Elevated Bioactivity of the Tolerogenic Cytokines, Interleukin-10 and Transforming Growth Factor-β, in the Blood of Acutely Malnourished Weanling Mice

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The main objective of this investigation was to determine the influence of acute deficits of protein and energy on the blood levels of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), physiologically the main anti-inflammatory and tolerogenic cytokines. In four 14-day experiments, male and female C57BL/6J mice, initially 19 days old, consumed a complete purified diet either *ad libitum* or in restricted daily quantities, or had free access to an isocaloric purified low-protein diet. A zero-time control group (19 days old) was included. In the first two experiments, serum IL-10 levels were assessed by sandwich enzyme-linked immunosorbent assay (ELISA) and bioassay. The mean serum IL-10 bioactivities were higher ($P \leq 0.05$) in both malnourished groups (low-protein and restricted intake: 15.8 and 12.2 ng/ml, respectively) than in the zero-time and age-matched control groups (6.3 and 7.3 ng/ml, respectively), whereas serum IL-10 immunoactivity was high only in the restricted intake group (e.g., second experiment: 17.0 pg/ml vs. 5.4, 3.7, and 3.1 pg/ml in the zero-time control, age-matched control and low-protein group, respectively). The third and fourth experiments centered on plasma TGF-β immunoactivity (sandwich ELISA) and bioactivity, respectively. The ELISA revealed a high mean plasma TGF-β1 level ($P < 0.05$) in the low-protein group only, but TGF-β bioactivity (β1 isoform, although 15% β2 in the restricted intake group) was high in both malnourished groups (8.7 and 9.3 ng/ml in the low-protein and restricted groups, respectively) relative to the age-matched control group (0.5 ng/ml). Thus, metabolically distinct weanling systems mimicking marasmus and incipient kwashiorkor both exhibit a blood cytokine profile that points to a tolerogenic microenvironment within immune response compartments. A model emerges in which malnutrition-associated immune competence, at least in advanced weight loss, centers on cytokine-mediated peripheral tolerance that reduces the risk of catabolically induced autoimmune disease, but this is at the cost of attenuated responsiveness to infectious agents. Exp Biol Med 231:1439–1447, 2006

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Reduced acquired immune competence and an attenuated inflammatory response are characteristic features of acute (i.e., wasting) deficits of protein and energy in the prepubescent stage of life (1). On theoretical grounds, this phenomenon has been proposed independently by two research groups to confer an adaptive benefit by reducing the risk of autoimmune reactions to catabolically released self-antigens (2, 3). A prediction stemming from this proposition is that immune suppression, critical to the maintenance of peripheral tolerance (4, 5), is a component of the immunodepression associated with acute protein and energy deficiencies. Two broadly immune-suppressive and anti-inflammatory cytokines, interleukin-10 (IL-10) (6, 7) and transforming growth factor-β (TGF-β) (8, 9), occupy dominant positions in current understanding of peripheral tolerance.

In the foregoing context it is interesting that the capacity of murine splenic mononuclear cells to produce IL-10 *in vitro* is unaffected by acute deficits of protein and energy sufficient to reduce infectious disease resistance (10, 11). Moreover, intracellular expression of IL-10 following polyclonal mitogen stimulation was greater in blood $CD4^+$ T cells taken from children exhibiting grades 2 and 3 acute protein-energy malnutrition than in the corresponding cells

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of age- and sex-matched, well-nourished subjects (12). However, several cell types in addition to T cells produce IL-10, notably mononuclear phagocytes, dendritic cells, and keratinocytes (6, 7), as well as adipocytes and stromal elements such as fibroblasts (13), and their relative importance as sources of the cytokine found in intercellular fluids is unknown. Consequently, no information is available to permit a judgment regarding the levels of IL-10 in the microenvironment that determines immune competence *in vivo* in acute forms of malnutrition.

TGF-β is, perhaps, even more pervasive than IL-10 as a tolerance-inducing hormone (8, 9). Mammals produce 3 homodimeric isoforms of TGF-β: β1, β2, and β3 (14). These molecules are highly conserved phylogenetically (14) and exhibit closely overlapping immune functions (8, 15), although in this regard TGF-β1 is the best-characterized isoform *in vivo* (8). High levels of TGF-β are reported in the blood of guinea pigs rendered protein deficient according to a protocol known to depress cell-mediated immune competence (16). However, this important observation has not been confirmed in other similar experimental systems or extended to other forms of acute malnutrition.

Cytokines are autocrine and paracrine hormones. Hence, blood levels constitute spillover which, although unlikely to provide an accurate representation of local extravascular concentrations, is often interpreted to reflect levels at sites of action (17, 18). This is analogous to the interpretation of endocrine hormone concentrations in the blood. Consequently, blood cytokine concentrations are useful in clinical practice and in research into the immunological characteristics of human and animal pathologies (19). The sandwich enzyme-linked immunosorbent assay (ELISA) is the most popular technique for estimation of cytokine concentrations in biological fluids (17). This is primarily because of the speed, simplicity, and specificity of the assay. However, cytokines in the blood are predominantly bound to numerous other proteins, and the sandwich ELISA is widely considered to only be able to detect the unbound fraction (17, 18). Consistent with this model, a sandwich ELISA detected only 2% of the IL-1, IL-6, and IL-10 present in human blood according to a competitive binding assay (18), and it could detect little more than 1% of the IL-10 bioactivity found in the blood of the mouse (19). In addition to problems of quantification, cytokine immunoactivity cannot be presumed to detect a representative fraction of bioactivity because of the inability of an immunoassay to discriminate between biologically active and inactive molecules bearing the epitope(s) that the assay is designed to detect (17).

