Immunology 1979 **38** 413

Effect of malnutrition and nutritional rehabilitation on tuberculin reactivity and complement level in rats

MOTOKO SAKAMOTO, K. NISHIOKA* & K. SHIMADA* *Department of Nutrition, Wayo Women's University Ichikawa, Chiba, Japan and *Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan*

Accepted for publication 17 May 1979

Summary. The purpose of this study was to clarify the effect of differing nutritional states on various components of the immune system, especially on the interplay of the complement system and cell-mediated immunity. Malnutrition was induced in Sprague–Dawley rats by feeding them diets containing 5% protein or 0·5% protein as compared with 18% protein in the diet of the controls. Nutritional rehabilitation was achieved in some experimental groups by transferring those fed 0·5% protein diet to the 18% protein diet. Malnutrition was confirmed by weight changes, biochemical findings in the sera, haematological observations and histological observation of the liver, and rehabilitation was confirmed by body weight increase and changes in other measurements. In rats suffering malnutrition, the tuberculin skin reactivity was suppressed. After feeding the 0·5% protein diet for 8 weeks, all the rats showed negative tuberculin skin reactions. In the malnourished rats, including those fed with 0·5% protein, the serum complement level decreased but did not show any significant differences as compared with the well nourished control group. After 1 week of nutritional rehabilitation, the tuberculin reactivity of six out of ten rats remained negative and after 2 weeks, all rats showed positive tuberculin reactions. After 1 week of nutritional rehabilitation, all the rats showed a normal or higher serum complement level. At this stage, two of the tuberculin-negative rats showed significantly higher titre of serum complement than even the controls.

Correspondence: Dr K. Nishioka, Tokyo Metropolitan Institute of Medical Science, 3–18 Honkomagome, Bunkyo-ku, Tokyo, 113 Japan.

0019-2805/79/1000-0413$02.00

INTRODUCTION

It has been observed that in the state of malnutrition, immunological competencies such as tuberculin reactivity, lymphoblast formation or antibody production are impaired (Smythe, Schonland, Brereton-Stiles, Coovadia & Loening, 1971; Edelman, Suskind, Olson & Shirishinha, 1973; Cooper, Good & Mariani, 1974; Munson, Franco, Arberter, Velez & Vitale, 1974; Dennis, Dudrick & Abdou, 1974; Chandra, 1975a,b; Good, Fernandes, Yunis, Cooper, Jose, Kramer & Hansen, 1976; Felnandes, Yunis & Good, 1976). These incompetencies have been considered closely related to the lowered resistance against a variety of infections in the malnourished state. On the other hand, malnutrition is considered to suppress the severity of infection in some cases despite the lowered state of immunological markers suggesting depressed cell-mediated immunity or antibody production (Murray & Murray, 1977).

Therefore, the analysis and interpretation of the lowered immunological surveillance system in malnutrition has not been well analysed and contradictions remain. We consider that it is important to analyse the change in various immunological systems in the mal-

nourished state and we believe that this can best be done with animal models. Rats have been employed in experimental nutrition study but measurements of complement activity and tuberculin skin reactivity in rats have been rather difficult. These difficulties have been overcome by employing Flax's method for tuberculin reactivity and Sakamoto's method for CH50 measurement in rats and the following experiments were undertaken; first, by comparing the malnourished rats with well nourished rats and second, by following the changes of these markers in malnourished rats after periods of nutritional rehabilitation. The results will be described in this paper.

MATERIALS AND METHODS

Five week old male Sprague–Dawley rats (SPF), purchased from Shizuoka farm, Shizuoka, Japan, averaging 80 g in weight, were used for all experiments. The animals were housed individually in stainless steel cages in an air-condition room at $24 \pm 2°$ temperature and $50 \pm 10\%$ humidity with lighting regulated to provide 12 h intervals of light and darkness.

