

## Blood serum interferon- $\gamma$ bioactivity is low in weanling mice subjected to acute deficits of energy or both protein and energy

Lyn M. Hillyer, Harry E. Maliwichi and Bill Woodward\*

Department of Human Health and Nutritional Sciences, University of Guelph, Ontario, Canada N1G 2W1

(Received 2 June 2006 – Revised 9 October 2006 – Accepted 13 October 2006)

The main objective of the present study was to determine the influence of acute deficits of protein and energy on the blood serum level of interferon- $\gamma$ , a signature type 1 polarising inflammatory cytokine. In two 14 d experiments, male and female C57BL/6J mice, initial age 19 d, consumed a complete purified diet *ad libitum* or in restricted daily quantities, or had free access to an isoenergetic purified low-protein diet. A zero-time control group (age 19 d) was included in the second experiment. Serum interferon- $\gamma$  was assessed in both experiments by sandwich ELISA and, in the second experiment, also by a bioassay based on inhibition of proliferation by WEHI-279 B lymphoma cells. The immunoassay detected interferon- $\gamma$  inconsistently in all groups (range 0–14 pg/ml; detection limits 1.5 and 0.7 pg/ml in experiments 1 and 2, respectively). By contrast, interferon- $\gamma$  bioactivity was found in all animals of each group (means 339, 499, 124 and 200 pg/ml in zero-time controls, age-matched controls, low-protein and restricted intake groups, respectively; detection limit, 12 pg/ml), and the mean serum bioactivity of each malnourished group was low compared with the age-matched control ( $P \leq 0.05$ ). The present study defines the physiological serum interferon- $\gamma$  bioactivity of the adolescent mouse. Moreover, to the extent achievable by way of the blood, the results reflect the influence of metabolically diverse forms of acute malnutrition on the polarising type 1 cytokine profile within lymphoid microenvironments wherein immune responses arise. Therefore, the results suggest a mechanism underlying the cell-mediated inflammatory incompetence that characterises acute, prepubescent malnutrition.

### Acute malnutrition: Interferon- $\gamma$ : Mice: Protein–energy malnutrition

Acute (i.e. wasting) prepubescent deficits of protein and/or energy consistently produce a profound depression in acquired cell-mediated immune competence, whereas humoral competence is less predictably affected (Woodward, 2001, 2004). Interferon- $\gamma$ , a signature type 1 polarising cytokine, promotes cell-mediated immune responses and the production of opsonising and complement-fixing subclasses of IgG antibody that support this type of response in defence against intracellular pathogens (Goldsby *et al.* 2000; Szabo *et al.* 2003). By contrast, type 2 cytokines such as IL-4 and IL-10 promote production of antibodies whose main function is to provide protection in the extracellular space (Szabo *et al.* 2003). *In vitro*, T cells from rodents subjected to acute protein and energy deficits exhibit a reduced capacity for production of interferon- $\gamma$  while sustaining the ability to produce type 2 cytokines (Woodward, 2001). These important findings pertain to the polarised effector stage of a T cell response; thus, they are consistent with the particular impact of acute malnutrition on cell-mediated immune competence, but provide little insight into the physiological basis for this phenomenon. In this connection, the polarising cytokine profile that prevails during antigen presentation determines the dominant effector T cell response that emerges, i.e. whether type 1 or type 2 cytokine-based (Szabo *et al.* 2003). It is of interest, therefore, to gain insight into the influence of acute malnutrition on the polarising cytokine profile of the immunological micro-environment.

Most cytokines function as autocrine and paracrine hormones and their blood serum or plasma concentrations represent spillover from sites of production. Although the blood cannot provide an accurate representation of cytokine concentrations within extravascular spaces, serum or plasma cytokine concentrations are reasonably interpreted to reflect cytokine levels at immunological sites of action (Bienvenu *et al.* 1998). This is analogous to the classic interpretation of blood endocrine hormone concentrations. Consequently, as discussed briefly elsewhere (Hillyer & Woodward, 2003), judiciously interpreted blood serum or plasma cytokine concentrations are useful in clinical practice and in research into the immunological characteristics both of diverse pathologies and of physiological conditions such as pregnancy, advancing age and the response to exercise. This point of view implicitly presupposes the acquisition of biologically meaningful estimates of serum or plasma cytokine concentrations.