The main objective of this investigation was to determine the influence of metabolically distinct forms of acute malnutrition (known to produce depression in adaptive immune competence and to be relevant to human wasting pathologies) on the blood concentrations of IL-10 and TGF-β in the weanling mouse. A secondary objective was to extend previous results pertaining to the assay of cytokines in the blood of the healthy mouse (19) by determining whether the sandwich ELISA might detect a representative fraction of IL-10 and TGF-β bioactivity reflective of the pathophysiology of acute protein and energy deficits.

Materials and Methods

Animals and Facilities. Four experiments were performed using male and female C57BL/6J mice from an in-house breeding colony (Department of Human Biology and Nutritional Sciences, Guelph, Canada). Caging and environmental conditions were exactly as described previously (20–23), and the investigations were approved by the Animal Care Committee of the University of Guelph in accordance with the Canadian Council on Animal Care.

Study Design, Diets, and Feeding Protocols. The mice were weaned at 18 days of age and acclimated to a complete, egg white–based, purified diet (24) for 1 day. At 19 days of age each mouse was randomly allocated to one of four groups: an age-matched control group, two malnourished groups, or a zero-time control group, with the latter examined at 19 days of age to permit discrimination between diet- and ontogeny-related phenomena. The age-matched control group was given free access to the complete diet for 14 days, and one malnourished group was fed the same diet in restricted daily quantities that were calculated according to each animal's pattern of weight loss as described elsewhere (20). The second malnourished group was given free access to a low-protein diet for 14 days. These long-established protocols of acute malnutrition consistently elicit a linear pattern of weight loss throughout the 14-day experimental period, during which the animals are weighed daily if subjected to the restricted intake protocol, or every 3 or 4 days if subjected to the low-protein protocol (20–24). Typically, the complete diet contains 19% crude protein, whereas the low-protein diet contains 0.6% crude protein by isocaloric replacement of egg white with starch (21). At the end of the 14-day feeding period, or at 19 days of age (zero-time control group), blood was taken from each mouse and the carcasses were stored at –20°C to await analysis.

Two experiments centered on IL-10, and two additional experiments were directed toward TGF-β. Serum IL-10 immunoactivity was assessed in the first experiment, and sample sizes for the age-matched control, zero-time control, low-protein, and restricted intake groups were 8, 6, 8, and 7, respectively. In turn, within each of the four experimental groups, each sample of animals was made by pooling 4 to 10 mice. In the second experiment the IL-10 concentration of each serum sample was determined by both immunoassay and bioassay. Sample sizes of 18, 16, 16, and 14 were achieved, respectively, for the age-matched control, zero-time control, low-protein, and restricted intake groups, and 2 to 3 mice were pooled to make each sample of malnourished and zero-time control animals. Pooling of animals was done

only in the studies of IL-10 and was maintained within sexes. Moreover, a pooled group of animals constituted a single degree of freedom for the purpose of statistical analysis. Plasma TGF-β immunoactivity and bioactivity were the focus of the third and fourth experiments, respectively, and sample sizes of 8 (third experiment) and 10 (fourth experiment) were achieved for each group of mice. In each of the four experiments similar numbers of males and females were included in each group.

Blood Collection. Blood was taken from the orbital plexus of each mouse under CO_2 anesthesia as described previously (25), and the animals were killed by cervical dislocation without recovering consciousness. For the purpose of the IL-10 assays, the blood was allowed to clot for 30 min at room temperature, and the resulting serum was stored at –80°C. By contrast, platelet-poor plasma was collected for assessment of TGF-β concentrations. This is the preferred procedure, because sample preparation, including blood coagulation, can produce high levels of TGF-β1 as an artifact of platelet activation (26, 27), although this does not always occur (28). Briefly, the first blood flow was discarded, after which 450 μl of blood was collected in tubes prechilled on ice and containing 50 μl of 1.5% EDTA in 0.01 *M* phosphate-buffered saline (pH 7.2). Within 20 min, the blood was centrifuged at 1200 *g* for 15 min at 4°C, and the top 70 μl of plasma was stored at –80°C to await analysis.

