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Monocyte factors modulate *in vitro* T-lymphocyte mitogenesis in protein malnutrition

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SUMMARY

This study investigated changes in T-lymphocyte mitogenesis and immunoregulatory cytokines during protein malnutrition. *In vitro* T cell response to concanavalin A was compared among protein deprived (PD), energy restricted pair fed control (PF), and *ad libitum* control (C) rabbits. Cell cultures were supplemented with crude monocyte supernatants (CMS) from PD, PF or C animals at either 1% or 8% final concentration in culture. Prostaglandin E_2 (PGE_2) concentration of unstimulated or stimulated lymphocyte culture supernatants and CMS was determined. Lymphocyte cultures from PD, PF and C animals had enhanced ^{3}H-thymidine incorporation when supplemented with C and/or PF derived CMS. Addition of 8% CMS from PD rabbits inhibited proliferation below levels observed in mitogen-only stimulated groups in all cultures. At the 1% concentration, inhibition was seen in PD and C derived cells cultures and modest enhancement was seen in PF cultures. PGE_2 concentration in supernatants from stimulated and unstimulated lymphocyte cultures from PD rabbits was higher than in C and PF cell cultures. These results suggest (a) that under appropriate culture conditions lymphocytes from PD donors are capable of enhanced proliferation and (b) that depressed T cell mitogenesis observed in protein malnutrition may reflect alterations in immunoregulatory signals. The role of interleukin 1 (IL-1) and PGE_2 in the modulation of this response is discussed.

Keywords protein malnutrition interleukin 1 prostaglandinE_2 T cell mitogenesis

INTRODUCTION

Experimental and clinical protein deficiency is characterized by numerous immunological dysfunctions including depressed *in vitro* T cell responsiveness to polyclonal mitogens and specific antigens (Chandra, 1979; Gross & Newberne, 1980). Recent evidence suggests that alterations in immunoregulatory signals may explain the depressed cellular immune response in malnutrition. For example, monocyte-macrophage derived prostaglandins (PG), especially E_2, control lymphocyte activation by directly blocking transformation (Webb, Rogers & Nowowiejski, 1979); although nutritionally stressed animals have increased activation of macrophages (Watson, Rister & Baehner, 1976), it is not known whether PGE_2 production is consequently elevated. Lymphokine production (e.g., leucocyte inhibitory factor (LIF), γ-interferon) is also known to be affected by protein malnutrition (Lomnitzer *et al.*, 1976; Schlesinger *et al.*, 1977) but the impact on immunoregulation has not been investigated. We (Hoffman-Goetz, Bell & Keir, 1985; Hoffman-Goetz & Marcon, 1983) as well as others (Keenan *et al.*, 1982) have shown that activated monocytes

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from malnourished donors produce less interleukin 1 (IL-1), a monokine required for T cell activation, compared with normally nourished donors.

In this report we examine the relative contributions made by IL-1 and PGE_2 in modulating *in vitro* T lymphocyte mitogenesis in experimental protein malnutrition. Our findings suggest that attenuated T cell responsiveness to mitogens in protein malnutrition may be linked to alterations in the balance of these mediators.

MATERIALS AND METHODS

Animals and diets. Male New Zealand white rabbits (*Oryctolagus cuniculus*), initially weighing 2·6–2·8 kg, were fed for 8 weeks on one of three experimental diets: (a) a 0% protein diet *ad libitum* (PD) (Ralston Purina no. 5765), (b) a 21% protein (casein) diet *ad libitum* (control, C) (Ralston Purina No. 5756), and (c) a 21% protein diet pair fed on a gram/kg body weight basis with the PD group (PF). Tap water was freely available. Fifteen animals maintained on these diets were used as donor sources of crude monocytic supernatants (CMS); a second cohort of 15 rabbits assigned to the same dietary regimens were used as the donor source of lymphocytes for mitogenesis assays. All rabbits were inspected periodically and any animal presenting with signs of clinical disease (i.e. snuffles, diarrhoea) was excluded from the study.

