were mixed and incubated for 5 min at room temperature. The contents were also transferred to a cryovial. Both sets of vials were immediately frozen at −70°C until used for assays. PKC activity was assayed by histone phosphorylation as described in the literature [Baliga and Borowirz, 1988; Pelch et al., 1986].

Measurement of Lymphocyte Proliferation

One million viable cells resuspended in 1 mL of culture medium, were mixed with either 2.5 mg Con A or 2.5 mg PHA/L for splenic lymphocytes or 20 µg anti-CD3 antibody/L for purified T cells. For background DNA synthesis, 25 µL culture medium was added to cells instead of

the mitogen. Mitogen-treated and untreated cells were incubated at 37°C, 5% CO_2 for 48 h before being pulsed with 1 μCi (37 kBq) ^{3}H-thymidine (specific activity 247.9 GBq or 6.7 Ci per mmol) per 200,000 cells. After 24 h, the cultures were harvested (PHD Mash Harvester, Cambridge Technology, Watertown, MA). Each filter disk was transferred to a scintillation vial containing 2 mL of cytoscint. The radioactivity incorporated into DNA was estimated by counting each vial for 1 min in an LKB ß scintillation counter (Turku, Swedden).

Calculations and Statistical Analysis

Descriptive analysis (mean ± SEM), analysis of variance (ANOVA), and Pearson's correlation coefficient were calculated by the use of the Microstatistical program (Ecosoft, Indianapolis, IN) and as described in the literature [Munro, 1993]. When ANOVA revealed significant differences among groups, Scheffé's test was used to determine which pairs of groups were significantly different [Munro et al., 1986]. The level of significance was set at $P < 0.05$.

RESULTS

Iron Status and General Nutritional Status

At the time of killing, mean hematocrits, hemoglobin concentrations, and liver iron stores of the ID group were approximately one third of those of control mice (Table I). The differences among the C, ID, and PF groups, or that among the six study groups, were statistically significant ($P < 0.005$). At the level of dietary restriction, undernutrition did not reduce iron status. Although the means of hemoglobin, hematocrit, and liver iron stores increased after 3 days of iron repletion, they were still significantly ($P < 0.05$) lower than those of C mice. In contrast, after 7 and 14 days of feeding the iron supplemented diet, mean hemoglobin and hematocrit concentrations returned to normal; and those of liver iron stores were restored to 60–73% of values of C mice.

There was a small (8%) but significantly ($P < 0.01$) lower food intake in ID mice (128 ± 1.86, g/53 days, mean ± SEM) and PF mice (128.6 ± 1.4) compared to that of C mice (139.5 ± 2.59). Although there was a significant ($P < 0.01$) difference in body weight among the six study groups, only the means of ID, R3, and R7 mice were significantly ($P < 0.05$) lower than those of C mice. Mice in the ID, R3, and R7 groups had significantly ($P < 0.05$) larger spleens but smaller thymuses (adjusted to body weight) than those from C, PF, and R14 groups. Except for R7 group that had higher ($P < 0.05$) mean liver weight, there was no significant difference among the other five groups.

Lymphocyte Proliferation

Lymphocyte proliferation in Con A or PHA-treated spleen cells, expressed as net Bq of ^{3}H-thymidine incorporation into DNA (total Bq in presence of mitogen minus background Bq) was significantly ($P < 0.005$) lower in the ID than PF and C groups (Fig. 1A). The proliferative responses increased with iron repletion such that by day 14, they returned to normal. While no significant difference was observed

TABLE I. Indicators of Iron Status, the Weights of Body and Various Organs in Control (C), Iron-Deficient (ID), Pairfed (PF) C57BL/6 Female Mice, and in Iron-Deficient Mice That Were Repleted for 3 days (R3), 7 days (R7), and 14 days (R14) at the Time of Sacrifice

