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Effects of protein deficiency on selective elicitation of lysosomal enzymes in rat peritoneal macrophages

U. R. IYENGAR and U. K. VAKIL

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

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Abstract. Young albino rats were fed *ad libitum* 4, 8 or 20 % (control) protein diet for 1–4 weeks. Total activities of some of the lysosomal enzymes, namely, acid phosphatase, aryl sulphatase, ß-glucuronidase and cathepsin D, were determined in resident and protease-peptone elicited peritoneal macrophages. Total cell number, protein content and the lysosomal enzyme activities were increased significantly in protease-peptone elicited macrophages; though at a lower rate in 4 % protein-fed group compared to control ones. However, the rate of induction of the tested hydrolases was selective and their response to the stimulant varied widely. Similarly, response of each enzyme to low protein diet also varied. Thus, at 4 weeks, cathepsin D and ß-glucuronidase activities, expressed per total number of elicited macrophages were reduced by 45 and 60 %, respectively, in 4 % protein-fed animals. These results indicate that the metabolic events related to lysosomal function in macrophages, are affected by dietary restriction of proteins.

Keywords. Rat; protein deficiency; peritoneal macrophages; elicitation; lysosomal enzymes.

Introduction

The macrophage is an integral component of the immune system and is involved in a number of immune functions, besides in phagocytosis and cell-killing (Bursuker and Goldman, 1983). Nutritional status of an animal is closely related to its resistance to infection (Chandra, 1980) due to a variety of factors. Protein malnutrition results in reduction in relative number of elicited peritoneal macrophages in response to proteose-peptone stimulation (Iyengar and Vakil, 1985). Similarly, IgG-FC receptor mediated phagocytic capability (Hamm and Winick, 1984) and bactericidal activity (Douglas and Schopfer, 1974) of the macrophages are reduced in protein deficiency. Macrophages also contain large number of lysosomal enzymes (Davies *et al.,* 1974) which are shown to control many immunological activities of the cells (Unanue, 1978). The enzymes play specific role in intracellular digestion and in destructive inflam matory processes (Goodrum and Spitznagel, 1982). It is shown that the mobility of macrophages to inflammatory sites is depressed in protein deficiency (Weeks and Kavas, 1979).

Abbreviations: (4-MU), 4-Methylumbelliferone; PBS, phosphate buffer saline solution.

In the present investigation, the effects of protein malnutrition on the activities of some selective lysosomal enzymes of resident and elicited rat peritoneal macrophages, were studied.

Materials and methods

Chemicals

4-Methylumbelliferone and its conjugated substrates, Phenolphthalein glucuronide and bovine haemoglobin were procured from Sigma Chemical Co., St. Louis, Missouri, USA. Eagles' minimal essential medium and proteose-peptone were Difco products and purchased from HIMEDIA, Bombay. Potassium [^{14}C]-cynate ($K^{14}CNO$, sp. activity 57 mCi/m mol) was obtained from Amersham, England. Other chemicals were of analar grade.

[^{14}C] -*haemoglobin preparation*

An aqueous solution of haemoglobin (400 mg/20 ml, pH 6·1) was incubated with 0·4 ml of 0·04 mCi of $K^{14}CNO$ for 2 h at 50°C (Peters *et al.,* 1972). The solution was kept for 16 h at 25°C and then incubated with 20 μmol of cysteine HCl (pH 6·1) for 2 h at 37°C. This was dialysed extensively against distilled water and 0·01 M Tris buffer (pH 7·4), till no radioactivity was detected in the washings. Specific activity of [^{14}C]-haemoglobin preparation was about 79,000 cpm/mg protein.

Animals

Young male albino rats of Wistar strain (about 100 g body wt) fed on animal house stock diet, (cereal-legume based diet containing 20% protein) were used. A number of animals were sacrificed before initiation of different dietary protein regimen to obtain the base line values for the parameters studied. Rest of the animals were randomly divided into three groups and fed *ad libitum* 4,8 or 20 % casein diet for 1–4 weeks. They were housed individually in cages at room temperature (25 ± 3°C). The composition of the diet (g per 100 g diet) was: corn starch (57), casein (20), sesame oil (8), sucrose (9), vitaminized sucrose (2) and salt mixture (4) (Adhikari *et al.,* 1972). In 8 and 4 % protein diets, casein was replaced by equivalent amounts of starch. Daily food intake and body wt of the animals were recorded.