The sandwich ELISA is the most popular technique for estimation of cytokine concentrations in biological fluids (Bienvenu *et al.* 1998). This is primarily because of the speed and simplicity of the assay combined with its specificity. However, cytokines in the blood are predominantly bound to numerous other proteins, and the sandwich ELISA is widely considered to detect only the unbound fraction (Bienvenu *et al.* 1998; Malone *et al.* 2001). Consistent with this model, a sandwich ELISA detected only 2% of the IL-1, IL-6 and IL-10 present in human blood plasma according to a competitive binding assay (Malone *et al.*

\* Corresponding author: Dr Bill Woodward, fax +1 763 5902, email wwoodwar@uoguelph.ca

2001), and could detect little more than 1 % of the IL-10 bioactivity found in the blood serum of the mouse (Hillyer & Woodward, 2003). Further, a 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 (Bienvenu *et al.* 1998). A validated bioassay, therefore, remains the standard against which all other assays should be assessed for the purpose of quantifying blood cytokine concentrations (Hillyer & Woodward, 2003).

The main objective of the present investigation was to determine whether metabolically distinct forms of acute prepubescent malnutrition influence the concentration of interferon- $\gamma$  in the blood serum of the mouse in a manner consistent with the known depression in interferon- $\gamma$ -dependent immune competence in these wasting pathologies. The sandwich ELISA has usually failed to detect interferon- $\gamma$  in the serum or plasma of the laboratory mouse (for example, Cowdery *et al.* 1996; Mukherjee & Talwar, 1996; Halford *et al.* 1998; Faggioni *et al.* 2000; Sass *et al.* 2002) and man (for example, Gonzalez-Quintela *et al.* 1999; Verbon *et al.* 1999; Sutas *et al.* 2000; Yoshizawa *et al.* 2002), although exceptions can be cited both for the mouse (Mooney *et al.* 2000) and for man (Szegedi *et al.* 2003). Moreover, a previous attempt to quantify murine blood serum interferon- $\gamma$  bioactivity also failed to detect the cytokine by means of a bioassay based on inhibition of a viral cytopathic effect *in vitro* (Solis-Pereyra *et al.* 1997). Nevertheless, in view of the ongoing production of interferon- $\gamma$  by several types of cells, notably natural killer cells and effector T cells (Goldsby *et al.* 2000), it must be expected that this cytokine is a physiological component of blood serum or plasma. Therefore, a second objective of the present investigation was to identify a bioassay for interferon- $\gamma$  suitable for application to blood serum.

## Experimental methods

### *Animals and facilities*

Male and female C57BL/6J mice were used from an in-house breeding colony derived from animals purchased several years earlier from the Jackson Laboratory (Bar Harbor, ME, USA). This colony is maintained under conventional conditions, but incoming air is filtered, each cage is supplied with a filter lid and positive pressure is maintained relative to the adjoining corridor. According to periodic analysis of sentinel mice maintained in cages without filter lids, the colony is free of common viral pathogens as well as *Mycoplasma pulmonis* and intestinal parasites. Caging and housing conditions were exactly as described previously (Konyer *et al.* 2003; Neyestani & Woodward, 2005), and the investigation was approved by the Animal Care Committee of the University of Guelph in accordance with the guidelines of the Canadian Council on Animal Care.

### *Diets, feeding protocols and experimental design*

Two experiments were conducted. Mice were weaned at age 18 d and acclimated for 1 d with free access to a complete purified diet used routinely in this laboratory (Konyer *et al.* 2003; Neyestani & Woodward, 2005). A typical proximate analysis

for this diet is 92.3 % DM, 18.8 % crude protein, 8.1 % diethyl ether extract, 2.6 % ash, 3.1 % crude fibre and 17.0 kJ gross energy/g (Neyestani & Woodward, 2005). At age 19 d, the mice were randomly allocated to experimental groups. These included an age-matched control group that consumed the complete diet *ad libitum*, a group that consumed the complete diet in restricted daily quantities, a group given free access to a low-protein purified diet and, in the second experiment, a zero-time control group that was examined at age 19 d to permit discrimination between diet- and ontogeny-related phenomena. The quantity of diet given the mice in the restricted intake group was calculated daily according to each animal's pattern of weight loss according to a standardised procedure in this laboratory (Neyestani & Woodward, 2005). The purified low-protein diet (Konyer *et al.* 2003) contained 0.6 % crude protein (as fed) and was prepared by replacement of most of the egg white (80 % crude protein; US Biochemical, Cleveland, OH, USA) of the complete diet with an equal weight of maize starch (St Lawrence Starch, Port Credit, Canada). These long-established protocols of acute malnutrition consistently elicit a linear pattern of weight loss, between 1.5 and 2 % of initial body weight daily, throughout the 14 d experimental period.