	C	PF	ID	R3	R7	R14
Hemoglobin, g/L	170.3 ± 3.2[a]	174.2 ± 4.74[a]	58.5 ± 3.54[b]	78.2 ± 8.2[b]	146.5 ± 6.98[a]	179.4 ± 6.3[a]
Hematocrit	0.49 ± 0.01[a]	0.49 ± 0.01[a]	0.19 ± 0.009[b]	0.25 ± 0.03[c]	0.44 ± 0.001[a,d]	0.53 ± 0.005[a]
Liver iron, μmol/g	0.53 ± 0.06[a]	0.60 ± 0.07[a]	0.17 ± 0.01[b]	0.26 ± 0.06[a,b]	0.32 ± 0.08[a,d]	0.39 ± 0.09[a,d]
Body weight	20.7 ± 0.36[a]	19.6 ± 0.59[a,b]	18.1 ± 0.32[b]	17.9 ± 0.49[b]	18.4 ± 0.47[b]	18.7 ± 0.42[a,b]
Spleen mg/g body weight	4.20 ± 0.27[a]	3.60 ± 0.30[a]	17.73 ± 0.01[b]	13.6 ± 2.2[b]	14.5 ± 1.73[b]	4.91 ± 0.3[a]
Thymus mg/g body weight	3.47 ± 0.22[a]	3.97 ± 0.32[a]	2.15 ± 0.21[b]	2.71 ± 0.57[b]	2.32 ± 0.39[b]	3.73 ± 0.35[a]
Liver g/g body weight	0.049 ± 0.002[a]	0.046 ± 0.002[a]	0.052 ± 0.001[a]	0.052 ± 0.002[a]	0.063 ± 0.002[b]	0.053 ± 0.002[a]

Values are mean ± SEM. n = 23 C, 18 PF, 24 ID, 14 R3, 17 R7, and 14 R14. Mean values followed by different letters are significantly different from each other (Scheffé's test, $p < 0.05$).

