J. Nutr. Sci. Vitaminol., **35**, 49–59, 1989

Effects of Dietary Restriction on Cellular Immunity in Rats

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(Received September 5, 1988)

Summary Phagocytosis of opsonized sheep red blood cells (SRBC) by alveolar macrophages (AM) was measured in rats fasted for 1 to 9 days or fed on diets restricted 20 to 95% compared to control group for 2 and 8 weeks. In rats fasted for 1 to 6 days, AM showed an increased phagocytosis at 2 days after fasting, but their phagocytic activity remarkably decreased afterwards. Furthermore, phagocytic activity of AM per rat revealed much more decrease at 3 to 6 days after fasting. Then the production of interleukin-1 (IL-1) by AM increased with prolonged fasting, but the production of prostaglandin E_2 (PGE_2) by AM cultured with lipopolysaccharide (LPS) conversely decreased in rats fasted for 2 days or longer. The proliferation of splenocytes increased with prolonged fasting. On the other hand, 20 to 95% restricted diets induced the increased phagocytosis of AM with prolonged experimental period. However, phagocytic activity of AM per rat showed significant increase only in rats on a 40% restricted diet. The findings suggest that differences in both duration and degree of dietary restriction modulate phagocytic function of AM, and may contribute to explaining, in part, conflicting observations which have been obtained on the immunologic state in malnourished animals.

Key Words dietary restriction, fasting, phagocytosis, alveolar macrophages, interleukin-1, prostaglandin E_2

It is well known that nutrition plays an important role in the host immune system. Some researchers have found significant impairments of cellular immune responses in calorie-restricted human and experimental animals, but others have reported an apparent enhancement of certain immunologic functions in moderately malnourished animals (*1–4*). Our previous studies (*5*) indicated that alveolar macrophages (AM) from protein-calorie-restricted rats showed an increased tumoricidal activity in week 3, but in weeks 5 and 7 they did not. We also reported that the

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phagocytosis of opsonized SRBC by AM from rats fed on a 5% casein diet or pyridoxine-deficient diet was higher than that of the control group (*6*, *7*). These studies suggested that macrophage function would be affected by the nutritional conditions depending on the degree and duration of dietary manipulation.

In this paper we attempted to examine the effects of various dietary restrictions on phagocytosis, production of IL-1 and PGE_2 by AM, and lymphocyte mitogenesis in rats.

EXPERIMENTAL

Animals and diets. Specific pathogen-free, inbred Fisher male rats, weighing 100 and 200 g, were used in this study. Rats were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and divided into four groups. Group 1 was fed on Purina chow diet *ad libitum* (control). Group 2 was fed on 20, 40, 60, and 80% restricted diets compared with group 1 (mild or moderate restriction) for 2 and 8 weeks. Group 3 was fed on 90 and 95% restricted diets compared with group 1 (severe restriction) for 3, 6, and 9 days. Group 4 was fasted for 1 to 9 days (fasting). The rats of each dietary group were given amounts of Purina chow diet equivalent to their designated restriction in reference to control. And they consumed their foods by next morning. All rats were given water *ad libitum* and housed individually in an air-conditioned room at 22 ± 2°C. Body weight of rats was measured daily.

Preparation and purification of AM. AM were obtained by tracheobronchial lavage as described previously (*7*). Briefly, the rats of each group were anesthetized by i.p. injection of sodium pentobarbital and exsanguinated by cutting both renal arteries. The chest cavity was opened and the trachea was cannulated with a cut tube from a Butterfly-21 infusion set. The lungs were then washed with 5 ml of sterilized saline at 37°C. This process was repeated several times to obtain 50 ml of lavage fluid per rat. Total number of cells collected was microscopically determined and the viability of nucleated cells was assessed by trypan blue dye exclusion. More than 97% of lavaged cells from normal rats consisted of AM, which were assessed by nonspecific esterase staining. AM were collected by centrifugation of the lavaged fluid and layered on wells of a Multiwell plate (Falcon Plastic, Oxnard, Calf.) containing 1 ml of RPMI 1640 medium with 5% fetal bovine serum (FBS). After 60 min, nonadherent cells including neutrophils were removed by washing the plates with the medium. AM were then used for each assay.