During the acclimation and experimental periods, animals were housed individually in cages without filter lids. In addition, all animals had free access to clean tap water, and coprophagy was permitted. The three groups other than the zero-time control were maintained on their respective regimens for 14 d, i.e. from age 19 d to age 33 d. In the first experiment, sample sizes were nine (four females and five males), ten (five females and five males) and ten (five females and five males) for the age-matched control, low-protein and restricted intake groups, respectively, and each sample within the malnourished groups was made by pooling two to three mice. In the second experiment, six mice (three females and three males) were included in each of the four groups, and each sample of malnourished animals was made by pooling three mice whereas each sample of zero-time controls was made by pooling two animals. In both experiments, pooling was maintained within sexes, and a pooled sample constituted a single degree of freedom for the purpose of statistical analysis. At the end of the 14 d experimental period, or at age 19 d (zero-time control group), blood was taken from each mouse and the carcasses were stored at  $-20^{\circ}\text{C}$  to await analysis.

### *Blood collection*

After measurement of body weight, blood was taken from the orbital plexus of each mouse under  $\text{CO}_2$  anaesthesia as described previously (Neyestani & Woodward, 2005), and the animals were killed by cervical dislocation without recovering consciousness. The blood was allowed to clot at room temperature for 30 min, and the resulting serum was stored at  $-80^{\circ}\text{C}$ .

### *Serum interferon- $\gamma$ immunoassay*

Serum interferon- $\gamma$  immunoactivity was determined using a commercial sandwich ELISA kit (BD Biosciences, Mississauga, ON, Canada) exactly according to the manufacturer's instructions. Outcomes were quantified by optical density at

450 nm, with background correction at 570 nm, using a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA, USA).

#### Serum interferon- $\gamma$ bioassay

The procedure was performed as described elsewhere (Reynolds *et al.* 1987) with minor modifications, and is based on the well-known capacity of interferon- $\gamma$  to inhibit B cell proliferation. Briefly, WEHI-279 mouse B lymphoma cells (CRL-1704; American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Canada, Oakville, ON, Canada) supplemented to contain 4 mM-L-glutamine, 1.5 g sodium bicarbonate/l, 4.5 g glucose/l, 0.11 g sodium pyruvate/l, 3.9 mg 2-mercaptoethanol/l and 10% heat-inactivated fetal calf serum (Sigma-Aldrich Canada). Assays were performed using ninety-six-well V-bottom microplates (Nunc 249662; VWR Canlab, Mississauga, ON, Canada) and each well included  $20 \times 10^3$  WEHI cells in 200  $\mu$ l medium to which had been added 15  $\mu$ l of either serum sample or recombinant mouse interferon- $\gamma$  standard (catalogue no. CL9209R; Cedarlane Laboratories, Hornby, ON, Canada), the latter to construct a standard curve. Cultures were maintained at 37°C for 3 d in a humidified atmosphere containing 5% CO<sub>2</sub>, and were pulsed, for the last 4 h, with 37 kBq per well methyl-[<sup>3</sup>H]thymidine (specific activity 250 GBq/mmol; MP Biomedicals, Solon, OH, USA). All samples were assayed in triplicate, and assay specificity was ensured by also including triplicate wells of each sample to which were added anti-mouse interferon- $\gamma$  (1  $\mu$ g/ml; clone RMMG-1, rat IgG1; BD Biosciences, Mississauga, ON, Canada).

#### Carcass composition

DM and total lipid concentrations were determined as described elsewhere (Konyer *et al.* 2003; Neyestani & Woodward, 2005).

#### Statistical analysis

Statistical analysis was performed according to the Statistical Analysis Systems (SAS) software package version 8.2 (SAS Institute, Cary, NC, USA), 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 Duncan's new multiple range test. The analyses were conducted with diet (including the zero-time control group in the second experiment) 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 ( $\chi^2$  approximation) which was applied to Wilcoxon rank sums followed, if justified by statistical probability ( $P \leq 0.05$ ), by  $\chi^2$  comparisons of Wilcoxon two-sample rank sums.