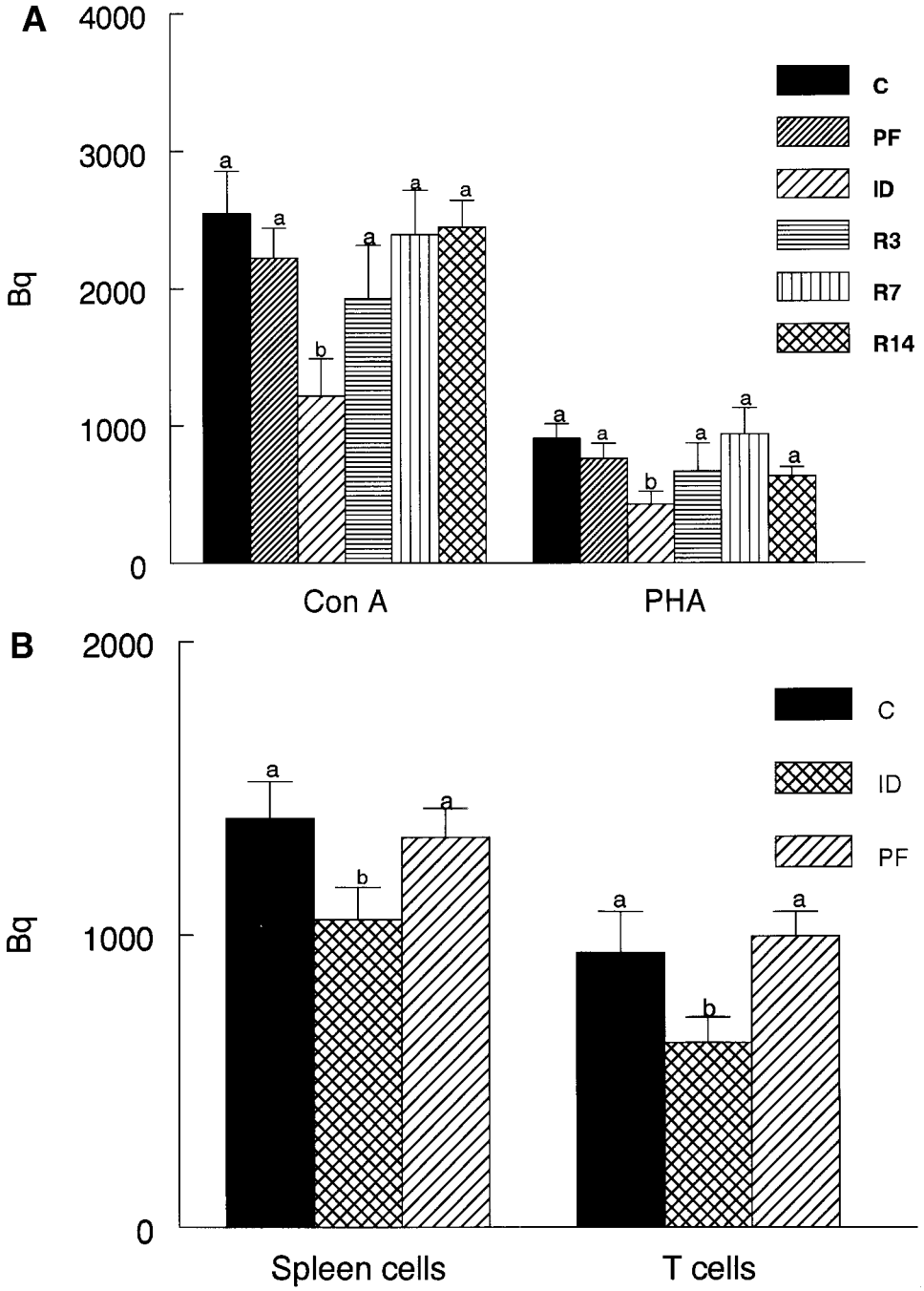

Fig. 1. Lymphocyte proliferation in spleen cells **(A)** and purified T cells **(B)** studied by ^{3}H-thymidine uptake in control (C), paired (PF), iron-deficient (ID), and iron deficient mice that were repleted (R) for 3 (R3), 7 (R7), or 14 (R14) days. Two hundred thousand viable cells were incubated with and without mitogens (Con A, concanavalin A; PHA, phytohemagglutinin, or anti-CD3 antibody) for 48 h before being pulsed with 37 Bq of ^{3}H-thymidine for 24 h as described in Materials and Methods. Results are expressed as net Bq = Total Bq minus background Bq. Values are mean ± SEM. For A, n = 18 C, 15 PF, 16 ID, 14 R3, 17 R7, and 14 R14. For B, n = 15 C, 11 PF, and 14 ID. ID group had significantly lower responses than the other study groups (a > b; $P < 0.05$).

between C, PF, and repleted groups, the ID group showed significantly ($P < 0.05$) lower mean proliferative responses to both mitogens than the other five groups. Lymphocyte proliferation in purified T cells was also significantly ($P < 0.05$) lower in cells from the ID mice than those from C and PF mice, but it was not different between cells from C and PF mice (Fig. 1B). Lymphocyte proliferation positively correlated with hemoglobin (r = 0.27, $P < 0.01$), hematocrit (r = 0.28, $P < 0.01$), and liver iron stores (r = 0.267, $P < 0.01$).

PKC Activity in Cells Incubated in Serum-Containing Medium

In resting and activated spleen cells, total PKC activity as well as membrane-bound activity was significantly different ($P < 0.01$) among the six study groups (Table II). The difference was mostly due to cells from ID mice that showed significantly ($P < 0.05$) lower activity than those from PF and C mice. In contrast, cells from C, PF, and repleted mice had similar total PKC activity. Spleen cells from ID mice also showed significantly lower membrane-bound activity than those from mice that were repleted for 3 and 7 days. While membrane-bound activity was not significantly different among resting cells from C and repleted mice, it was significantly ($P < 0.05$) lower in mitogen-treated spleen cells from repleted mice than in those from C mice that also showed significantly ($P < 0.05$) lower activity than cells from PF mice. Although the cytosol PKC activity of resting cells was also different ($P < 0.05$) among the six groups, being higher in PF and C groups, only the difference between PF and ID groups was significant ($P < 0.05$).

To rule out the possibility that the difference between cells from ID and iron sufficient mice was due to differences in the proportion of immunocompetent T cells, total PKC activity was also measured in purified T cells along with whole spleen cell suspensions. While total PKC activity was also significantly ($P < 0.05$) lower in cells from ID mice than those from C and PF mice, no significant difference was observed among cells from C and PF mice (Table III). In resting and mitogen-treated T cells, membrane-bound activity was significantly lower in cells from ID mice than those from C and PF mice. Cytosol PKC activity was also significantly lower in resting T cells from ID mice than those from C and PF mice. In contrast slightly, though not significantly, higher cytosol activity was observed in T cells from ID cells than those from iron-sufficient mice, suggesting reduced translocation.

In resting spleen cells (Fig. 2) and purified T cells (Fig. 3), there was no significant difference in the mean ratios of membrane-bound over cytosol PKC activity among study groups of mice (Fig. 2). These ratios varied between 0.4 and 0.63. In mitogen-treated cells, however, while the ratios increased in C and PF groups by approximately four-fold in Study 1, by seven-fold in Study 2 (Fig. 3), and nearly 11-fold in purified T cells (Fig. 3), they remained below 1 in ID group. The differences among study groups were significant ($P < 0.01$). Cells from ID mice showed significantly ($P < 0.05$) lower mean ratios than did those from C, PF, and repleted mice. Reduced translocation in cells obtained from ID mice cannot be attributed to underfeeding since it was not observed in PF mice. In fact no significant difference was observed between C and PF groups. The ratios returned to normal after repletion with dietary iron (3 days for Con A, 7 days for PHA).