Collection of macrophages

For the collection of resident macrophages, the animal was injected intraperitoneally with 20 ml of 0·1 M phosphate buffer saline (PBS, pH 7·2) solution, massaged lightly and killed under ether anesthesia. The peritoneal fluid was collected, centrifuged (600 *g,* 10 min) and the cell pellet suspended into Eagles' minimal medium containing 100 μg/ml each of streptomycin and penicillin. This was transferred asceptically into plastic petri dish (9·5 cm diameter) and incubated for 2 h at 37°C. The nonadherent cells were removed by washing the adherent cell monolayers three times with 0·9 % NaCl and suspended into fresh solution. The cells were stained with Giemsa, examined under

light microscope and counted. They were identified (95%) as medium to large mononuclear cells. Viability (about 90 %) of the cells was determined by exclusion of trypan blue.

Elicited exudate cells were collected as above from rat peritoneum, three days after intraperitoneal injection of 10 % proteose-peptone (5 ml). Nonionic detergent triton X-100 (1 %) in 0·9% NaCl was added to the cells and kept at 4°C for 1 h. Appropriate volume of the cell-lysates were stored at — 20°C until analysed.

Biochemical determinations of lysosomal enzymes

Acid phosphatase (EC 3.1.3.2) and aryl sulphatase (EC 3.1.6.1) were assayed by fluorimetric method, using 4-methylumbilliferone (4-MU)-conjugated derivatives, namely, 4-MU-phosphate (0·4 mM) and 4-MU sulphate (10 mM), respectively, as the substrates (Mead *et al.,* 1955). An aliquot of the macrophage lysate (0·10 ml) was incubated with appropriate substrate (in acetate buffer, pH 5·0) at 37°C for 90 min. The reaction was stopped by addition of 2·5 ml glycine buffer (0·4 M, pH 10·5). The 4-MU liberated was determined fluorometrically in a Hitachi 203 fluorescence spectrophotometer at an excitation and emission wavelengths of 365 and 450 nm, respectively. A standard curve was drawn using known concentrations of 4-MU. The enzyme activity was expressed as nmol 4-MU liberated per min per 10^6 cells.

To measure *β*-glucuronidase (EC 3.2.1.31) activity (Stahl and Touster, 1971), lysed cell suspension (≡ approx. $1{\cdot}5 \times 10^6$ cells) was incubated with 0·625 mmol Phenolphthalein *β*-glucuronide in 0·1 M acetate buffer (pH 5·0) at 37°C for 60min. The reaction was terminated by the addition of 4 ml of a solution (pH 10·7) containing 0·133 M glycine/0·067 M NaCl/0·083 M Na_2CO_3. The colour intensity was measured at 550 nm. One unit of the enzyme was defined as one nmol of Phenolphthalein liberated per min per 10^6 cells.

For the measurement of cathepsin D (EC 3.4.4.23) activity (Roth and Locty, 1971), [^{14}C] -haemoglobin (about 100,000 counts), 1·4 ml of 0·2 M acetate buffer (pH 3·8), and 1 ml of cell lysate, were incubated at 37°C for 90 min. The reaction was stopped by the addition of 5 % trichloro acetic acid and then centrifuged. An aliquot of the supernatant was added to 10 ml of dioxane containing 10% naphthalene and 0·5% 2,5-diphenyl oxazole as scintillation fluid. Radioactivity was counted in a Beckman liquid scintillation counter. Corrections were made for quenching and background counts. The enzyme activity was expressed as cpm liberated per min per 10^6 cells.

Total proteins in macrophage lysate were determined (Miller, 1959) using bovine serum albumin as standard. The results were statistically analysed using Student's *'t'* test (Snedecor and Cochran, 1967). Differences in mean values yielding $P \prec 0{\cdot}05$ were considered significant.