Serum IL-10 assay. Serum IL-10 immunoactivity was determined using a commercial sandwich ELISA kit (BD Biosciences, Mississauga, Canada) exactly as described elsewhere (19). In turn, serum IL-10 bioactivity was assessed as described previously (19) on the basis of the ability of IL-10 to inhibit interferon-γ production by mitogen-stimulated T cells *in vitro*. Briefly, a single-cell suspension of splenic mononuclear cells was made in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Sigma Chemical Co.), 1 m*M* HEPES (ICN Biomedicals Inc., Aurora, OH), 10^5 U/L penicillin, and 100 mg/L streptomycin (complete medium). Concanavalin A (Type V; Sigma Chemical Co.) and antihuman TGF-β1 (clone: A75–2, rat IgG2a; BD Biosciences) were added to the mononuclear cell suspension to achieve concentrations of 4 μg/ml and 10 μg/ml, respectively. Cultures were set in 96-well V-bottom plates (catalog #249662, Nalge Nunc International, Roskilde, Denmark), and each well contained 10^6 viable cells (eosin Y exclusion) in 80 μl complete medium plus either 20 μl serum or, for the purpose of a standard curve, 20 μl complete medium containing recombinant murine IL-10 (BD Biosciences). The cultures were incubated for 24 hrs at 37°C in a humidified atmosphere containing 5% CO_2, after which the interferon-γ concentration of cell-free culture fluids was determined by ELISA (OptEIA kit; BD Biosciences). The specificity of the bioassay when applied to mouse serum has been demonstrated by this laboratory using an anti-murine IL-10 monoclonal antibody that was able to eliminate detectable inhibition of interferon production (19).

Plasma TGF-β1 Immunoassay. A commercial sandwich ELISA kit for assay of human TGF-β1 (100% cross-reactivity with murine TGF-β1; BD Biosciences) was applied to acid-activated plasma samples exactly as described by the manufacturer. To achieve acid activation, samples were first diluted with 5 volumes of 0.01 *M* phosphate-buffered saline (pH 7.2), after which 1 volume of 1 *M* HCl was added to 25 volumes of the diluted plasma for incubation at 4°C for 1 hr. To complete their preparation for assay the activated samples were neutralized by addition of 1 volume of 1 *M* NaOH to 29 volumes of activated plasma. Outcomes were quantified by optical density at 450 nm, with background correction at 570 nm, using a Vmax kinetic plate reader (Molecular Devices Corp., Menlo Park, CA).

Plasma TGF-β Bioassay. Plasma samples, which were not subjected to acid activation, were assayed using the Mv-1-Lu mink lung cell line (CCL-64; American Type Culture Collection, Rockville, MD), which exhibits reduced proliferative activity when exposed to any of the three isoforms of mammalian TGF-β (29). The procedure was as described elsewhere (16), with minor modifications. Briefly, the cells were maintained in Eagle's minimal essential medium containing 1.0 m*M* sodium pyruvate and 0.1 m*M* nonessential amino acids (Sigma-Aldrich Canada, Oakville, Canada) and supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich Canada). The cells were subcultured 1 day prior to use to ensure that they were in exponential growth at the outset of the assay, which was conducted in 96-well V-bottom plates (Nunc 249662 VWR Canlab, Mississauga, Canada). Each plasma sample was assayed in 10-μl volumes that were added to wells containing 20×10^3 Mv-1-Lu cells in 90 μl RPMI 1640 medium (Sigma-Aldrich Canada) supplemented to include 10% heat-inactivated fetal calf serum (Sigma-Aldrich Canada), 1 m*M* HEPES (ICN Biomedicals, Irvine, CA), 10^5 U/L penicillin, and 100 mg/l streptomycin. Cultures were incubated for 24 hrs at 37°C in a humidified atmosphere containing 5% CO_2, after which 37 kBq of ^{3}H-thymidine (methyl-^{3}H, specific activity 250 GBq/mmol; ICN Biomedicals) was added to each well for a further 4-hr incubation. Recombinant human TGF-β1 (BD Biosciences, catalog #559119) was used to generate a standard curve.

Carcass Composition. Dry matter, crude protein, and total lipid concentrations were measured as described elsewhere (21).

Statistical Analysis. The SAS system (SAS Institute, Cary, NC) for Windows (version 8.2) was used for statistical analysis (30), and a predetermined upper limit of probability of $P \leq 0.05$ was applied for statistical significance. Data were subjected to two-way ANOVA followed, if justified by the resulting statistical probability value (i.e., $P \leq 0.05$), by Tukey's Studentized Range test. The analyses were conducted with diet (including the zero-

Table 1. Experiment 1: Initial and Final Body Weights, Food Intakes, Carcass Compositions, and Serum IL-10 Immunoactivity of C57BL/6J Mice[a]

Index	Dietary group[b]				SEM
	B	C	LP	R	
Initial body weight (g)[c]	8.4	8.6	8.7	8.8	0.01
Final body weight (g)	—	19.2^{A}	6.8^{B}	6.6^{B}	0.38
Food intake (g consumed in 14 days)[c]	—	65.9^{A}	21.8^{B}	12.6^{C}	0.02
Food intake (g food/g body weight/day)	—	0.34^{A}	0.20^{B}	0.12^{C}	0.01
Carcass composition (% wet weight)					
Dry matter[c]	31.7^{A}	30.5^{AB}	27.8^{BC}	27.1^{C}	0.03
Crude protein[d]	16.8^{B}	15.8^{B}	16.0^{B}	18.2^{A}	12.51
Lipid	9.6^{A}	10.5^{A}	4.3^{B}	1.8^{C}	0.58
Serum IL-10 immunoactivity (pg/mL)[e]	8.4^{B}	3.8^{B}	6.9^{B}	40.4^{A}	—