TABLE II. Protein Kinase C Activity in Spleen Cells Incubated in Serum-Containing Medium and That Were Obtained From Control (C), Iron-Deficient (ID), Pairfed (PF) Mice, and Iron-Deficient Mice That Were Repleted by Feeding the Iron-Supplemented Diet for 3 days (R3), 7 days (R7), and 14 days (R14)

	C	PF	ID	R3	R7	R14
Total						
No mitogen	15.6 ± 1.97[a]	22.0 ± 3.1[a]	11.4 ± 1.12[b]	15.6 ± 1.43[a,b]	14.3 ± 1.57[a,b]	14.1 ± 2.37[a,b]
Con A	11.6 ± 1.34[a,b]	17.6 ± 3.14[a]	9.9 ± 0.94[b]	12.3 ± 1.0[a,b]	11.3 ± 1.25[a,b]	10.2 ± 0.69[a,b]
PHA	11.6 ± 1.5[a,b]	19.7 ± 3.15[a]	9.8 ± 0.82[b]	12.3 ± 1.62[a,b]	13.5 ± 1.86[a,b]	9.94 ± 1.28[a,b]
Bound[b]						
No mitogen	4.29 ± 0.47[a,c]	5.92 ± 0.86[a]	3.93 ± 0.49[b]	4.29 ± 0.38[c]	4.93 ± 0.66[c]	4.15 ± 0.51[b]
Con A	7.74 ± 0.95[a,b]	11.9 ± 2.0[a]	4.21 ± 0.62[c]	7.21 ± 1.1[b]	3.59 ± 0.40[c]	4.43 ± 0.46[c]
PHA	7.50 ± 1.3[b]	13.3 ± 2.65[a]	3.66 ± 0.8[c]	5.89 ± 1.38[b,c]	9.26 ± 1.67[b]	5.20 ± 0.82[c]
Cytosol						
No mitogen	10.4 ± 1.52[a,b]	16.1 ± 2.28[a]	7.50 ± 0.71[b]	11.3 ± 0.4[a,b]	9.01 ± 1.03[a,b]	9.98 ± 1.90[a,b]
Con A	3.85 ± 0.5	5.69 ± 1.16	5.6 ± 0.46	5.1 ± 0.79	7.69 ± 0.97	5.79 ± 0.45
PHA	4.11 ± 0.5	6.46 ± 0.82	6.11 ± 0.24	5.36 ± 1.14	4.26 ± 0.25	4.93 ± 0.78

[a]Values are mean ± SEM. n = 18 C, 15 PF, 16 ID, 14 R3, 17 R7, 14 R14. In any row, values followed by different letters are significantly different ($P < 0.05$).
[b]Bound, membrane-bound activity.

TABLE III. Protein Kinase C Activity in Spleen Cells and Purified T Cells by Dietary Iron Treatment Group[a]

	Mitogen	Control	Iron-deficient pmol/10^6 cells/30 min	Pairfed
Spleen				
Total	none	35.6 ± 1.76[a]	26.5 ± 2.23[b]	37.3 ± 0.66[a]
Bound[b]	none	10.4 ± 0.75	8.5 ± 0.73	12.7 ± 1.33
Cytosol	none	25.2 ± 1.85[a]	18.0 ± 2.1[b]	24.7 ± 0.66[a]
Total	Anti-CD3	41.2 ± 2.42[a]	28.2 ± 3.37[b]	40.7 ± 0.67[a]
Bound	Anti-CD3	30.8 ± 2.33[a]	17.8 ± 3.41[b]	32.7 ± 0.66[a]
Cytosol	Anti-CD3	10.4 ± 2.61	10.3 ± 1.10	8.0 ± 1.16
T cells				
Total	none	50.4 ± 1.17[a]	27.3 ± 2.53[c]	39.3 ± 2.4[b]
Bound	none	14.8 ± 1.50	9.75 ± 0.80	14.0 ± 1.16
Cytosol	none	35.6 ± 0.75[a]	18.5 ± 3.51[c]	25.3 ± 2.4[b]
Total	Anti-CD3	54.8 ± 7.79[a]	30.3 ± 3.50[b]	62.0 ± 3.01[a]
Bound	Anti-CD3	42.0 ± 8.15[a]	15.5 ± 3.42[b]	48.0 ± 2.31[a]
Cytosol	Anti-CD3	10.8 ± 1.20	14.8 ± 1.46	14.0 ± 2.0

[a]Values are mean ± SEM (n = 5 control, 8 iron-deficient, 3 pairfed). Within a row, means followed by unlike letters are significantly different ($P < 0.05$; a > b > c).
[b]Bound, membrane-bound.