Nutritional indices. Nutritional status of all rabbits was assessed at 8 weeks of dietary restriction by determination of plasma albumin and liver L-alanine aminotransferase activity. Plasma albumin concentration was measured by a standard bromocresol green colorimetric assay. Liver tissue excised from rabbits at the termination of the experiment was stored at −60°C until assayed for enzyme activity. L-alanine aminotransferase activity was determined by a method adapted from Segal and Matsuzawa (1970). Briefly, approximately 200 mg of liver was homogenized in cold water 1:10, wt:vol) and centrifuged (15 min, 1500 ***g***, 4°C). Supernatant (10 μl) was added to a reaction mixture (3·0 ml) containing potassium phosphate buffer, α-ketoglutarate, L-alanine, lactate dehydrogenase, NADH, and water. The change in absorbance at 340 nm was recorded for 7 min by a chart recorder interfaced with a spectrophotometer (Bausch and Lombe).

Preparation of CMS. Crude monocytic supernatants (CMS) were prepared as previously described (Hoffman-Goetz *et al.*, 1985). In summary, rabbits were exsanguinated by cardiac puncture, the blood diluted with equal volumes of sterile phosphate buffered saline (PBS), and the mononuclear cells separated by Ficoll Hypaque centrifugation. The mononuclear cells were washed with PBS and minimum essential medium (MEM) containing 2 mM L-glutamine, Earle's salts and antibiotics (100 U penicillin/ml). The total numbers of cells were counted, differential counts performed and cell viability determined by trypan blue staining. The mononuclear cells ($20·7 \pm 1·1$% monocytes) were suspended at 5×10^6 cells/ml in MEM together with heat-killed *Staphylococcus epidermidis* (ATCC 12228) in a ratio of 40 bacteria: 1 mononuclear cell. The cell suspensions were incubated for 18 h at 39°C. Following centrifugation (1 h, 10,000 ***g***, 4°C) pooled supernatants were sterile filtered (0·2 μm). All CMS were determined to be endotoxin free (to < than 3 ng/mL) as assessed by LAL tests (Sigma Chemical Co., St Louis, Missouri, USA).

Bioassay of CMS. CMS was assessed for IL-1/EP activity by bioassay of the fever response in rabbits. One microlitre of CMS derived from C donors elicited a 0·6–0·7°C rectal temperature rise within 1 h of intravenous (i.v.) injection into rabbits and was equivalent to one Rabbit Pyrogen Dose (Rosenwasser & Dinarello, 1981).

Lymphocyte mitogenesis. Unfractionated lymphocyte-monocyte populations were obtained from C, PF and PD rabbits and prepared as described above (*see* Preparation of CMS). RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum was used in place of MEM and cell concentrations were adjusted to 1×10^6 cells/ml. There were no statistically significant differences among the groups with respect to monocyte number. Cells (200 μl) were seeded in triplicate into 96 well flat bottomed microtiter plates (Flow Labs, Mississauga, Ontario, Canada) and polyclonal T cell mitogenesis was induced with 1·5/ml of concanavalin A (Con A). This dose of Con A has been reported to be suboptimal in normal rabbit lymphocyte blastogenesis (Hoffman-Goetz *et al.*, 1985). CMS from C, PF and PD donors was added at a concentration of 1 or 8% (final volume in culture).

The design of the experiment is shown in Table 1. The cultures were incubated 68 h at 39°C in a humidified 5% CO_2:95% air atmosphere, pulsed with 0·5 μCi of [methyl ^{3}H]thymidine (2·0 Ci/mmol specific activity) (New England Nuclear, Boston, MA, USA) for 6 h, and harvested onto glass fiber filters using a semiautomated multiple microculture harvester (Skatron A.S., Lierbyen, Norway). Radiolabelled thymidine (^{3}H-TdR) incorporation into lymphocytes was measured by liquid scintillation spectrometry (Beckman LS 1801) using commercially available cocktail (Beckman HP/b, Beckman Instruments, Toronto, Canada). ^{3}H-TdR incorporation was expressed as the average ct/min of triplicate samples.