Phagocytosis by AM. AM (2×10^5) were incubated with opsonized SRBC labeled with 200 μCi $Na_2{}^{51}CrO_4$ (Japan Atomic Energy Research Institute, Tokyo) for 2 h at 37°C. The cultures were rinsed once for 10 s with distilled water to lyse nonphagocytosed SRBC and washed twice with saline. Then, all remaining adherent cells were lysed with 0.1 N NaOH, and radioactivity of the lysate was measured in a gamma counter.

Interleukin-1 (IL-1) production by AM. IL-1 production by AM was assessed

by proliferation of normal thymocytes of C3H/He mouse as described previously (*8*). AM (1×10^6/ml) were cultured for 24 h in the presence or absence of 10 μg/ml of lipopolysaccharide (LPS—*E. coli* 055 : 135, Difco Lab., Detroit, Mich.). Supernatant of this culture was harvested and assayed for IL-1 activity. Thymocytes (1×10^7/ml) of normal mouse were incubated with 50 μl of cell culture supernatant for 72 h; 1 μCi of [^{3}H]thymidine was added 20 h prior to culture termination. Cells were harvested with a Mash II Harvester and the incorporated radioactivity was measured by using a scintillation counter.

Prostaglandin E_2 (PGE_2) production by AM. AM were resuspended in RPMI 1640 medium plus 5% FBS and placed in a flat-bottomed Microtiter plate at a density of 10^5 cells/0.2 ml. After incubation for 24 h, the supernatant was removed and frozen. Using the method of Shono *et al.* (*9*) and Young *et al.* (*10*), the amount of PGE_2 in the supernatant was measured. Anti-PGE_2 (0.1 ml) was mixed with 0.1 ml of ^{125}I-labele PGE_2 and 0.1 ml of either known amounts of PGE_2 or supernatant of cultured AM. After 24 h, immune complex was precipitated with 1 ml of a 16% polyethylene glycol solution (6,000 mol wt.) and the ratioactivity in the precipitate was counted by using a gamma counter. The amount of PGE_2 in the AM culture was estimated from standard curve.

Preparation and mitogenesis of splenocytes. After the rats were killed, the spleen was removed and minced with scissors. Suspension of lymphocytes was harvested by being passed through a stainless-steel sieve in medium and adjusted to 1×10^6 cells/ml. Then, 0.1 ml of each cell suspension was cultured in wells of tissue culture Multiwell plates and immediately pulsed with 1 μCi of [^{3}H]thymidine (specific activity 25 μCi mmol, New England Nuclear). After 20 h, the cells were harvested on glass fiber filters with a Mash II Harvester, and the incorporated radioactivity was counted by using a liquid scintillation counter.

Scanning electron microscopy (SEM) of AM. Plates with attached AM were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C and washed with the same buffer. They were then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h and dehydrated in an ethanol series. Ethanol was removed from the sample with amyl acetate and then the samples were dried in a critical point apparatus (Hitachi HCP-2, Tokyo) under CO_2 and coated with gold. The specimens were examined in a Hitachi S-800 field emission scanning electron microscope at 25 kV.

Statistical analysis. Results were analyzed for satistical significance by Student's *t*-test.

RESULTS

Changes in body and lymphoid organ weights

Body weight gain was significantly lower in food-restricted rats as compared with that of the corresponding *ad libitum*-fed control rats. In mildly or moderately restricted rats (group 2), body weight gain was reduced depending on the degree of

food restriction. Rats fed the 60% restricted diet did not show any gain of body weight throughout the experiment and maintained initial body weight. Fasting for 6 days (group 4) resulted in a decline in body weight to 60% of that before fasting. The weight of thymus also decreased with increasing food restriction (group 2) and began to decrease from 2 days after fasting (group 4). The spleen weight of group 2 was maintained throughout the experiment, regardless of a large decrease of body weight; when it was compared per 100 g body weight, the spleen weight thus appeared to increase. However, the change of spleen weight in fasting (group 4) showed a similar tendency to that of change in body weight.

Numbers of thymocytes, splenocytes, and AM

In rats fed on a mildly or moderately restricted diet (group 2), numbers of thymocytes showed a similar change to that of body weight, but splenocytes showed downward trends in number with increasing food restriction. In fasting (group 4), the number of thymocytes decreased to 50% of control group at 4 days after fasting, but splenocytes were not significantly different in number from that of control group. There were no significant changes in number of AM per 100 g body weight among experimental groups (groups 2, 3, 4).