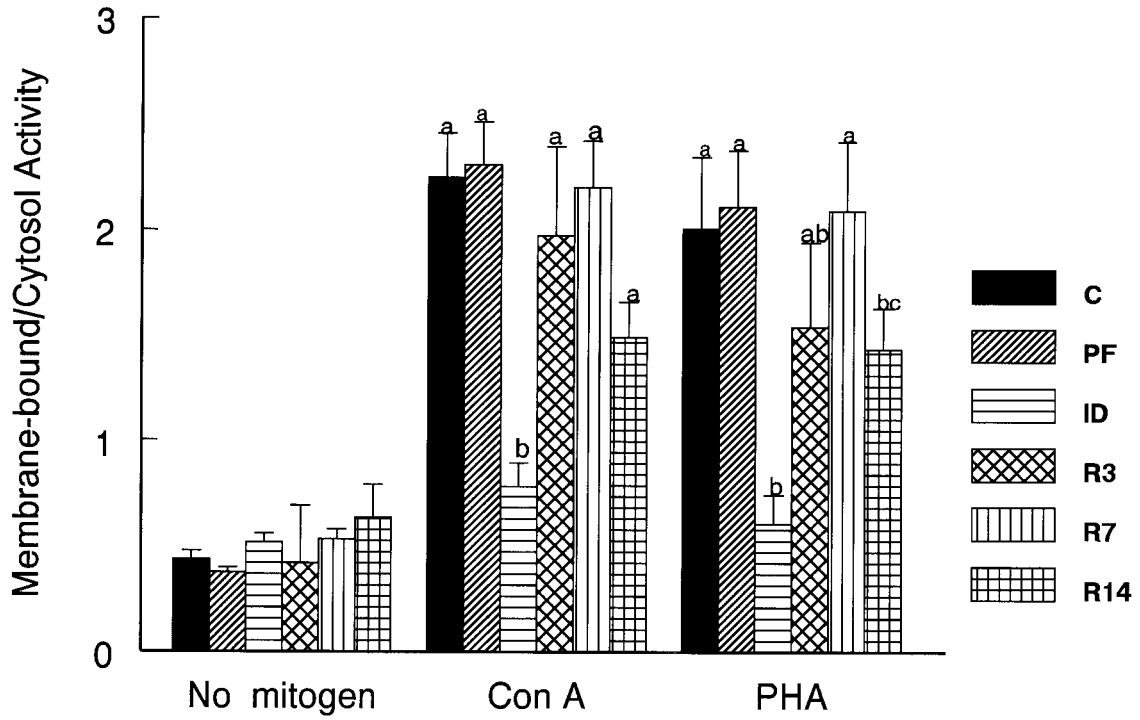

Fig. 2. Protein kinase C translocation from the cytosol to the particulate fraction after cell activation for 30 min. Values are mean ± SEM. n = 18 controls (C), 15 pairfed (PF), 16 iron deficient (ID), ID mice that were repleted (R) for 3, (14 R3), 7 (17 R7), and 14 (14 R14) days. Con A, concanavalin A; PHA, phytohemagglutinin. Bars followed by different letters are significantly different (a > b > c; $P < 0.05$).

PKC Activity in Cells Incubated in Serum-Free Medium With and Without Deferoxamine

In C, ID, and PF mice, incubation of spleen cells in serum-free medium without deferoxamine (DFO) had no significant negative effect on total PKC activity or the translocation from cytosol to membrane-bound fractions in mitogen-treated cells (no data shown). However, as for cells incubated in complete medium, PKC activity and translocation were reduced in cells