PGE_2 determination. The concentration of PGE_2 present in supernatants of stimulated and unstimulated lymphocyte cultures was measured by radioimmunoassay (Seragen Inc., Boston, MA, USA). 100 μl of supernatants were added to 100 μl of rabbit antiserum against PGE_2 and 100 μl of tracer (^{3}H-PGE_2). After incubation for 2 h at 25°C, 700 μl of dextran-coated charcoal was added. The mixture was centrifuged (100 *g*, 20 min, 4°C) and 500 μl of supernatants was collected. The ct/min of ^{3}H-PGE_2 present in the supernatants was evaluated by liquid scintillation spectrometry and used as a measure of antibody bound ^{3}H-PGE_2. The concentration of nonradioactive PGE_2 present in the samples was calculated from a standard curve and regression analysis. The same RIA procedure was used to determine PGE_2 concentrations in CMS.

Statistical analysis. Lymphocyte proliferation data were analysed by a mixed model analysis of variance with unequal *n*. Nutritional parameters and PGE_2 data were analysed by one way analysis of variance. Post hoc analysis was done using Scheffe's test. A *P* value of 0·05 was accepted as demonstrating statistical significance. Values are reported as group means ± 1 s.e. unless otherwise noted. Delta values were calculated as the difference between Con A stimulation and Con A + CMS stimulation.

Table 1. Design of experiment to determine effect of protein deficiency on CMS stimulation of lymphocyte mitogenesis*

Lymphocyte donor group	CMS donor group	Concentration of IL-1(%)†
C	C	1
C	C	8
C	PF	1
C	PF	8
C	PD	1
C	PD	8
PF	C	1
PF	C	8
PF	C	8
PF	C	1
PF	PF	8
PF	PD	1
PD	C	1
PD	C	8
PD	PF	1
PD	PF	8
PD	PD	1
PD	PD	8

* 2×10^5 cells in response to 1·5 μg/ml of Con A. C *ad libitum* 21% protein diet; PD ad *libitum* 0% protein diet; PF pair fed 21% protein diet.

† Final volume in cell culture.

RESULTS

Nutritional Assessment

Results of the nutritional assessment of rabbits assigned to C, PF and D dietary groups are presented in Table 2. After 8 weeks of dietary restriction, plasma albumin concentration of the PD group was significantly lower than plasma albumin concentrations of PF and C rabbits ($P < 0{\cdot}01$). Liver L-alanine aminotransferase activity has been used to differentiate protein malnutrition from energy restriction in mice (W. Woodward, personal communication). A 10-fold decrease in mean L-alanine aminotransferase activity was seen in the PD rabbits compared with the PF and C groups; this difference was significant ($F(2,12) = 74{\cdot}09$; $P < 0{\cdot}001$).

Lymphocyte mitogenesis

Incorporation of ^{3}H-TdR into lymphocyte cultures stimulated with Con A (1·5 μg/ml) is presented in Table 3. Proliferation to mitogen was significantly depressed in the PD group compared with proliferation responses of lymphocytes from C and PF groups ($P < 0{\cdot}01$).

CMS Lymphocyte Responses

Figures 1–3 show the changes in Con A induced proliferative responses of lymphocytes from PD, PF and C rabbits on culture with CMS. Addition of CMS to cell cultures resulted in significant changes in lymphocyte mitogenesis in PD lymphocytes ($F(2,24) = 11{\cdot}75$, $P < 0{\cdot}0005$) (Fig. 1), in PF lymphocytes ($F(2,26) = 9{\cdot}91$, $P < 0{\cdot}0009$) (Fig. 2), and in C lymphocytes ($F(2,23) = 17{\cdot}8$, $P < 0{\cdot}0115$) (Fig. 3). Supplementation of PD, PF and C cell cultures with CMS derived from PD rabbits did not significantly enhance mitogenic responses and generally inhibited T lymphocyte proliferation. This inhibition of proliferation was most apparent when cells from the three donor sources were cultured with 8% CMS. In order to evaluate whether this pattern of inhibition reflected differences in