Phagocytic activity of AM

In food-restricted rats (group 2), AM ability to phagocytose ^{51}Cr-opsonized

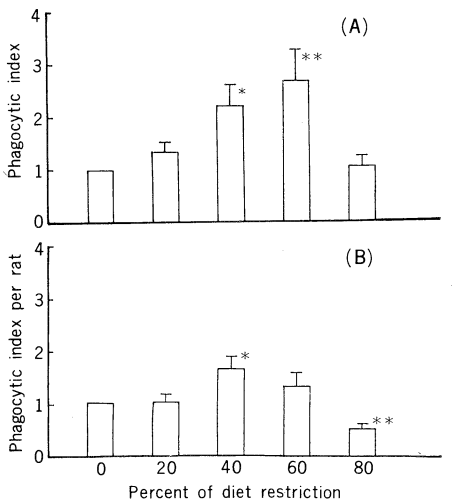

Fig. 1. Phagocytic activity of AM (2×10^5) in rats (A) or AM per rat (B) fed on mildly or moderately restricted diets (20 to 80% restriction to control) (group 2) for 2 weeks. Phagocytic index was calculated by assigning 1 to phagocytic activity of control group and comparing this to phagocytic activity of other groups. Values are means $\pm$ SD of triplicate cultures. Statistically significant *vs.* controls at $*p<0.05$, $**p<0.005$.

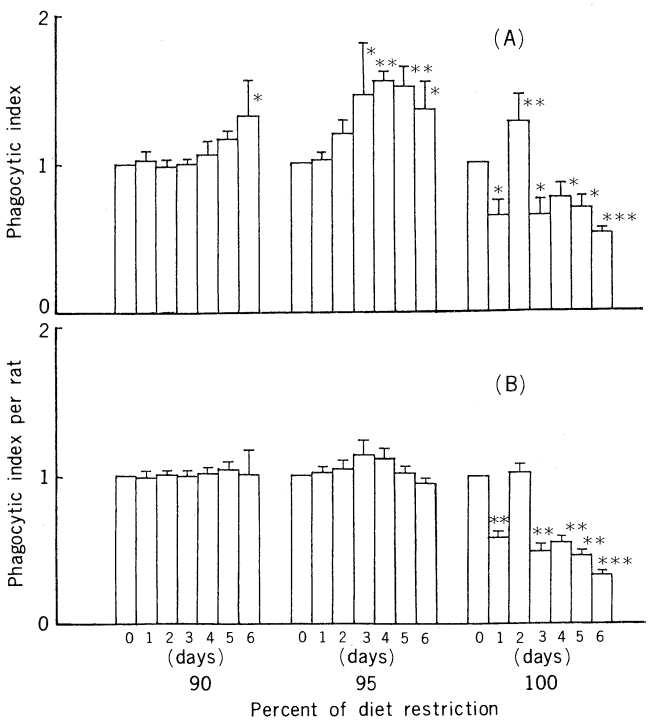

Fig. 2. Phagocytic activity of AM (2×10^5) in rats (A) or AM per rat (B) fed on severely restricted diets (90 to 100% restriction to control) (groups 3 and 4) for 1 to 6 days. Phagocytic index was calculated by assigning 1 to phagocytic activity of control group and comparing to phagocytic activity of other groups. Values are means ± SD of triplicate cultures. Statistically significant *vs.* controls at $*p<0.05$, $**p<0.01$, $***p<0.005$.

SRBC was enhanced with increasing degree of food restriction. The activity of AM from rats fed on the 60% restricted diet for 2 weeks was enhanced 2.5-fold compared with that of controls. But phagocytic activity of AM from rats fed on the 80% restricted diet was not increased and revealed the similar activity to that of control group (Fig. 1A). In phagocytic activity per rat, AM from rats fed on the 40% restricted diet showed a significant increase ($p<0.05$) and AM from rats fed on the severely restricted diet (group 3, 90 or 95% restriction compared to control group) showed a significant increase of phagocytosis at 3 days or 3 to 6 days, respectively, after onset of the experiment. However, AM from rats fasted for 1 to 6 days (group 4, 100% restriction compared to control group) showed a significant increase of phagocytic activity only at 2 days after fasting (Fig. 2A). And in all days except the second day after fasting, phagocytosis of AM was significantly reduced. Phagocytic activity of AM per rat was unaffected by severe food restriction such as 90 or 95% restricted diet and was almost equivalent to that of control group (Fig.