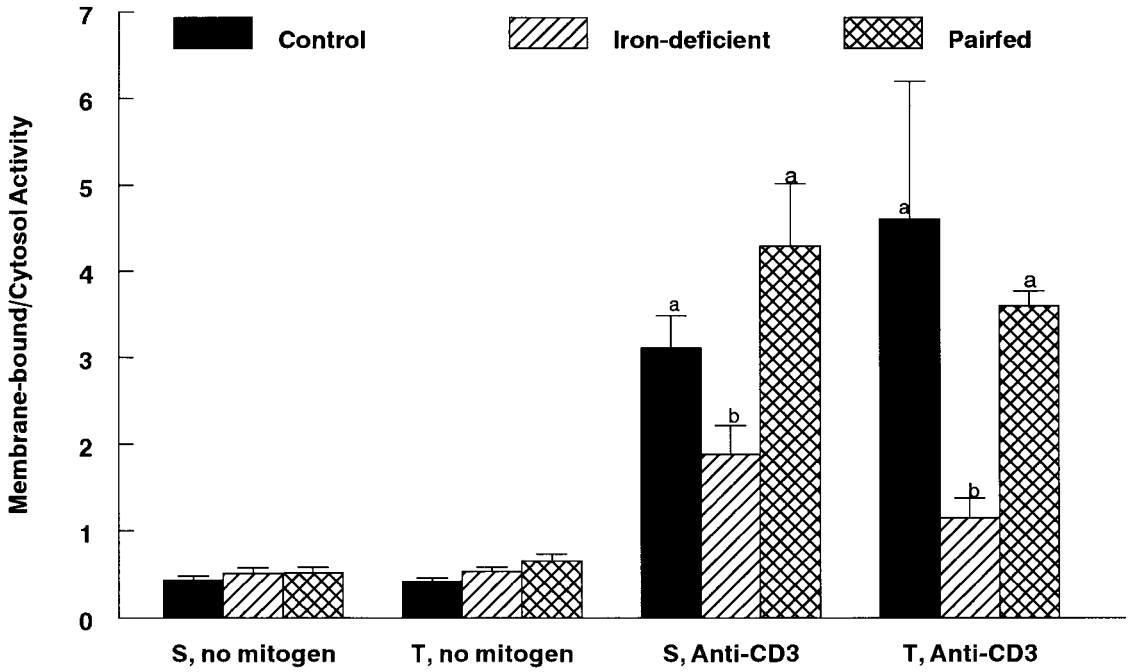

Fig. 3. Ratios of membrane-bound to cytosol PKC activity in purified T cells and spleen cells by dietary treatment groups (n = 5 controls, 8 iron-deficient, and 3 pairfed). Values are mean ± SEM. Within each sets of bars, values followed by different letters are significantly different (a > b; $P < 0.05$).

TABLE IV. Protein Kinase C Activity in Spleen Cells and Purified T Cells Incubated in the Absence and Presence of 250 mg/L Deferoxamine (DF0) 120 min Prior to Cell Activation[a]

		Control	Iron-deficient pmol/10^6 cells/30 min.	Paired
Spleen				
DFO	Mitogen			
Without	None	35.6 ± 1.73[a]	26.5 ± 2.23[b]	37.3 ± 0.66[a]
With	None	16.4 ± 1.94	15.5 ± 2.12	16.0 ± 3.46
Without	Anti-CD3	41.2 ± 2.42[a]	28.3 ± 3.37[b]	40.7 ± 0.66[a]
With	Anti-CD3	22.4 ± 1.17	18.3 ± 1.83	20.7 ± 0.66
T cells				
Without	None	50.4 ± 1.17[a]	27.3 ± 2.54[c]	39.3 ± 2.40[b]
With	None	20.4 ± 1.27[a]	12.8 ± 1.6[b]	16.0 ± 3.0[a,b]
Without	Anti-CD3	54.8 ± 7.8[a]	30.3 ± 3.39[b]	62.0 ± 3.0[a]
With	Anti-CD3	26.4 ± 1.17	20.0 ± 2.67	25.33 ± 3.5

[a]Values are mean ± SEM (n = 5 controls, 8 iron-deficient, 3 pairfed). Within each column, means followed by unlike letters are significantly different ($P < 0.05$; a > b > c).

from ID mice that were incubated in serum-free medium. Because of the puzzling observation of the lack of effect of serum free medium on PKC activity, a pilot study was conducted to study the effects of iron chelation by DFO on total PKC activity and translocation. In spleen cells and purified T cells from C, PF, and ID mice, iron chelation for 2 h prior to cell activation reduced PKC activity by 46–60% in C and PF groups and by 27.7–53.2% in the ID group (Table IV). In fact, in DFO-treated cells, the difference between the three groups of mice disappeared. DFO-treatment also reduced the ratio of membrane-bound over cytosol PKC activity in spleen cells (Fig. 4A) and purified T cells (Fig. 4B). However, the decrease was larger in cells from C and PF mice than those from ID mice.