[a] Values are means, $n=6$, $n=8$, $n=8$, and $n=7$ for groups B (4 males, 2 females), C (4 males, 4 females), LP (4 males, 4 females), and R (3 males, 4 females), respectively. Within a row, values not sharing a superscript letter differ ($P \leq 0.05$) according to Tukey's test, unless a different statistical procedure is indicated. Diet main-effect P values were as follows: initial body weight, 0.064; final body weight, 0.0001; food intake (per 14 days), 0.001; food intake (per g body weight), 0.0001; carcass dry matter, 0.0022; carcass crude protein, 0.0003; carcass lipid, 0.0001; serum IL-10 level, 0.0006.
[b] B, zero-time control (i.e., 19 days of age); C, group consuming complete diet *ad libitum*; LP, group consuming low-protein diet *ad libitum*; R, group fed complete diet in restricted daily quantities.
[c] From ANOVA of natural log-transformed data. Mean values are antilogs of log means.
[d] From ANOVA of square-transformed data. Mean values are square roots of squared means.
[e] Kruskal-Wallis test of Wilcoxon rank sums that were as follows: B, 78; C, 77; LP, 98; R, 182.

time control group) and sex as main effects. Data sets that failed to exhibit normal distribution according to each of the four tests applied by the SAS program ($P \leq 0.05$) were subjected to transformation to bring them into conformity with this basic assumption of parametric testing. Where transformation attempts failed, data were subjected to the Kruskal-Wallis test (χ^2 approximation), which was applied to Wilcoxon rank sums followed, if justified by statistical probability ($P \leq 0.05$), by χ^2 comparisons of Wilcoxon two-sample rank sums. Finally, the association between the IL-10 immunoactivity and bioactivity was assessed by Pearson correlation analysis.

Results

The Malnutrition Protocols Elicited Differing Weight Loss Pathologies. Growth indices for the first experiment are shown in Table 1. Initial body weights did not differ among groups, and the food intakes and gains in fat and lean tissue exhibited by the age-matched control group were comparable to previous results pertaining to C57BL/6J weanlings given free access to the same complete purified diet (21–23, 31). Weight loss did not differ between the two malnourished groups that exhibited deficits in both lean and fat tissue. However, the restricted intake protocol induced a greater loss of carcass lipid (and, hence, a greater deficit in carcass energy) than the low-protein protocol. Further, both malnourished groups exhibited low food intakes relative to the age-matched control, including low levels of intake on a body weight basis. In summary, as discussed previously (23), the low-protein protocol elicited a wasting deficit of both protein and energy, whereas the restricted intake protocol produced mainly a deficit of energy. Growth indices pertaining to the second, third, and fourth experiments (not shown) were comparable to those presented in Table 1.

Serum IL-10 Levels Detected by ELISA and Bioassay. The serum IL-10 immunoactivities of the first experiment are shown in Table 1. No ontogeny-related influence was apparent, nor did the low-protein protocol affect IL-10 immunoactivity. However, the restricted intake group exhibited a higher level of serum IL-10 than the other three groups. These results were confirmed in the second experiment (Fig. 1A), but the bioassay (Fig. 1B) yielded two important differences in outcome from the immunoassay. First, serum IL-10 bioactivities were orders of magnitude higher than the immunoactivities of the same samples. Thus, the results of this investigation confirm a previous report (19) that IL-10 is found at ng/ml levels (of bioactivity) in the blood of the mouse as is also reported in humans on the basis of competitive immunoassay (18). Second, the bioassay revealed high serum IL-10 levels in both malnourished groups, which, in turn, did not differ from one another. Furthermore, correlation analysis revealed no association between IL-10 concentrations determined by ELISA and those assessed by bioassay ($r = -0.02$, $P = 0.87$). Finally, sex influenced neither IL-10 immunoactivity ($P = 0.29$ and 0.96 in the first and second experiments, respectively) nor IL-10 bioactivity ($P = 0.65$). Quality characteristics of the assays, specifically the linearity of standard curves, the intraassay reliabilities (coefficients of variation), and the estimates of detection limits, were comparable to those reported previously by this laboratory (19) and are not shown.

Plasma TGF-β Levels Detected by ELISA and Bioassay. The plasma TGF-β1 immunoactivities from the