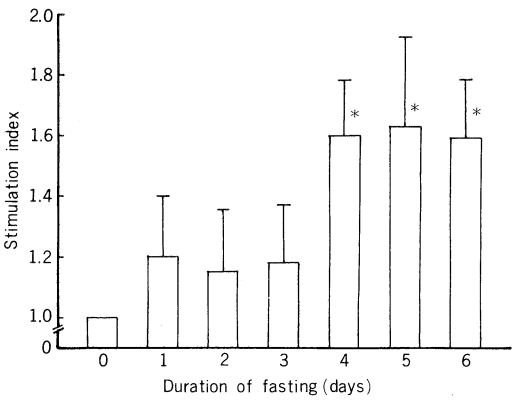

Fig. 3. IL-1 production by AM from rats fasted for 1 to 6 days. IL-1-rich supernatant was harvested from culture of AM incubated with LPS (10 μg/ml) for 24 h and assayed for IL-1 activity. Data are expressed as the ratio of cpm of [^{3}H]thymidine within thymocytes of C3H/He mouse cultured with the supernatant of experimental groups to corresponding cpm of controls. Values are means $\pm$ SD of triplicate cultures and the radioactivity of control group represents 982 cpm. Statistically significant *vs.* controls at $^*p < 0.05$.

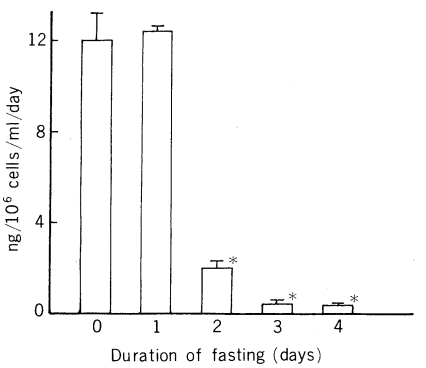

Fig. 4. PGE_2 production by AM from rats fasted for 1 to 4 days. Values are means $\pm$ SD of triplicate cultures. Statistically significant *vs.* controls at $^*p < 0.001$.

2B). In fasting, a significant decrease of AM phagocytosis per rat was seen on days 1, 3, 4, 5, and 6.

IL-1 and PGE_2 production by AM

As shown in Fig. 3, IL-1 production by AM of rats fasted for 4 to 6 days (group 4) significantly increased and showed about 1.6-fold increase compared with that of control rats. The production of PGE_2 by AM of rats fasted for 2 days or longer significantly decreased (Fig. 4).

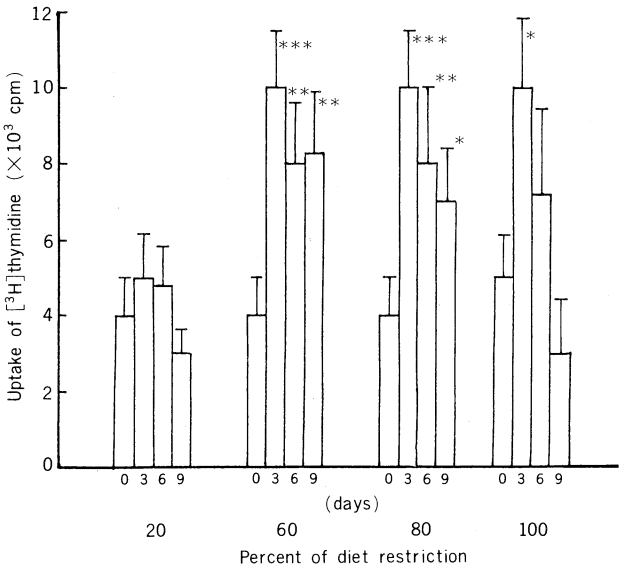

Fig. 5. Proliferation of splenocytes of rats fed on moderately or severely restricted diets (20 to 100% restriction compared to control) for 3, 6, and 9 days. Values are means ± SD of triplicate cultures. Statistically significant *vs.* controls at $*p<0.01$, $**p<0.005$, $***p<0.001$.