Results

Growth

Food intake and body wt record of the animals, fed 20,8 or 4 % protein diet are depicted in figure 1. The consumption of food (g/rat/day) by 20 % and 8 % protein-fed groups

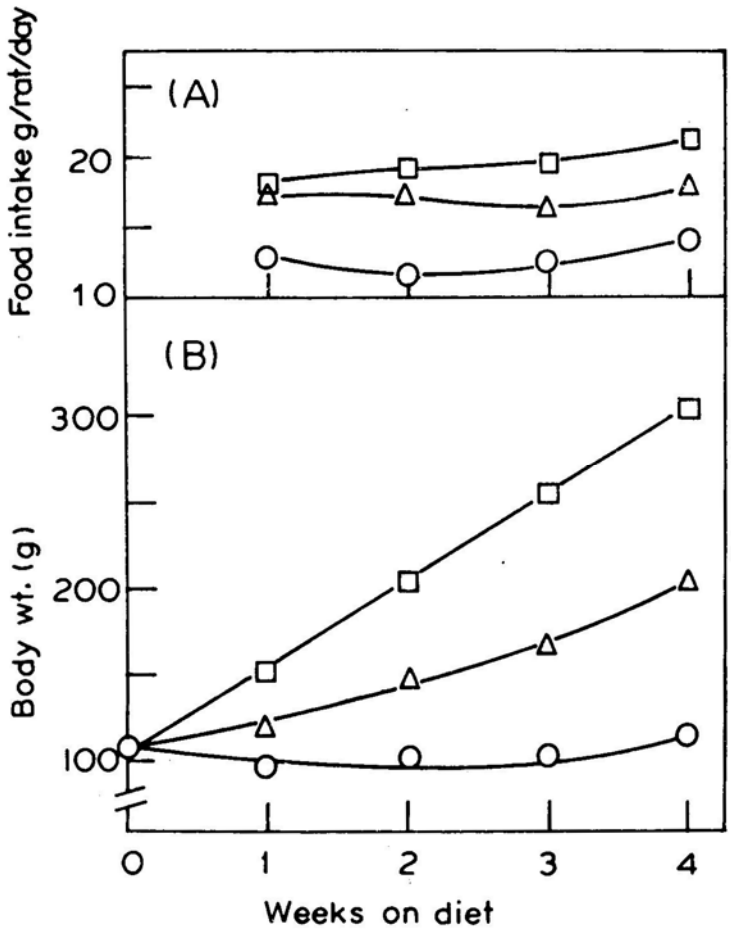

Figure 1. Food intake (g) and body wt gain (g) by rats, fed different levels of dietary proteins. Young male rats were fed 4 % (O) or 8 % (Δ) or 20 % (□) protein diet. Food intake and weight gain were recorded and plotted against number of weeks of feeding. Each point represents an average value for 8 rats.

was higher than that by the 4% group (figure 1A). Animals fed adequate (20%) and marginal (8 %) protein diets gained weight throughout the experimental period, though at a lower rate in the latter group (figure 1B). Animals fed inadequate (4 %) protein diet, did not gain weight but maintained essentially the initial level during 4 weeks.

Effects of protein deficiency on rat peritoneal macrophages

In the preliminary experiments, time course studies were carried out (figure 2) to determine the maximum response of the animals (fed 20, 8 or 4 % protein diets for 4 weeks) to an injected stimulant, namely, proteose-peptone solution. The total number of elicited macrophages isolated from peritoneal cavity showed a rapid increase with time and reached a peak value at 72 h; it decreased almost to base line value at 120 h. It