## Results

### The malnutrition protocols elicited distinct weight-loss pathologies

Growth indices for the two experiments are shown in Table 1 and reflect similar outcomes in the separate studies. Initial

**Table 1.** Initial and final body weights, food intakes and carcass compositions (Mean values with their pooled standard errors)

Index	Dietary group				SEM
	B	C	LP	R	
Experiment 1					
Initial body weight (g/mouse)	–	8.1	8.7	8.7	1.00
Final body weight (g/mouse)*	–	17.6 <sup>a</sup>	6.5 <sup>b</sup>	6.5 <sup>b</sup>	0.04
Food intake (g/mouse per 14 d)*	–	48.1 <sup>a</sup>	20.1 <sup>b</sup>	10.3 <sup>c</sup>	0.03
Food intake (g/g body weight per d)*	–	0.27 <sup>a</sup>	0.19 <sup>b</sup>	0.10 <sup>c</sup>	0.01
Carcass composition (g/100 g wet weight)					
DM	–	28.6 <sup>a</sup>	26.8 <sup>b</sup>	24.9 <sup>c</sup>	0.42
Lipid	–	8.5 <sup>a</sup>	3.9 <sup>b</sup>	2.0 <sup>c</sup>	0.33
Experiment 2					
Initial body weight (g/mouse)	7.8	8.2	8.0	7.9	0.29
Final body weight (g/mouse)*	–	17.9 <sup>a</sup>	6.2 <sup>b</sup>	6.1 <sup>b</sup>	0.05
Food intake (g/mouse per 14 d)*	–	69.0 <sup>a</sup>	22.5 <sup>b</sup>	10.9 <sup>c</sup>	0.03
Food intake (g/g body weight per d)*	–	0.38 <sup>a</sup>	0.23 <sup>b</sup>	0.11 <sup>c</sup>	0.02
Carcass composition (g/100 g wet weight)					
DM	29.1 <sup>b</sup>	31.9 <sup>a</sup>	28.2 <sup>c</sup>	27.0 <sup>c</sup>	0.45
Lipid	8.0 <sup>b</sup>	10.2 <sup>a</sup>	4.6 <sup>c</sup>	2.5 <sup>d</sup>	–

B, zero-time control group, age 19 d; C, age-matched control group given free access to complete diet; LP, group given free access to low-protein diet; R, group fed complete diet in restricted daily quantities.

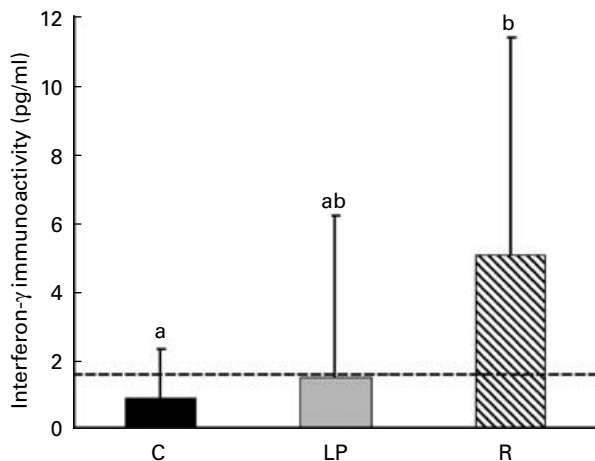
<sup>a,b,c,d</sup> Within a row, values with unlike superscript letters were significantly different ( $P \leq 0.05$ ) according to Duncan's new multiple range test or (experiment 2, carcass lipid) according to a Kruskal–Wallis test of Wilcoxon rank sums that were as follows: B, 92.0; C, 124.0; LP, 63.0; R, 21.0. Outcomes pertain to diet main effect, only. In both experiments, main effects of sex (not shown) were apparent only on final body weights (males greater than females) and food intakes expressed on a body-weight basis (females greater than males).

\*From ANOVA of natural log-transformed data. Mean value is antilog of log mean.

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 with previous results pertaining to C57BL/6J weanlings given free access to the same complete purified diet (Konyer *et al.* 2003; Neyestani & Woodward, 2005). Weight loss did not differ between the two malnourished groups, which 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 (Konyer *et al.* 2003), the low-protein protocol elicited a wasting deficit of both protein and energy, whereas the restricted intake protocol produced mainly a deficit of energy.