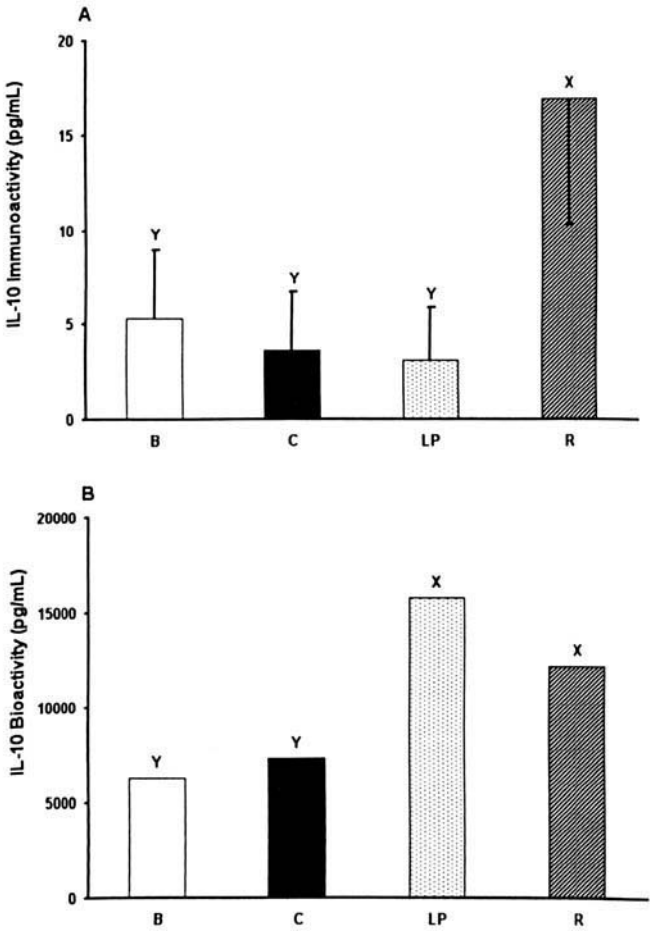

Figure 1. Serum IL-10 concentrations determined by (A) sandwich ELISA and (B) bioassay in male and female weanling C57BL/6J mice given free access to a complete purified diet (group C, age-matched control, 9 males plus 9 females), given free access to a low-protein diet (group LP, 7 males plus 9 females), fed the complete diet in restricted daily quantities (group R, 6 males plus 8 females), or examined at 19 days of age as zero-time controls (group B, 8 males plus 8 females). Groups C, LP, and R were fed for 14 days beginning at 19 days of age, and the malnourished groups lost approximately 1.5% of initial body weight daily. Bars represent mean values, and within each assay bars not sharing an upper case letter differ ($P \leq 0.05$) according to Tukey's test (immunoassay, pooled SEM = 1.058), or according to the Kruskal-Wallis test of Wilcoxon rank sums (bioassay), which were 333 (group B), 433 (group C), 698 (group LP), and 616 (group R). Diet main-effect P values were 0.0001 for both the immunoassay and the bioassay.

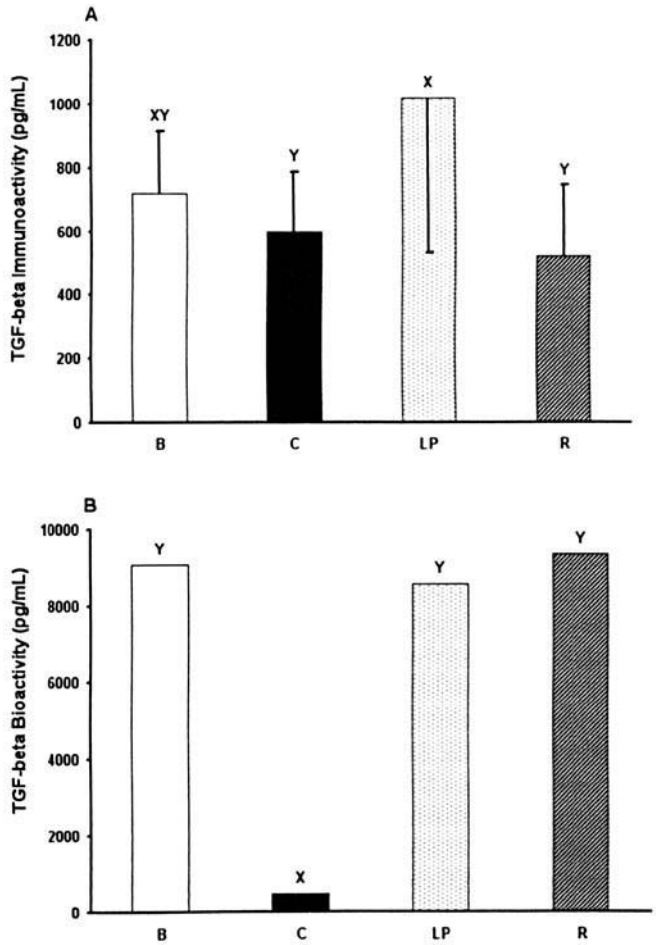

Figure 2. Plasma TGF-β concentrations determined by (A) sandwich ELISA specific for the β1 isoform (4 males plus 4 females in each group, except 5 males and 3 females in group B) and (B) bioassay (5 males plus 5 females in each group), in which all 3 TGF-β isoforms are active. Animals included male and female weanling C57BL/6J mice with free access to a complete purified diet (group C, age-matched control), given free access to a low-protein diet (group LP), fed the complete diet in restricted daily quantities (group R), or examined at 19 days of age as zero-time controls (group B). Groups C, LP, and R were fed for 14 days beginning at 19 days of age, and the malnourished groups lost approximately 1.5% of initial body weight daily. Bars represent mean values, and bars not sharing an upper case letter represent groups that differ ($P \leq 0.05$) according to Tukey's procedure in the case of the immunoactivity assessments (pooled SEM = 0.127), or according to the Kruskal-Wallis test of Wilcoxon rank sums, which were 261 (group B), 56 (group C), 237 (group LP), and 266 (group R) in the study of cytokine bioactivities. Means of immunoactivity measurements are antilogs of log-transformed means. Diet main-effect P values were 0.0053 (immunoassay) and 0.0001 (bioassay).

third experiment are shown in Figure 2A. No ontogeny-related influence was apparent, nor did the restricted intake protocol affect TGF-β1 immunoactivity. However, the ELISA detected a higher level of plasma TGF-β1 in the low-protein group than in either the age-matched control or the restricted intake groups. A sex effect was not found on the plasma TGF-β1 immunoactivity ($P = 0.88$), and the influence of the low-protein diet was independent of sex (P for interaction = 0.23). The standard curve for the assay was linear ($R^2 = 0.992$) up to 2000 pg/ml, and all samples yielded optical density readings within the linear range of the curve. According to procedures described previously for the bioassay of IL-10 (19), the intraassay coefficient of variation (providing a measure of the reliability of the assay) was found to be 3.8%, and the detection limit of the assay was estimated to be 21.4 pg/ml.