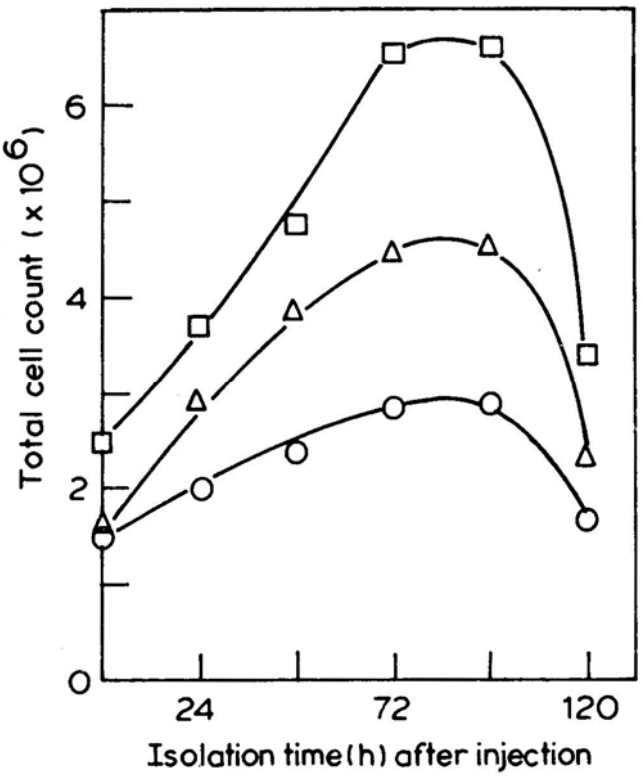

Figure 2. Total number of elicited macrophages collected at different time intervals following the administration of stimulant. Animals, fed for 4 weeks on experimental diets, were injected proteose-peptone and macrophages were collected after 24–120 h, as described in the text. (o), 4% protein; (Δ), 8% protein;(□), 20% protein.

can be observed that the pattern of exudative response to the stimulant was similar in all the groups, though the rate of increase in the number of cells was very slow in protein-deficient group compared to the control. Hence in all subsequent experiments, the animals were sacrificed at 72 h.

The results on the total number of elicited peritoneal macrophages, recovered from the animals fed different dietary protein levels for 1–4 weeks, are shown in table 1. In 20% protein fed group, the total number of macrophages were $4{\cdot}64 \times 10^6$ cells. This value was comparable to the basal control value ($4{\cdot}5 \times 10^6$ cells) on 0 day, obtained from the animals, fed stock diet, before initiation of dietary protein regimen. However, the value for 8% ($3{\cdot}88 \times 10^6$) and 4% ($3{\cdot}58 \times 10^6$) protein fed groups were slightly lowered during this period. Further, total population of elicited macrophages increased steadily in the control group, whereas it was reduced markedly ($P < 0{\cdot}05$) in the protein-deficient (4 %) animals at 4 weeks compared to that in 20 % protein-fed animals. Thus, at 4 weeks, the total number of elicited cells were only $2{\cdot}5 \times 10^6$ at 4 % level and $8{\cdot}32 \times 10^6$ at 20 % protein level. Protein content (table 1) remained more or less the same in

Table 1. Effects of dietary protein levels on total elicited peritoneal macrophages.

Week on diet	Dietary protein level (%)					
	20	8	4	20	8	4
	(Total cells × 10^6)			(μg protein/10^6 cells)		
0	4·50 ± 0·50			210·3 ± 5·46		
1	4·64 ± 0·83	3·88 ± 1·07	3·58 ± 0·30	197·5 ± 46·91	184·30 ± 19·24	174·0 ± 53·20
2	7·68 ± 1·04	4·92 ± 0·29	3·20 ± 0·41	227·0 ± 25·70	147·30 ± 27·10	141·6 ± 9·92
4	8·32 ± 1·56	3·97 ± 0·74*	2·50 ± 0·55*	200·8 ± 19·62	145·15 ± 22·60*	138·7 ± 17·35*

Macrophages were separated from peritoneal exudate, 72 h after the injection of the stimulant. Each value is the average of 8 individual determination ± S.E.
* P <0·05

control group during the experimental period of 4 weeks. However, this was reduced significantly (P <0·05) in the 4%. protein fed group compared to control.

Protein status and macrophage lysosomal enzymes

In preliminary experiments, optimum assay conditions for each macrophage enzyme studied, namely, acid phosphatase, aryl sulphatase, β-glucuronidase and cathepsin D, were established individually. Time course and kinetic studies showed (data not given) that all the enzyme assays were linear with time, though having different peak activities (up to 3 h). These were also proportional to the amount of enzyme concentration (sample size up to 2 × 10^6 cells) at 37°C and at their specific pH optimum (3·8 for cathepsin D and 50 for other enzymes). These enzymes exhibit latent activity and are released from the lysosomes by incubation with triton X-100