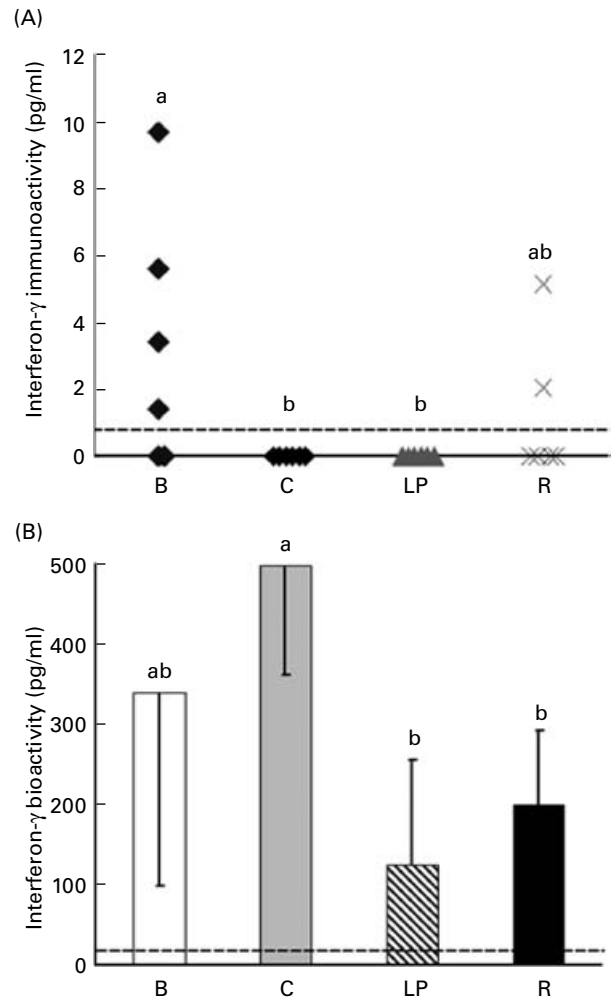
*Blood serum interferon- $\gamma$  immunoactivity: physiological levels and the influence of acute forms of protein and energy deficit*

The serum interferon- $\gamma$  immunoactivities revealed by sandwich ELISA are shown in Fig. 1 for the first experiment and in Fig. 2 (A) for the second experiment. The linearity of the standard curve of the assay, expressed as  $R^2$ , exceeded 0.99 in both experiments. In addition, the reliability (intra-assay CV) and detection limit of the assay were estimated as described elsewhere (Hillyer & Woodward, 2003) and were, respectively, 5.2% (experiment 1) and 1.5% (experiment 2) and 1.5 pg/ml (experiment 1) and 0.7 pg/ml (experiment 2). Interferon- $\gamma$  levels near the detection limit of the assay were found in all groups together with evidence of a decline in interferon- $\gamma$  immunoactivity during post-weaning prepubescent ontogeny. In both experiments, the healthy adolescent mouse exhibited an immunoactivity below the detection



**Fig. 1.** Serum interferon- $\gamma$  concentrations determined by sandwich ELISA. Weanling C57BL/6J mice had free access to a complete purified diet (group C; four females and five males), or an isoenergetic low-protein diet (group LP; five females and five males) or were fed the complete diet in restricted daily quantities (group R; five females and five males). The mice were fed for 14 d beginning at 19 d of age and the malnourished groups lost approximately 1.8% of initial body weight daily. Values are means, with standard deviations represented by vertical bars. <sup>a,b</sup> Mean values with unlike letters were significantly different ( $P \leq 0.05$ ) according to Duncan's new multiple range procedure (pooled SEM 0.29). (---), Detection limit of the assay.

limit of the ELISA, as did the mice subjected to a combined protein and energy deficit by consumption of the low-protein diet. By contrast, energy deficit apart from protein insufficiency (i.e. the restricted intake model) inconsistently supported a detectable level of interferon- $\gamma$  immunoactivity such that the group level detected differed statistically from that of the age-matched control group only in the first experiment. Results pertaining to sex are not shown because this factor did not affect the level of interferon- $\gamma$  immunoactivity



**Fig. 2.** Interferon- $\gamma$  concentrations determined by (A) sandwich ELISA and (B) bioassay in the same samples of blood serum. Weanling C57BL/6J mice had free access to a complete purified diet (group C), or an isoenergetic low-protein diet (group LP), or were fed the complete diet in restricted daily quantities (group R). Zero-time controls (group B; age 19 d) were also included. Groups C, LP and R were fed for 14 d beginning at 19 d of age and the malnourished groups lost approximately 1.6% of initial body weight daily. Each of the four groups included three females and three males. (A) The diet main-effect for the immunoassay was significant ( $P = 0.024$ ) based on a Kruskal-Wallis test of Wilcoxon rank sums which were 106.0 (group B), 57.0 (group C), 57.0 (group LP) and 80.0 (group R). <sup>a,b</sup> Groups with unlike letters were significantly different in serum interferon- $\gamma$  immunoactivity ( $P \leq 0.05$ ) according to  $\chi^2$  comparisons of Wilcoxon two-sample rank sums. (B) The diet main-effect for the bioassay was significant ( $P = 0.038$ ) and the values, with standard deviations, are antilogs of means from natural log-transformed data. <sup>a,b</sup> Mean values of serum interferon- $\gamma$  bioactivity with unlike letters were significantly different ( $P \leq 0.05$ ) according to Duncan's new multiple range procedure (pooled SEM 0.059). (---), Detection limit of the ELISA (A) or bioassay (B).