The standard curve for the bioassay applied to the samples taken in the fourth experiment was linear (counts per minute versus log TGF-β concentration; $R^2 = 0.998$) over the concentration range from 15 to 1000 pg/ml, and all samples fell within the linear portion of the curve. In addition, the bioassay had a coefficient of variability of

12.9% and a detection limit of 132 pg/ml, each of which was estimated as described elsewhere (19).

The bioassay revealed high plasma TGF-β levels in both malnourished groups relative to the age-matched controls, and the plasma TGF-β bioactivities of the malnourished groups did not differ from one another (Fig. 2B). Two additional differences from the outcome of the immunoassay (Experiment 3) are also noteworthy. First, plasma TGF-β bioactivities of the malnourished groups were approximately one order of magnitude higher than the corresponding immunoactivities. Second, the bioassay revealed an ontogeny-related decline in plasma TGF-β concentration (by comparison of zero-time and age-matched control groups). However, in agreement with the outcome of the ELISA, a sex effect also was not apparent on the plasma TGF-β bioactivity ($P = 0.19$), nor was the influence of malnutrition on this index dependent on sex (P for interaction = 0.52).

The specificity of the bioassay was tested with anti-human TGF-β1 (clone: A75–2, rat IgG2a; BD Biosciences), which was included at a concentration of 50 μg/ml in the assay of 3 plasma samples from age-matched control mice. This procedure eliminated 99% ± 0.6% (mean ± SD) of the detectable bioactivity associated with TGF-β. Moreover, monoclonal antibodies specific for TGF-β2 (capture antibody for E_{max} Immunoassay System, catalog #G7600; Promega Corp., Madison, WI) and TGF-β3 (clone: 44922, mouse IgG1; R&D Systems Inc., Minneapolis, MN) failed to affect the TGF-β bioactivity in plasma from the age-matched control animals which, therefore, exhibited detectable levels of TGF-β1 only. A similar outcome pertained to mice fed the low-protein diet *ad libitum* ($n = 4$), in which 96% ± 2.9% of the TGF-β bioactivity was eliminated by the anti–TGF-β1 antibody and no β2 or β3 isoform was detected. By contrast, anti–TGF-β1 removed only 85% ± 8.4% of the TGF-β bioactivity from the plasma of the mice fed according to the restricted intake protocol ($n = 4$), and a combination of anti–TGF-β1 and anti–TGF-β2 (clone: 8607.211, mouse IgG2b; R&D Systems, Inc.) was required to eliminate TGF-β bioactivity (99% ± 0.5%; $n = 4$) from the plasma of this malnourished group.

A blood TGF-β1 bioactivity comparable to that reported herein for the adolescent mouse also is reported for the adult of this species (32), albeit based on assay of serum rather than of platelet-poor plasma as in the present investigation. A TGF-β1 concentration of approximately 0.5 ng/ml, therefore, may be considered to define the blood bioactivity of this cytokine in the normal laboratory mouse. Moreover, as reported previously in a study of healthy adult humans (26), only β1 isoform–associated bioactivity was detectable in the blood of the healthy mouse. In the present investigation, the sandwich ELISA and the bioassay detected similar levels of TGF-β1 in the blood of the normal mouse. Interestingly, however, the level of cytokine detected in the mouse was only 10% to 20% of the concentration reported in platelet-poor plasma of adult humans (26, 27) on the basis of both an ELISA and a bioassay.

Plasma TGF-β Bioactivity Falls Precipitously Following Weaning. Milk of many species, including the rodent, contains both β1 and β2 isoforms of TGF (33), and rapid intestinal absorption of intact TGF-β is reported in the suckling mouse (34). Consequently, it was possible that the high plasma TGF-β concentration exhibited at weaning (Fig. 2) reflected an uptake of maternal cytokine rather than endogenous synthesis. Attempts to knock out plasma bioactivity with specific monoclonal antibodies (as described for the other groups of mice) identified both β1 and β2 isoforms in the plasma of newly weaned, 19-day-old mice ($n = 4$), a finding consistent with the proposition that milk is a dominant source of TGF-β at this stage of life. Therefore, in view of the short blood plasma half-life of TGF-β in the mouse (35), the prediction emerged that the plasma concentration of this cytokine would fall rapidly after weaning, and it was important to the primary objective of this investigation to determine whether this occurs. To that end, a cohort of male and female C57BL/6J mice was weaned and acclimated according to the procedure applied to the preceding four experiments, and their plasma TGF-β bioactivity was assessed at 19, 21, 23, and 33 days of age (3 males and 3 females at each age tested). The mice were given free access to the complete purified diet used in the preceding experiments of this investigation, and their initial body weights, rates of weight gain, and food intakes were comparable to the age-matched control groups of the preceding experiments. Mean plasma TGF-β bioactivities were 5733, 539, 297, and 113 pg/ml at 19, 21, 23, and 33 days of age, respectively, and the cytokine bioactivity found at 19 days of age differed from that at all subsequent ages tested, which, in turn, did not differ from one another (two-way ANOVA followed by Tukey's test, pooled SEM of log transformed data = 0.74). No sex effect was apparent on plasma TGF-β bioactivity in this cohort of animals ($P = 0.27$), and the decline in cytokine concentration associated with time after weaning was independent of sex (P for interaction = 0.49).