Table 2. Assessment of nutritional status of rabbits after 8 weeks on the experimental diets

Dietary group	*n*	Plasma albumin (g/dl)	L-alanine aminotransferase activity (μmol NADH oxidized/mg protein/min)
PD	5	2·80 ± 0·25*	$6{\cdot}29 \times 10^{-4} \pm 4{\cdot}5 \times 10^{-5}$†
PF	5	3·72 ± 0·04	$1{\cdot}61 \times 10^{-3} \pm 7{\cdot}7 \times 10^{-5}$
C	5	4·25 ± 0·12	$1{\cdot}25 \times 10^{-3} \pm 4{\cdot}7 \times 10^{-5}$

*$P < 0{\cdot}01$ *v* C.
†$P < 0{\cdot}0001$ *v* C.

Table 3. Baseline incorporation of ^{3}H-TdR into lymphocyte cultures (2×10^5 cells/well) stimulated with con A*

Diet group	*N*	Unstimulated cultures (ct/min ± s.e.)	Stimulated cultures (ct/min ± s.e.)
PD	5	3,342·7 ± 279	44,101 ± 13615†
PF	5	1,569·6 ± 247	122,972 ± 13061
C	5	963·2 ± 103	132,976 ± 2350

* 2×10^5 cells were incubated with 1·5 μg/ml Con A for 74 h to induce proliferation.
† $P < 0{\cdot}0001$ *v* C.

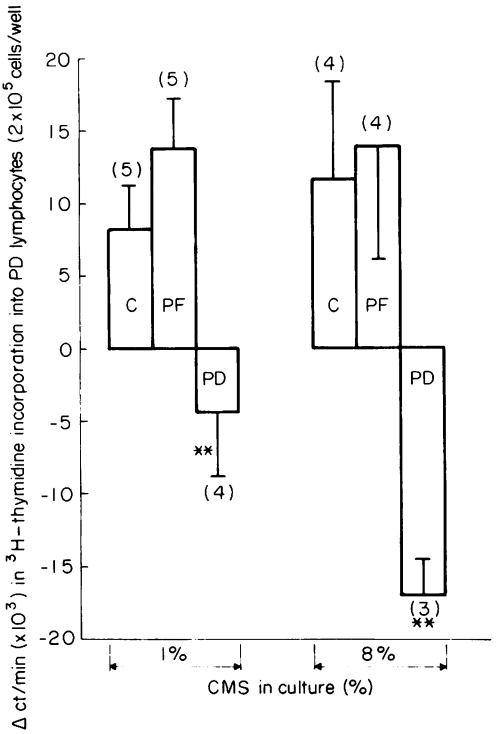

Fig. 1. The change in Con A response of lymphocytes from PD from PD rabbits on culture with CMS. Value = group mean ±1 s.e.m. Numbers in parenthesis = number of animals tested for lymphocyte responsiveness at each concentration of CMS and with each type of CMS. (C) CMS from C animals; (PF) CMS from PF animals; (PD) CMS from PD animals; (**) $P < 0{\cdot}01$ compared with C.