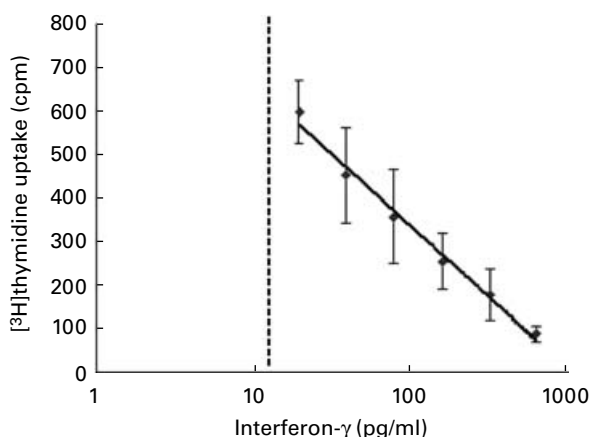
( $P=0.64$  and  $0.97$ , respectively, in the first and second experiments). Moreover, the parametric analysis to which the data from the first experiment could be subjected revealed that the influence of diet on serum interferon- $\gamma$  immunoactivity was independent of sex (interaction  $P=0.77$ ).

#### Application of the WEHI-279 bioassay to blood serum

A typical standard curve obtained in connection with the WEHI-279 proliferation inhibition bioassay is shown in Fig. 3. The curve conformed to a logarithmic function up to an in-well concentration of  $625$  pg/ml, representing dilutions of  $15:100$ . Averaging across the assays used in the present investigation, the linearity of the standard curve, expressed as  $R^2$ , was  $0.99$ , and the reliability (intra-assay CV) and in-well detection limit of the assays, estimated as described elsewhere (Hillyer & Woodward, 2003), were  $3.0\%$  and  $12$  pg/ml, respectively. Correction for non-interferon- $\gamma$  inhibitory activity proved essential to the specificity of the assay when applied to blood serum. The proportion of inhibitory activity attributable to interferon- $\gamma$  was  $44$  (SD  $15$ ) % across dietary groups and was independent of both diet ( $P=0.59$ ) and sex ( $P=0.61$ ).

#### Blood serum interferon- $\gamma$ bioactivity: physiological levels and the influence of acute forms of protein and energy deficit

Serum interferon- $\gamma$  bioactivities are shown in Fig. 2 (B). All groups exhibited bioactivities far above the detection limit of the assay and, comparison with the results in Fig. 2 (A) (pertaining to the same samples), revealed that the sandwich ELISA detected no more than  $1\%$  of the interferon- $\gamma$  bioactivity in the blood serum of the adolescent mouse either in health or in wasting disease. By further contrast with the results obtained by ELISA, both malnourished groups exhibited a low interferon- $\gamma$  bioactivity relative to the age-matched control group which, in turn, did not differ from the zero-time



**Fig. 3.** A representative standard curve for the interferon- $\gamma$  bioassay. Quality-control measures include: linearity of standard curve between  $20$  and  $625$  pg/ml interferon- $\gamma$ , representing in-well concentrations ( $R^2$   $0.99$ ); intra-assay CV as an estimate of reliability ( $2.8\%$ ); in-well detection limit represented by the vertical dashed line ( $14.4$  pg/ml). The assay was based on inhibition of proliferation of WEHI-279 mouse B lymphoma cells *in vitro* and the outcome measure was uptake of [ $^3$ H]thymidine (counts/min; cpm). Values are the means of three replicates, with the vertical bars representing the range of cpm values obtained at the corresponding cytokine concentration.

control group in terms of this index. Sex did not influence serum interferon- $\gamma$  bioactivity ( $P=0.72$ ) nor did the influence of diet on this index depend on sex (interaction  $P=0.86$ ). Hence, results pertaining to sex are not shown.