Effect of protein deficiency on lysosomal enzyme activities

Data on the effects of protein status of an animal on the activities of acid phosphatase, aryl sulphatase (table 2), β -glucuronidase and cathepsin D (table 3) in resident and elicited macrophages are compiled. Data at 0 time represent the basal control values obtained from the animals, fed stock diet before initiation of protein dietary regimen. The activities of the four lysosomal enzymes (units/10^6 cells) in resident macrophages were comparable to the initial (0 time) values and did not change significantly (P <0·1) in the 20 and 8 % protein fed groups in 4 weeks, except, aryl sulphatase activity which showed an increase (P <0·01). Though at 4weeks, acid phosphatase and aryl sulphatase activities were reduced significantly (P< 0·02) in the 4 % protein- fed group compared to that in control, cathepsin D activity showed some increase during this period.

The total lysosomal enzyme activities (units/10_6 cells) were increased markedly in elicited macrophages in all the groups. However, at 4 weeks, acid phosphatase, aryl sulphatase (table 2) and β -glucuronidase (table 3) activities were significantly low (P < 0·02) in the 4% group compared to the elicited macrophages from the 20% protein group, though, cathepsin D activity was comparable in both the groups (263 units/10^6 cells).

Further, the intensity and the response of each enzyme to the stimulant was

Table 2. Effects of protein deficiency on lysosomal enzyme activities of resident and elicited rat peritoneal macrophages.

		Dietary protein level (%)					
	Week on	20		8		4	
Enzyme	diet	Resident	Elicited	Resident	Elicited	Resident	Elicited
Acid phosphatase		(units/10^6 cells)					
	0	7·16 ± 0·90	31·43 ± 2·45				
	1	5·91 ± 0·86	27·32 ± 4·64	5·24 ± 0·36	20·67 ± 1·61	6·41 ± 0·65	30·24 ± 9·91
	3	6·16 ± 0·72	30·05 ± 1·97	5·55 ± 0·66	12·62 ± 2·37	4·98 ± 0·47	13·93 ± 1·43
	4	7·43 ± 0·77	29·26 ± 3·32	6·17 ± 0·83	17·82 ± 3·69	4·84 ± 0·44[a]	17·94 ± 1·77[b]
Aryl sulphatase							
	0	10·75 ± 0·40	22·10 ± 2·86				
	1	9·20 ± 1·30	22·71 ± 2·71	8·77 ± 2·18	24·06 ± 5·66	7·38 ± 1.40	23·70 ± 1·24
	3	11·10 ± 0·76	24·79 ± 2·61	7·11 ± 0·93	18·63 ± 4·51	9·60 ± 0·39	19·85 ± 1·58
	4	13·20 ± 1·47	26·05 ± 2·90	13·18 ± 1·46	20·26 ± 3·58	8·80 ± 0·78[a]	18·76 ± 1·18[b]

Lysosomal enzyme activities were measured in the resident and elicited macrophages as described in 'Methods'. 1 unit of acid phosphatase and aryl sulphatase activity relates to nmol of 4-MU liberated/min/10^6 cells. Each value is the average of 8 determinations ± S.E.

[a] $P < 0.02$; [b] $P < 0.01$.

Table 3. Effects of dietary protein deficiency on lysosomal enzymes in resident and elicited rat peritoneal macrophages.

		Dietary protein level (%)					
	Week on	20		8		4	
Enzyme	diet	Resident	Elicited	Resident	Elicited	Resident	Elicited
		(units/10^6 cells)					
β-Glucuronidase	0	1·85 ± 0·12	3·35 ± 0·15				
	1	1·72 ± 0·32	2·91 ± 0·06	1·52 ± 0·19	2·44 ± 0·35	1·32 ± 0·10	3·64 ± 0·83
	3	1·61 ± 0·07	3·61 ± 0·41	1·31 ± 0·18	2·23 ± 0·51	1·34 ± 0·22	2·41 ± 0·41
	4	1·51 ± 0·13	3·19 ± 0·58	1·64 ± 0·13	2·61 ± 0·20	1·40 ± 0·18	2·26 ± 0·15[a]
Cathepsin D							
	0	78·91 ± 4·64	244·4 ± 29·41				
	1	69·95 ± 12·90	297·3 ± 56·85	72·93 ± 6·10	342·8 ± 24·24	73·25 ± 9·31	339·7 ± 83·60
	3	82·45 ± 13·80	271·8 ± 23·74	86·50 ± 7·50	308·4 ± 52·70	73·98 ± 10·72	280·9 ± 32·82
	4	77·70 ± 14·40	264·6 ± 42·30	84·50 ± 12·70	284·0 ± 38·70	94·35 ± 14·91	263·3 ± 41·30