Discussion

The present investigation revealed high bioactivities of IL-10 and TGF-β (predominantly the β1 isoform) in the blood, independently of sex, in advanced stages of metabolically diverse forms of acute, prepubescent protein, and/or energy deficit. By contrast, the influence of these pathologies on blood immunoactivities of the same cytokines, as revealed by sandwich ELISA, depended on the metabolic characteristics of the nutritional deficits. In studies of biological fluids, assays that detect total cytokine levels must be preferred over the sandwich ELISA that detects only a tiny unbound fraction (18) of unknown biological potency (17), and a recent report pertaining to IL-10 in the mouse (19) highlights the bioassay as the gold standard for

quantification of blood cytokine concentrations. Interpretation of the results of the present investigation, therefore, centers on the outcomes of the bioassay procedures. Importantly, numerous previous studies demonstrated depressed primary acquired immune competence in both models of acute malnutrition used in this investigation (21, 22). Moreover, the low-protein and restricted intake protocols used herein impose pathologies that closely mimic and are relevant to the well-defined human conditions of incipient kwashiorkor and marasmus (20, 22, 23, 31). Consequently, this investigation gives rise to the proposition that immune competence in acute protein and energy deficiencies is shaped, in part, by broadly immune suppressive and anti-inflammatory cytokines widely regarded (4, 6–8, 15, 36) as key mediators in the physiologic maintenance of self-tolerance. Furthermore, analysis of the zero-time control group included in the design of these experiments shows that the tolerogenic cytokine profile identified herein reflects more than a biologically trivial delay in weanling ontogeny. Finally, as a point of perspective, this investigation identifies two candidate molecules relevant to the longstanding observation that the blood serum of acutely protein-energy malnourished humans contains factors that inhibit T-cell functions, including proliferation and cytokine production (37).

This investigation confirms previous reports (18, 19) that the sandwich ELISA detects only a tiny fraction of the IL-10 bioactivity in the blood. In addition, the sandwich ELISA detected an unrepresentative fraction of the bioactivity of both IL-10 and TGF-β in this investigation. This was apparent through correlation analysis in the case of IL-10 and, more importantly for the present purpose, through incongruent outcomes regarding the impact of acute protein and energy deficits on the blood cytokine levels as revealed by immunoassay and bioassay. Thus, it was apparent only by way of bioassay that high blood concentrations of IL-10 and TGF-β are characteristic of acute protein and energy deficits independent of metabolic form. By means of validated bioassays for two independent cytokines, therefore, this investigation extends the emerging body of evidence that the sandwich ELISA, despite its convenience-based popularity (17), yields blood cytokine concentrations that cannot be trusted to provide insight into physiologic or pathologic conditions. This evidence is consistent with reservations based on the biochemistry of the sandwich ELISA (17) and on direct comparisons between the sandwich ELISA and the competitive binding immunoassay (18). Similar caution is warranted with regard to biological fluids other than the blood.