In resting and activated cells, total PKC and membrane-bound activity positively and significantly ($P < 0.05$) correlated with Hb (0.27–0.54), Hct (0.29–0.54), and liver iron stores (0.28–0.47). In activated cells, an inverse corre-

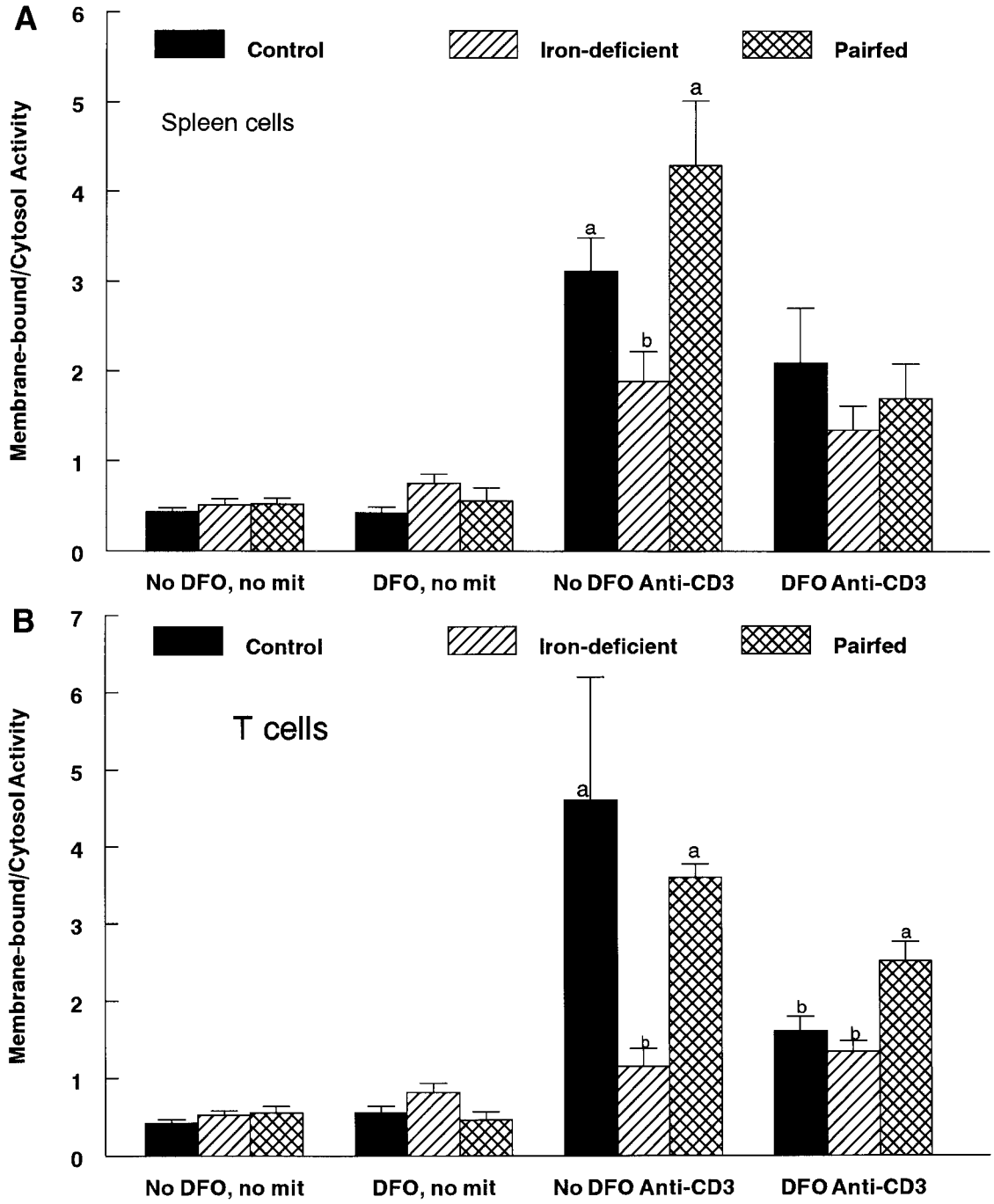

Fig. 4. Effects of deferoxamine (DFO) on PKC translocation in mitogen-treated and untreated spleen **(A)** and purified **(B)** cells (n = 5 controls; 8 iron-deficient, and 3 pairfed). Within each set of bars, values followed by different letters are significantly different (a > b; $P < 0.05$).

lation was observed between cytosolic PKC and both indices of iron status (−0.03–−0.46). This is what we would expect with reduced translocation with low Hb and liver iron stores. In Con A treated cells, ^{3}H-thymidine uptake positively and significantly correlated with the ratio of membrane-bound to cytosol PKC activity (r = 0.335, $P < 0.01$). In PHA treated cells, a similar trend was observed [r = 0.330, r = 0.476, r = −0.305 ($P < 0.05$), for ratio of membrane to cytosol activity, membrane-bound, and cytosol activity, respectively].