#### Discussion

The present investigation identifies a bioassay suitable for quantification of interferon- $\gamma$  concentrations in blood serum. The assay reveals a low bioactivity of interferon- $\gamma$  in this biological fluid, independently of sex, in advanced stages of metabolically diverse forms of acute, prepubescent protein and/or energy deficit. Mean serum bioactivities were  $25$  and  $40\%$  of age-matched control in the low-protein and restricted intake groups, respectively. By contrast, the influence of these pathologies on blood serum immunoactivities of the same cytokine, as revealed by sandwich ELISA, depended on the metabolic characteristics of the nutritional deficit. 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 (Malone *et al.* 2001) of unknown biological potency (Bienvenu *et al.* 1998). Also, a recent report pertaining to IL-10 in the mouse (Hillyer & Woodward, 2003) highlights the bioassay as the gold standard for quantification of blood cytokine concentrations. Interpretation of the results of the present investigation, therefore, centres on the outcomes of the bioassay procedure. Importantly, numerous previous studies demonstrate depressed primary cell-mediated immune competence in both models of acute malnutrition used in the present investigation (Woodward, 2001). Moreover, the low-protein and restricted intake protocols used here impose pathologies closely mimicking, and relevant to, the well-defined human conditions of incipient kwashiorkor and marasmus (Konyer *et al.* 2003; Neyestani & Woodward, 2005). Consequently, low concentrations of interferon- $\gamma$  within the lymphoid microenvironment may contribute to depression in cell-mediated immune competence in acute prepubescent protein and energy deficiencies, at least in their advanced stages. Analysis of the zero-time control group included in the design of these experiments permits the conclusion that the cytokine bioactivity profile identified here in association with acute malnutrition reflects more than a biologically trivial delay in weanling ontogeny.

The results of the present investigation are relevant to a growing body of evidence that adaptive immune competence in acute malnutrition features a new balance between type 1 and type 2 cytokines. This proposition (Woodward, 2004; Neyestani & Woodward, 2005) is based on several types of evidence. In the first place, cell-mediated immune competence is consistently depressed by acute malnutrition, whereas humoral responses are less predictably affected (Woodward, 2001, 2004). Moreover, the serum immunoglobulins of mice subjected to malnutrition according to the experimental protocols used in the present investigation are skewed toward a class and subclass profile consistent with systemic type 2 cytokine polarisation (Neyestani & Woodward, 2005). Some evidence pertaining directly to cytokines is also available. In particular, T cells from acutely malnourished rodents have exhibited a depressed capacity to produce interferon- $\gamma$  in response to antigen and polyclonal mitogen stimulation, while production of IL-4 remained unaffected (Woodward, 2001). Similarly, a type 2 cytokine shift was apparent when IL-4 and interferon- $\gamma$  production was

assessed, *in vitro*, in mitogen-stimulated blood T cells from rheumatoid arthritis patients subjected to a short-term fast (Fraser *et al.* 1999). Now, the present results demonstrate low serum bioactivities of a signature type 1 cytokine, thereby constituting the first evidence of the impact of acute malnutrition on the net ability to sustain a polarising type 1 or type 2 cytokine *in vivo*. In addition, high blood serum bioactivities of the type 2 cytokine, IL-10 (albeit not a polarising cytokine), are reported in the experimental systems used in the present investigation (Hillyer *et al.* 2006). Therefore, to the extent that extrapolation from the blood to extravascular fluids is meaningful, as discussed in a substantial literature elsewhere (for example, Bienvenu *et al.* 1998), evidence is accruing that acute deficits of protein and energy elicit type 2 polarisation on the part of the cytokine microenvironment within which immune responses arise. These results reflect the polarising milieu influencing initiation of both primary responses, i.e. within lymphoid organs, and memory responses, i.e. in the non-lymphoid periphery. The type 2 polarised endpoint of adaptive immune competence in acute malnutrition, therefore, may have its origins at least partly in the polarising cytokine microenvironment that prevails at the moment of antigen encounter.