Lysosomal enzyme activities were measured in the resident and 72 h elicited macrophages as described in 'Methods'. 1 unit of β-glucuronidase activity is equivalent to nmol of phenolphthalein liberated/min/10^6 cells. One unit of cathepsin D activity relates to the radioactivity in supernatant liberated from labelled haemoglobin (cpm/min/10^6 cells). Each value is the average of 8 determinations ± S.E.

[a] $P < 0{\cdot}02$.

examined. Varied and selective elevation of individual lysosomal enzymes in elicited macrophages was observed. Thus, at 4 weeks, cathepsin D showed 3·5 fold increase in total activity in proteose-peptone elicited cells compared to the resident ones in 20 % protein-fed group. Whereas for *ß* -glucuronidase, the stimulation effect was of low significance with only 2-fold increase under similar conditions. Changes in 4 % protein-fed groups were similar, though the increment in the enzyme activities were slightly lower. Thus, cathepsin D and *ß*-glucuronidase registered 2·8 and 1·6-fold increase, respectively, in elicited cells at 4 weeks. The pattern was more or less the same and not statistically (P <0·1) different during the 4 weeks in all the groups.

To have a better insight into the action of the stimulant, absolute increase in total enzyme units (per 10^6 cells) in elicited macrophages over that of resident ones was calculated (figure 3). At first week, increase in the number of enzyme units were comparable, or slightly higher in the 4 % protein-fed group. However, at 4 weeks, all the enzymes were adversely affected, and their response to stimulation (in terms of total units/10^6 cells) was reduced.

Since the total number of elicited cells were reduced with progressive protein deficiency (table 1), the decrease in lysosomal enzyme activities in total peritoneal macrophages was more pronounced than when expressed per 10^6 cells. When the time related changes were examined, the decrease ranged from a minimum of about 45 % in cathepsin D and *ß*-glucuronidase activities to a maximum of about 60% in acid

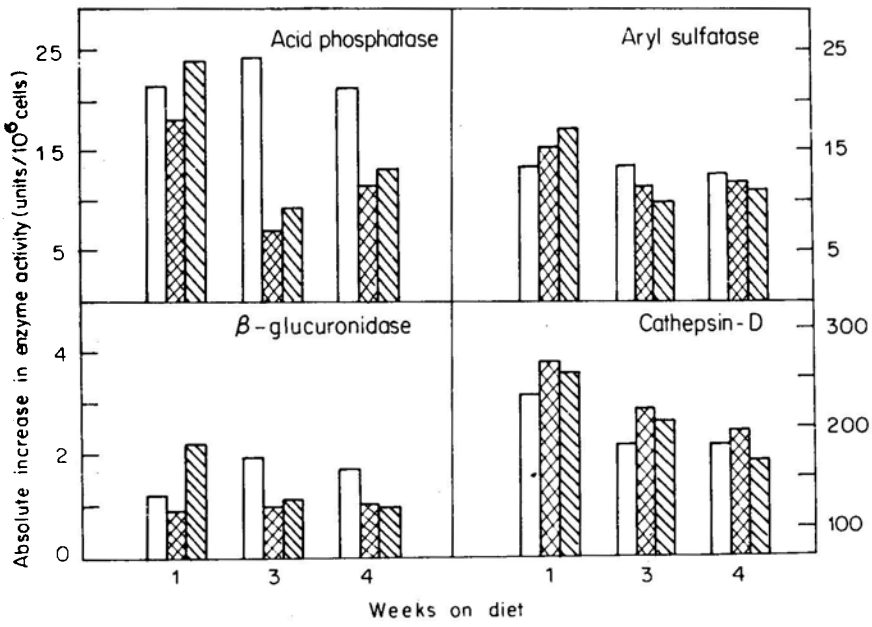

Figure 3. Absolute increase in the lysosomal enzyme activities in elicited macrophages. Three sets of bars indicate the time in weeks, for which the animals were fed 4 %(□), 8 %(▩) or 20 %(▧) protein diet. Each bar (mean of 8 values) represents increase in the enzyme activity (units/10^6 cells) in elicited macrophages in relation to the values of resident cells (expressed as 0).