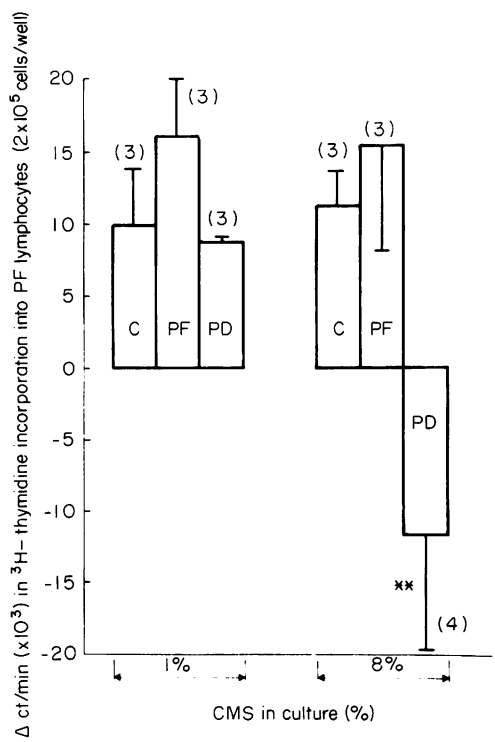

Fig. 2. The change in Con A responses of lymphocytes from PF rabbits on culture with CMS. Value = group mean ±1 s.e.m. Numbers in parenthesis = number of animals tested for lymphocyte responsiveness at each concentration of CMS and with each type of CMS. (C) CMS from C animals; (PF) CMS from PF animals; (PD) CMS from PD animals; (**) $P < 0{\cdot}01$ compared with C.

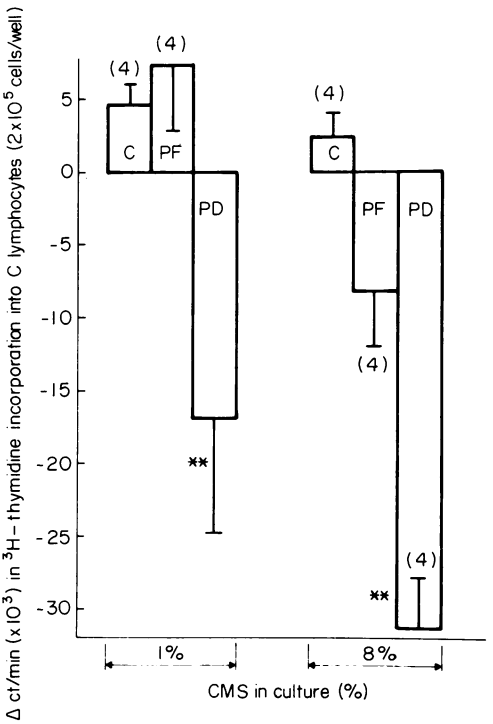

Fig. 3. The change in Con A responses of lymphocytes from C rabbits on culture with CMS. Value = group mean ±1 s.e.m. Numbers in parenthesis = number of animals tested for lymphocyte responsiveness at each concentration of CMS and with each type of CMS. (C) CMS from C animals; (PF) CMS from PF animals; (PD) CMS from PD animals; (**) $P<0{\cdot}01$ compared with C.

mitogen sensitivity among lymphocyte donor groups, cells were cultured in the presence of a higher concentration of Con A (2·5 μg/ml). Addition of PD-derived CMS was associated with an inhibition of proliferation irrespective of donor source of lymphocytes (Table 4).

In contrast, supplementation of PD, PF and C cell cultures with CMS derived from PF and C rabbits significantly enhanced proliferation above Con A (1·5 μg/ml) baseline levels and above levels observed in cultures containing PD-derived CMS ($P<0{\cdot}01$). A notable exception was observed in C derived lymphocytes cultured with 8% CMS. Here, the proliferation responses were significantly greater than in cultures supplemented with PD-derived CMS ($P<0{\cdot}01$) but not different from C-derived cultures stimulated with Con A alone (Fig. 3).

PGE_2 concentrations

The concentrations of PGE_2 found in the supernatants of unstimulated and Con A stimulated lymphocytes from PD, PF and C rabbits are shown in Fig. 4. Stimulated and unstimulated lymphocyte cultures (1×10^6 cells) obtained from PD rabbits produced significantly more PGE_2 compared with PGE_2 production by C and PF lymphocyte cultures following a 74 h incubation ($F(2,11)=23{\cdot}56$, $P<0{\cdot}0001$). There was no significant difference in PGE_2 concentrations of stimulated PF and C cultures. However, unstimulated PF cultures contained significantly more PGE_2 than unstimulated C cultures ($P<0{\cdot}01$). PD and C derived CMS were also tested for PGE_2 concentration. PD derived CMS contained 2890 pg/0·1 ml of PGE_2 compared with C derived CMS (1550 pg/0·1 ml).