DISCUSSION

The present data are in agreement with our previous observation that showed that iron deficiency altered lymphocyte proliferation, total PKC activity, and PKC translocation from the cytosol to particulate fraction upon cell activation with mitogens [Kuvibidila et al., 1991]. Since in PF mice, neither lymphocyte proliferation, nor overall activity and the ratios of membrane-bound over cytosol activity were different from those of C mice, the alteration observed in cells from ID mice cannot be attributed to underfeeding, but rather to lack of iron. Decreased PKC translocation was not due to a simple difference in the proportion of immunocompetent T cells since the difference between ID and C, or ID and PF was maintained even after purifying T cells from the spleen.

Our data are in agreement with those of Alcanra et al. [1994] and Phillips [1987] who showed that lack of iron in incubation medium had a negative effect on total PKC activity in a human T lymphoblastic leukemic cell line. In contrast to our results from C and PF mice, Alcanra et al. [1991, 1994] found that incubation of human T lymphoblastoid cell line in iron-free medium for about 60 min resulted in a significant decrease in total PKC activity. No information was provided on the distribution of PKC (between the cytosol and membrane-bound fractions) or translocation, which is an index for cell activation. The discrepancy between our study and that of Alcanra et al. could be due to the fact that they used a human tumor cell line, whereas we used normal murine spleen cells. However, a more likely explanation of the lack of effects of serum-free medium on PKC activity and translocation in C and PF mice was the presence of residual iron (1.23 µmol/L) in the basal medium and apotransferrin that was added to the medium to maintain adequate viability in all samples. Another possible explanation is that the intracellular iron contents of C and PF cells were high enough to satisfy the needs of these cells during the 2.5 h incubation period. To test this possibility, spleen cells and purified T cells were incubated with DFO for 2 h prior to cell activation. Interestingly, iron chelation significantly decreased PKC activity in both spleen cells and purified T cells from iron-sufficient and iron-deficient mice. However, the decrease was greater in cells from C and PF mice than those from ID mice suggesting higher iron levels in cells from C and PF mice than those from ID mice. Results of the pilot study clearly suggest that iron is essential for PKC activity as well as translocation process. In the ID group, the addition of serum into the culture medium did not result in a significant increase in PKC activity compared to cells that were incubated in medium without serum. The results suggest that whatever the changes that took place in ID cells probably required more than 2.5 h of incubation to be fully corrected.

PKC is not an iron-containing or iron-dependent enzyme, but rather a diacylglycerol and calcium dependent enzyme. The effect of iron deficiency on PKC activity is therefore indirect and may be exerted at two possible levels: at mRNA synthesis and translation, and second at the level of production of diacylglycerol. At least in tumor cells, PKC mRNA content is reduced when cells were incubated in the presence of deferoxamine [Alcanra et al., 1991]. Measurement of PKC mRNA levels in splenic lymphocytes of ID and PF mice is underway. Based on our data on the hydrolysis of cell membrane phospholipids, we believe that reduced diacylglycerol (DAG) production due to impaired hydrolysis of cell membrane phosphatidyl inositol 4,5-bis phosphate (PIP2) may be responsible of impaired PKC translocation [Kuvibidila et al., 1998].

Earlier, we reported that impaired PIP2 hydrolysis in ID cells was not fully corrected even after 14 days of repletion with dietary iron [Kuvibidila et al., 1998]. In contrast, the present study suggests that total PKC activity and translocation returned to control levels within 3 days of repletion and before complete correction of iron deficiency anemia. If we assume that for each molecule of PIP2 that is hydrolyzed, one molecule of DAG and inositol triphosphate (IP3) are produced, then we also expect reduced DAG production in parallel to IP3 in ID and mice repleted for 3 days. Our data on normal PKC activity and translocation in mice repleted for 3 days therefore suggest that iron is also altering PKC activity independently of the changes in PIP2 hydrolysis. The observation made by Alcanra et al. [1991, 1994] on PKC mRNA production tends to support this speculation.

In summary, we hypothesize that lymphocyte proliferation is impaired in iron deficient cells

due to interrelated multiple factors. First, there are changes in cell membrane composition that affect ligand binding and cell membrane phospholipid hydrolysis. These changes lead to a decrease in the production of second messengers inositol triphosphates and diacylglycerol, which are known to influence PKC translocation and perhaps other forms of kinases. Reduced PKC activity affects the phosphorylation of regulatory proteins including transferrin receptor and interleukin-2 receptor. As we [Kuvibidila et al., 1992] and other investigators [Galan et al., 1992] previously reported, there is also reduced secretion of interleukin-2, a cytokine which is essential for optimal lymphocyte proliferation. It is therefore very likely that the altered PKC activity together with reduced activity of ribonucleotide reductase activity and interleukin-2 secretion are all responsible of impaired lymphocyte proliferation and associated functions.

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