A compelling body of evidence suggests that the reduced acquired immune competence associated with acute, prepubescent deficits of protein and/or energy is a regulated pathophysiology under hormonal governance (1, 38). The results of the present investigation suggest that a broadly based cytokine-mediated suppression contributes to this phenomenon, at least in the advanced stages of weight loss. Thus, IL-10 and TGF-β silence T lymphocytes both through direct influences on these cells and indirectly through antigen-presenting cells, natural killer cells, and regulatory T cells. For example, IL-10 inhibits proliferation by activated $CD4^+$ T cells by suppressing transcription of IL-2 (6), and TGF-β exerts a similar influence on naive T cells of both major subsets (8). In addition, TGF-β inhibits differentiation of naive T cells toward Th1, Th2, and $CD8^+$ cytotoxic effector status by way of direct influences on several critical transcription factors (8, 15, 39). Moreover, through less well-characterized mechanisms, IL-10 directly downregulates synthesis of cytokines, notably IL-5 and tumor necrosis factor, by polarized effector $CD4^+$ T cells (6, 36). Thus, TGF-β and IL-10 exert their primary direct influences in a complementary manner by acting on naive and activated T cells, respectively. More than this, a potently suppressive synergy is noteworthy between IL-10 and TGF-β (15, 40). For example, IL-10 renders activated T cells responsive to TGF-β by promoting their expression of the critical RII portion of the TGF-β receptor (8). A major indirect influence of TGF-β and IL-10 on T cells comes by way of antigen presentation. Both TGF-β (8, 15) and IL-10 (5, 6) inhibit the differentiation of dendritic cells and macrophages to the maturity required (4) for promoting development of effector T cells. In fact, maturational arrest yields a population of immature dendritic cells potently suppressive and tolerogenic *vis-à-vis* the naive T cell (4, 5, 38). In addition, both IL-10 and TGF-β suppress the synthesis of key cytokines, notably IL-12, by mature antigen-presenting cells, and thereby inhibit inflammatory Th1-type responses. IL-10 exerts this influence, possibly its most important suppressive function, as a direct action at least in part by downregulating activation of nuclear factor κB (6), whereas TGF-β acts indirectly by suppressing the interferon-γ production of natural killer cells (39). Finally, both TGF-β and IL-10 can induce development of subsets of regulatory T cells currently regarded as critical to peripheral tolerance (6, 8), and the influence of TGF-β in this regard is at least partly a direct action on the T cells (8). In the context of the multiple anti-inflammatory, suppressive, and tolerogenic actions attributed to TGF-β, it is interesting that combined energy and protein deficiency produced high blood levels of only the β1 isoform, whereas energy deficit without accompanying deficiency of nitrogen resulted in high levels of both β1 and β2 isoforms. However, this outcome does not influence the immunologic implications of the findings, because the three isoforms of TGF-β exhibit essentially indistinguishable immunologic activities, at least *in vitro*, presumably as a reflection of their shared target cell receptor complex (15).

The present investigation can identify neither the cellular origin(s) of the IL-10 and TGF-β found in the blood, nor the influences that initiate and sustain high blood concentrations of these cytokines during acute protein and energy deficits. However, several possibilities are noteworthy. In the first place, high levels of glucocorticoids

characterize acute protein and energy deficits both in humans (1, 38) and in the experimental systems used in this investigation (41). In this connection, human T cells and mononuclear phagocytes, widely regarded as major producers of IL-10 (6), increase their production of this cytokine when exposed to high levels of glucocorticoids *in vitro* (42, 43). Likewise, essentially all cells can produce one or another isoform of TGF-β (14), and several types of cells, such as T cells, macrophages, and fibroblasts, respond to glucocorticoids *in vitro* by increasing their production of this cytokine (44). In this context, it is also of interest that moderate caloric restriction increased the expression of TGF-β1 mRNA and protein in the salivary glands of aging autoimmune mice (45). A second possibility relates to apoptosis, a dominant feature of the noninflammatory lymphoid involution that characterizes acute protein and energy deficits (1, 46). Mononuclear phagocytes produce large quantities of both IL-10 (6) and TGF-β (8, 9) following engulfment of apoptotic cells. Moreover, IL-10–producing cells, such as T cells and keratinocytes, release this cytokine in quantity while undergoing apoptosis (6), and release of TGF-β appears to be a general characteristic of apoptotic cells (47). In addition, many types of cells enter apoptosis when exposed to TGF-β at the concentrations found in the blood of the malnourished animals of this investigation (48, 49). Thus, a TGF-β–centered positive feedback loop appears possible in acute malnutrition, and such a phenomenon could underlie the particularly large impact observed herein on the blood levels of this cytokine. By contrast, the levels of TGF-β found in the blood of our age-matched control animals appear insufficient to induce apoptosis (48). Finally, an interaction between IL-10 and TGF-β is reported, in which TGF-β1 directly stimulates IL-10 production by T cells through Smad 4 and the IL-10 promoter (8). Thus, several plausible physiologic mechanisms may conspire to effect the wasting-associated elevation in blood IL-10 and TGF-β concentrations identified in this investigation.

A tolerance-centered model of wasting-associated immune depression has been proposed (2) and recently reintroduced (3) on theoretical grounds. In this model, a reduced capacity to elicit acquired immune responses confers the benefit of protection against autoimmune disease at the cost of susceptibility to infection. Evidence that wasting malnutrition imposes an immunologic challenge in the form of self-antigen derives, for example, from studies of malnutrition-related diabetes in adults (50, 51). In addition, the tolerance model provides an accommodating framework for findings relating protein and energy deficits to autoimmunity and immune regulation. For example, subtotal fasting is reported to alleviate symptoms of rheumatoid arthritis in human adults (52), and protein deficiency facilitated induction of oral tolerance in the young adult mouse (53). IL-10 and TGF-β are considered key mediators of peripheral tolerance (4, 6–8, 15, 36), in which TGF-β is ascribed a dominant role (6, 8). Thus, the results of the present investigation focus attention on peripheral (i.e., extrathymic) tolerance-inducing actions, perhaps most notably the creation of a predominantly immature, tolerogenic dendritic cell population through maturational blockade. This action may be expected not only of IL-10 and TGF-β (5, 6, 8, 15) but also of the glucocorticoid hormones (54), which, likewise, are found at high levels in all forms of acute malnutrition (1, 38), including the experimental systems used herein (41).

It is intriguing that immune depression associated with pathologies in which energy supply is limiting may, nevertheless, reflect energy-requiring suppressive actions. This expectation must be at the core of any proposition that malnutrition-associated immune depression reflects physiologic regulation rather than a biologically trivial disintegrative process.

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