Proliferation of splenocytes

A significant increase in proliferation of splenocytes was observed in rats fed on the 60 or 80% restricted diet (group 2) for 3 to 9 days and in rats fasted for 3 days (Fig. 5).

Scanning electron micrograph of AM

AM from rats fasted for 2 days had abundant pseudopodia, which showed ruffling shape and adhered strongly to the glass (Fig. 6A). On the other hand, AM from rats fasted for 6 days were smaller in size and their pseudopodia became less in number and thinner than those of the control group (Fig. 6B).

DISCUSSION

Macrophages are an integral component of the immune system and are involved in a variety of immune functions in addition to phagocytosis and cell killing. In the present experiment, we were able to detect a temporary enhancement of AM ability to phagocytose opsonized SRBC when rats were fasted or fed severely restricted diets. Furthermore, the rats fed on mildly or moderately restricted diets exhibited a steady increase in their phagocytic activity except for the 80% restricted group. Morphological studies also showed that there were some changes similar to those observed in macrophages stimulated with various agents (*11*, *12*) in the cell

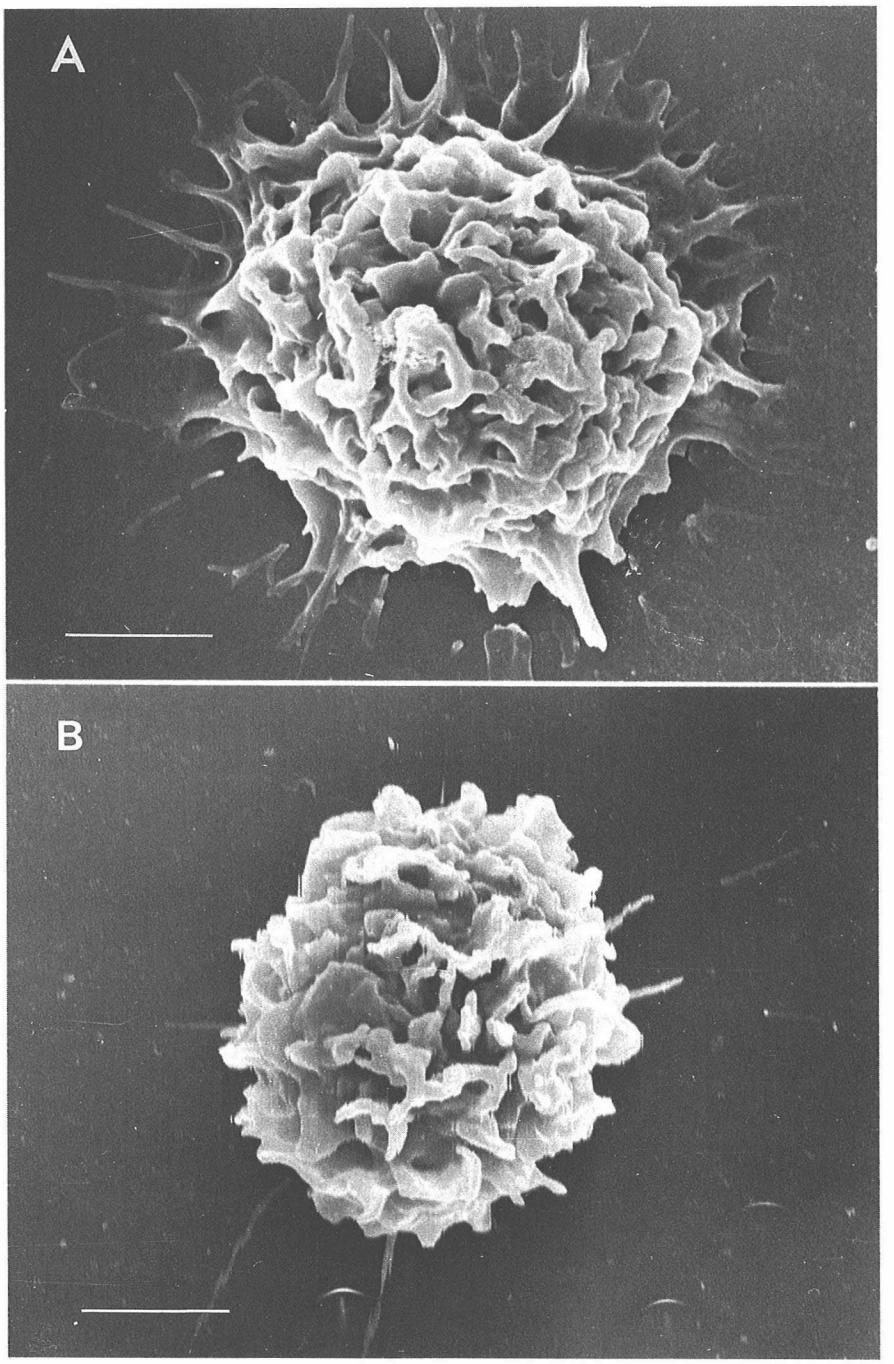

Fig. 6. Scanning electron micrographs of AM from rat fasted for 2 days (A) or 6 days (B). Bar, 3 μm.

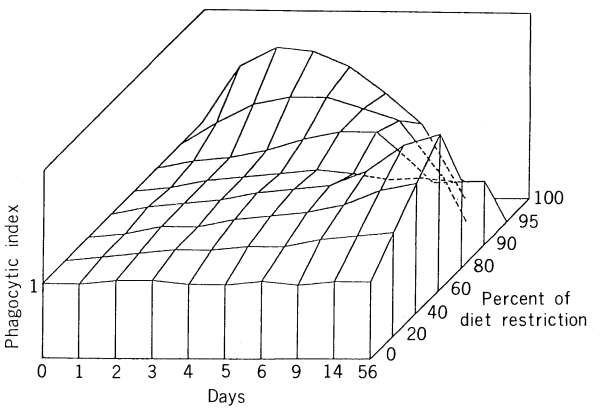

Fig. 7. A three-dimensional model summarizing the alteration of phagocytic activity of rat AM following various food restrictions. Phagocytic index was calculated by assigning 1 to phagocytic activity of control and comparing to phagocytic activity of other experimental groups.

surface of AM fasted for 2 days. Among the data concerning nutritional deprivation and immune functions there are many discrepancies in the literature reported previously. For example, Passwell *et al.* (*13*) and Gautam *et al.* (*14*) reported a decreased clearance of colloidal carbon in protein-deficient animals at 3 and 6 weeks. On the other hand, Cooper *et al.