The present investigation does not address the mechanism underlying the low blood serum levels of interferon- $\gamma$  in acute protein and energy deficits. However, several reasonable possibilities can be cited. Numerous inflammatory cytokines, including interferon- $\gamma$ , are positively regulated through the transcription factor NF- $\kappa$ B (Ghosh *et al.* 1998), which is reported to resist activation in one model of murine combined protein and energy deficiency known to produce cell-mediated immune depression (Anstead *et al.* 2003). The high blood serum levels of IL-10 reported in the experimental systems used in the present investigation (Hillyer *et al.* 2006) are relevant in this context. This cytokine is a primary anti-inflammatory mediator that indirectly down regulates production of interferon- $\gamma$  by polarised effector T cells both by inhibiting dendritic cell maturation and by inhibiting IL-12 production by mature antigen-presenting cells (Moore *et al.* 2001). In fact, the latter action, probably the main anti-inflammatory influence of IL-10, is mediated through inhibiting activation of NF- $\kappa$ B (Moore *et al.* 2001). In addition, the experimental protocols used here induce high serum levels of a second potentially anti-inflammatory cytokine, namely transforming growth factor- $\beta$  (Hillyer *et al.* 2006). This cytokine suppresses production of interferon- $\gamma$  by natural killer cells, the main source of this type 1 polarising activity early in an adaptive immune response (Laouar *et al.* 2005). Transforming growth factor- $\beta$  also suppresses expression of T-bet and Stat4, which are transcription factors that promote differentiation of naive T cells to become effectors secreting interferon- $\gamma$  (Ming *et al.* 2006). Thus, several mechanisms appear likely to conspire to reduce the synthesis of interferon- $\gamma$  in acute malnutrition. The possibility cannot be dismissed that an influence on turnover is also a factor although, using the acute-phase proteins as a guide (Woodward, 2001), reduced turnover of interferon- $\gamma$  would be anticipated during wasting disease.

The results of the present investigation are consistent with the consensus of the published literature that the sandwich ELISA fails to detect interferon- $\gamma$  in the blood of the healthy laboratory mouse (for example, Cowdery *et al.* 1996; Mukherjee & Talwar, 1996; Halford *et al.* 1998; Faggioni *et al.* 2000; Sass *et al.* 2002)

or human subjects (for example, Gonzalez-Quintela *et al.* 1999; Verbon *et al.* 1999; Sutas *et al.* 2000; Yoshizawa *et al.* 2002). Previous attempts to quantify murine blood serum interferon- $\gamma$  bioactivity were based on inhibition of a viral cytopathic effect *in vitro* and also failed to detect the cytokine (Solis-Pereyra *et al.* 1997). Moreover, unidentified blood serum inhibitors preclude the use of macrophage-based bioassays centred on stimulation of NO production or expression of major histocompatibility antigens (LM Hillyer and B Woodward, unpublished results). The present findings using the WEHI-279 bioassay, therefore, assume significance as a successful attempt to define the blood serum interferon- $\gamma$  bioactivity of the healthy adolescent mouse maintained in a clean, conventional environment. Interferon- $\gamma$  appears to be a normal component of murine blood, and a level of 0.5 ng/ml emerges as the first estimate of the physiological serum bioactivity of this cytokine after taking appropriate measures to ensure the specificity of the assay. Importantly, the sandwich ELISA failed to detect interferon- $\gamma$  immunoactivity in serum from the same animals, an outcome that poignantly extends previous findings pertaining to IL-1, -6 and -10 illustrating the unsuitability of this type of assay for determining cytokine levels in the blood (Malone *et al.* 2001; Hillyer & Woodward, 2003; Hillyer *et al.* 2006) and, presumably, in other tissue fluids either in health or in disease.

A malnutrition-associated depression in serum interferon- $\gamma$  bioactivity, as reported in the present paper, is consistent with the cell-mediated inflammatory incompetence that characterises acute forms of protein and energy deficit (Woodward, 2001, 2004) and suggests a mechanism likely to underlie this well-established immunological phenomenon. In addition, the present investigation identifies a bioassay that can be applied to interferon- $\gamma$  in the blood and, presumably, in other tissue fluids. Significantly, this assay produced results consistent with the immunopathology of the experimental systems under study, whereas the ever-popular sandwich ELISA did not. If attention is centred on the information provided by the bioassay, the results of the present investigation lend support to the proposition (Woodward, 2004) that adaptive immune depression in acute forms of malnutrition reflects physiological regulation rather than a biologically trivial disintegrative process.

### Acknowledgements

The present study was supported by a grant awarded to B. W. by the Natural Sciences and Engineering Research Council of Canada and by a scholarship awarded to H. E. M. by the Association of Commonwealth Universities. A portion of the investigation comprised part of the MSc research of H. E. M. For laboratory quality control and setting up the bioassay procedure, L. M. H. took primary responsibility. B. W. served as principal investigator.

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