phosphatase activity from the first to the fourth week on protein-deficient diet. The total enzyme activities in 20 % protein group increased from 60 % (cathepsin D) to about 100% (aryl sulphatase) during the same period.

Discussion

The observations in the present study indicate that reduction in food intake in protein-deficient animals, produced a combined protein-calorie malnutrition. The growth of the animal was almost arrested due to inefficient utilization of low protein diet (figure 1).

As the severity of dietary protein malnutrition increased, the number of resident and elicited macrophages decreased proportionately. It is known that stimulated rat peritoneal macrophages synthesize lysosomal enzymes at higher rates (Brown and Swank, 1983). Recent evidences suggest that both, a mannose-6-phosphate recognition marker on most of the lysosomal enzymes (*e.g.* on *ß*-glucuronidase, cathepsin D, etc.) (Hasilik and Neufeld, 1980) and phosphomannosyl receptor, an integral membrane glycoprotein (Creak and Sly, 1983) play a significant role in the intracellular transport of the newly synthesized enzymes from microsomal organelles to lysosomes. Overall decrease in total sugars, including mannose, has been observed in protein deficient macrophages (Iyengar and Vakil, 1985). Thus, low activities of the lysosomal enzymes in macrophages collected from protein-deficient rat at 4 weeks, may be attributed to (a) overall diminution in cell protein synthesis (table 1) and/or (b) impaired shuttling of newly synthesized lysosomal precursors from the site of synthesis to the lysosomes in mature form. Similar decrease in lysosomal enzymes in spleen and thymus was observed in protein deficient animals (Munoz *et al.,* 1981). These enzymes are shown to be involved in restructuring of lymphoid cells during their transformation in the presence of antigen (Hirschhorn and Hirschhorn, 1965).

However, it was noticed that cathepsin D activity (expressed per 10^6 cells) in elicited macrophages was comparable in both the groups (table 3). The significance of this observation is not clear. It can be speculated that comparatively higher proteolytic activity per cell, in protein deficient rat might be the expression of the mechanisms responsible for excess protein breakdown. This phenomenon may be stimulated by lack of appropriate amount of amino acids in the diet. Proteolytic enzymes in rat liver are shown to be involved in protein catabolism during malnutrition (Umana, 1967).

On scrutinizing the results more closely, it could be observed that proteose-peptone injection caused selective and differential stimulation of the enzymes studied. Interaction of stimulant with the intact cells and its subsequent endocytosis, are required for the induction of the lysosomal enzymes in macrophages (Tomino, 1979). The stimulant used being protein in nature, it could induce proteolytic enzymes like cathepsin D much more (3·5 fold) compared to *ß* -glucuronidase and aryl sulphatase (about 2-fold) in control. Similarly, incubation of macrophages with liposomes is shown to induce *ß* -glucuronidase but not *ß* -glucosidase in lysosomes (Takano *et al*., 1983). Such selective stimulation with digestible (Edward and Stanton, 1975) and undigestible (Morland and Morland, 1978) endocytic stimuli *in vitro* also occurs in the mouse peritoneal macrophages.

Many proteases, including cathepsin D augment immune response *in vitro* and *in vivo* and play important role in the regulation of phagocytosis by macrophages and in antibody production (Ohnishi, 1984). Malnourished subjects are more susceptible to infection than normal healthy one, since many aspects of immunocompetence are affected (Chandra and Newberne, 1977). Humbor *et al.* (1983) have shown that the migratory ability of the peritoneal macrophages is decreased in protein deficiency. Thus, reduced availability of macrophages, and consequently of the lysosomal enzymes at inflammation sites, may be one of a variety of factors, affecting the host-parasite interaction in protein deficiency.

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