Table 4. Proliferation responses to Con A and change from baseline on culture with CMS from PF and PD rabbits*

Lymphocyte donors	Con A	ct/min (with PF-CMS)	ct/min (with PD-CMS)
PD	78,287 ± 20187 (3)	12,636 ± 5523 (3)	−18,042 ± 6528** (3)
PF	150,903 ± 15408 (3)	5,628 ± 4286 (3)	1,853 ± 5010 (3)
C	150,903 ± 11274 (3)	6,558 ± 4165 (3)	−13,839 ± 816† (3)

* 2×10^5 cells/well were incubated for 68 h with 2·5 μg/ml of Con A and 8% CMS from PF control and PD rabbits. Cultures were pulsed for 6 h with 0·5 μCi of ^{3}H-TdR. Number in parentheses is number of rabbits tested.

† difference is significant, $P < 0{\cdot}01$ compared with culture containing PF-CMS.

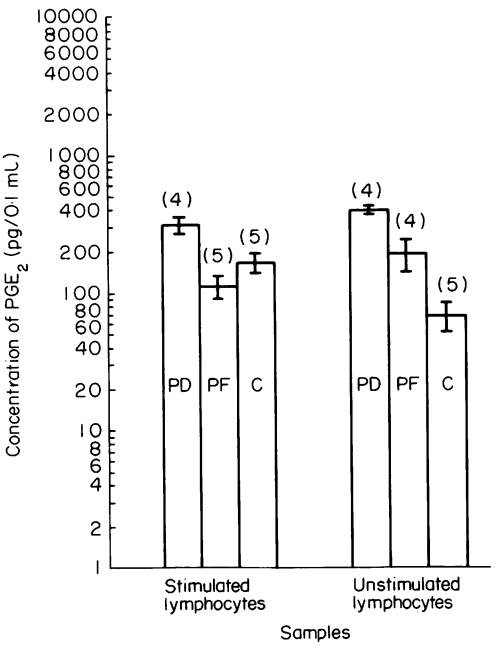

Fig. 4. Concentration of PGE_2 in supernatants of unstimulated and Con A stimulated lymphocyte cultures from C, PF and PD rabbits after 74 h incubation. Values = group means ± 1 s.e.m. Note semi-log scale.

DISCUSSION

Control of the cellular immune response involves a balance of positive and negative regulatory signals. These signals originate from macrophage and lymphocyte populations and involve chemical mediators, such as IL-1, local hormones, such as PGE_2 (Osheroff, Webb & Paulsrud, 1975; Rocklin, Bendtzen & Greineder, 1980) and cell-cell interactions (Hercowitz *et al.*, 1979). The net balance of signals drive the system toward enhancement or suppression of the cellular immune

response. For example, Hudspith *et al.* (1974) have reported reduced IL-1 production and enhanced PGE_2 production by mononuclear cells of sarcoid patients resulting in suppression of *in vitro* lymphocyte mitogenesis. Herman and Rabson (1984) have shown that elevated PGE_2 levels inhibit IL-1 production by natural killer (NK) cells. It has been documented that PGE_2 increases cAMP levels in lymphocytes just before they enter the S phase (Oppenheim *et al.*, 1980), and inhibits lymphoproliferative events.