* (*15*) described an increased phagocytosis of bacteria by macrophages from mice fed on 8% protein diet. The latter results would agree with our present data in rats fed on mildly restricted diets. Probably such conditions would produce both protein and calorie deficiencies in rats. Hamm and Winick (*16*) also suggested that the difference in the duration of dietary deficiency affected the immunologic functions. Synthetic results of our experiments are shown as a three-dimensional model in Fig. 7, in which phagocytic activity of AM changed depending on both the duration and the degree of dietary restriction.

AM from rats fasted for 1 to 6 days showed 1) temporary enhancement of phagocytic activity, 2) increased production of IL-1, and 3) a marked decrease of PGE_2 production as shown in Figs. 2, 3, and 4, respectively. The different results between IL-1 and PGE_2 might be explained by the reports of Khansari *et al.* (*17*), Kunkel *et al.* (*18*), and Hayari *et al.* (*19*). They found that IL-1 and PGE_2 could be produced from separate subsets of monocytes or macrophages, and that production of IL-1 by AM was inhibited by the addition of exogenous PGE_2. And it is well known that IL-1 is called lymphocyte activating factor (LAF) and acts on T-lymphocytes to induce interleukin-2 (IL-2) receptor (*20*). Furthermore, there is some evidence that PGE_2 is a potent inhibitor of several lymphocyte functions including mitogenesis, cytolysis, and antibody production (*21*, *22*). From the above data on IL-1 and PGE_2 it may be suggested that AM of fasted rats effectively act to enhance lymphocyte functions even though their phagocytic function was re-

markably reduced. In fact, the proliferation of splenocytes significantly increased in rats fed on 60 or 80% restricted diet and in fasted rats, as shown in Fig. 5.

In conclusion, the present study suggests that both the duration and the degree of dietary restriction affect phagocytic activity of AM, which act to enhance lymphocyte function even in a severely restricted condition such as fasting.

The authors wish to thank Prof. S. Yamamoto, Department of Biochemistry, School of Medicine, The University of Tokushima, for his valuable assistance and advice in measuring PGE_2 in the media.

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