Our results show that mononuclear cell cultures from protein malnourished rabbits produce factors which reduce *in vitro* T cell mitogenetic responses. The inhibition of T lymphocyte proliferation to mitogen in cultures supplemented with CMS derived from protein malnourished donors suggests little, if any, functionally active IL-1 is present in the crude monocytic supernatants. Depressed proliferation to Con A in cultures from PD rabbits also suggests that IL-1 is not produced by the contaminating monocytes. Although equivalent numbers of monocytes were found in the unfractionated cultures from PD, PF and C donors, it is possible that changes in monocyte subpopulations may have occurred. Shellito and Kaltreider (1984) have shown marked functional heterogeneity of rat macrophages, with high density subpopulations producing more IL-1 and intermediate density macrophages exerting suppressive effects on lymphocyte proliferative responses to mitogen. A shift in the proportion of regulatory mononuclear phagocytes during protein malnutrition might, therefore, contribute to the observed higher levels of immunosuppressive PGE_2. Shifts have been demonstrated for lymphocyte subsets in clinical protein energy malnutrition (Chandra, 1983).

The data presented here show elevated levels of PGE_2 both in CMS and stimulated lymphocyte cultures of protein malnourished donors. Recently, Kunkel and Chensue (1985) reported that arachidonic acid metabolites are potent autoregulators of mitogenesis, with PGE_2 suppressing macrophage IL-1 production in a dose-dependent manner. Thus, elevated PGE_2 levels observed in our study may be involved in suppression of IL-1 synthesis by monocytes. Our findings suggest a role for PGE_2 in both the suppression of mitogenesis in control cultures supplemented with PD derived CMS as well as the lower proliferative responses of PD cell cultures to mitogen alone (Table 3).

Under appropriate culture conditions, it is possible to drive the lymphocytes from protein malnourished donors toward enhanced proliferation. Supplementation of cell cultures with CMS derived from control donors (C & PF), which contain IL-1, is apparently sufficient to override inhibitory factors (i.e. PGE_2) present in cell culture. This finding supports the hypothesis that depressed *in vitro* lymphocyte mitogenesis observed in protein malnutrition is, at least in part, due to alteration in the balance of helper to suppressor factors rather than simply the ability of lymphocytes to respond to these factors.

Supplementation of C derived lymphocytes with 8% CMS from PF rabbits inhibited ^{3}H-TdR incorporation into these lymphocytes. There are two potential explanations for this phenomenon. First, the addition of 8% CMS, coupled with the IL-1 produced by the unfractionated monocyte-lymphocyte population, may exceed the optimal IL-1 concentration required for mitogenesis and, thus, lead to a blunted proliferative response. In this regard, Duff and Durum (1983) reported inhibition of murine splenic and thymocyte-blast formation to Con A at high concentrations of IL-1 in cell culture. Second, the effect of PGE_2 is more pronounced on less differentiated T cells and has been implicated in promoting lymphocyte maturation and differentiation (Oppenheim *et al.*, 1980). Since lymphocyte recirculation and replacement occurs continuously in the intact healthy animal, it is possible that C cultures contain different proportions of mature to immature subpopulations compared with PD and PF cultures. Which of these possibilities can explain the observed inhibition in responsiveness to PF derived CMS is not clear from our data.

The findings presented in this study raise several questions about the mechanisms of nutritionally induced changes in immunoregulation regarding: (a) the uncoupling of depressed IL-1 and enhanced PGE_2 production: would administration of indomethacin and control IL-1 further augment lymphocyte mitogenesis in protein malnutrition?, (b) the mode of action of PGE_2 on lymphocytes from malnourished donors: does PGE_2 work through the adenyl cyclase-cAMP system in these lymphocytes? (c) the level of nutritional stress required to alter the balance of

immunoregulatory signals: is it possible to titrate the effect independently for helper and suppressor factors? and (d) the kinetics of the putative PGE_2-mediated immunosuppression.

Regulation of the cellular immune response depends on the interaction of enhancing and suppressing signals originating from mononuclear phagocytes and lymphocytes. The results presented here suggest that chronic protein malnutrition alters the balance towards immunosuppression by changing the production of PGE_2. This, and other factors which may account for the depressed T-cell proliferative response, can be partially overcome by the provision of IL-1-containing CMS from healthy donors.

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