Each group of rats was offered a limited diet supplying 20 g/day/rat (National Academy of Sciences, 1968) of a diet containing 18, 5 or 0·5% protein. The diet was given three times per week in Experiment 1. In Experiment 2, the rats were fed only twice weekly. Water was available to the rats at all times. The composition of the diets is shown in Table 1 (Philbrick & Hill, 1974).

Records were kept of the food intake, and the body weight gain was measured two or three times a week.

During the experiment, haematological changes, biochemical components of blood serum (creatinine, total protein, albumin, globulin, urea-nitrogen, lactic dehydrogenase), liver lipids, wet weights of organs, tuberculin skin reactivity and complement activity were observed. The Student's *t* test (Arkin & Colton, 1961) was used throughout.

Haematological analysis was carried out by an Automatic Blood Cell Counter (MEK 1100, Nihon-Kohden, Japan).

Blood serum was separated in a low temperature centrifuge and the serum was used for biochemical analysis and titration of complement activity.

Biochemical analysis of serum was carried out on an Acu Chem Microanalyzer (Ortho Instrument, U.S.A.).

Liver total lipid was extracted according to the method of Folch (Folch, Lee & Sloanestanley, 1956) and the volume of triglycerides in the liver tissues was counted by the Weibel method (Weibel, 1963) using the integrating eyepiece I of Zeiss. Liver tissues were

Table 1. Composition of experimental diets

	Protein in diet*					
	18%		5%		0·5%	
	g/Kg	cal	g/Kg	cal	g/Kg	cal
Vitamin-free-casein	180	720	50	200	5	20
DL-Methionine	3		0·5		0·05	
Corn starch	632	2528	765	3060	810	3240
Salt mixture†‡	50		50		50	
Cellulose	25		25		25	
Vitamin mixture†§	10		10		10	
Corn oil	100	900	100	900	100	900
Total		4148		4160		4160

* Diets were prepared after Philbrick & Hill (1974).

† Prepared by Oriental Co. (Japan)

‡ Salt mixture (%): $Ca(H_2PO_4)_2\ H_2O$ 14·56, KH_2PO_4 25·72, $NaH_2PO_4H_2O$ 9·35, NaCl 4·66, Ca-lactate 35·09, Fe-citrate 3·18, $MgSO_43H_2O$ 7·17, $ZnCO_3$ 0·11, $MnSO_4$ 0·12, $CuSO_4$ 0·03, KI 0·01.

§ Vitamin mixture (per 100 g): VA acetate 5,000 iu, VD_3 10,000 IU, VB_1 120 mg, VB_2 400 mg, VB_6 80 mg, VB_{12} 0·05 mg, folic acid 20 mg, Ca-panthothenate 10·0 mg, *p*-aminobenzoic acid 500 mg, niacin 600 mg, inocitol 600 mg, cholin-Cl 2000 mg, cellulose powder.

stained by haematoxylin eosin and the volume of triglyceride to liver cells and sinusoids in the liver tissues were counted.

As the indicator of the cell-mediated immunity system, tuberculin reactivity was measured (Flax & Waksman, 1962; Lefford, 1974). Heat-killed tubercle bacilli, Aoyama B strain (obtained through the courtesy of Dr T. Tokunaga, Division of Tuberculosis, NIH of Japan) 0·15 mg in 0·05 ml of liquid paraffin, were injected into the footpad of the rats in order to sensitize them and more than 12 days after the injection, purified protein derivative of tuberculin (PPD) in phosphate-buffered saline (PBS) (pH 7·36) was injected intradermally into the back skin of the rats. After 24 h, skin reactions were observed and the diameter of erythema and induration were measured by inspection and palpation respectively. For proper induration of the tuberculin skin reaction, careful intradermal injection of the optimal amount of PPD into depilated rat skin is very important, and only in the presence of both erythema and induration was skin reaction considered positive. Actually, throughout our examination, the diameters of erythema and induration were the same when intradermal injection of PPD was carried out correctly.

Preliminary tests, with doses of PPD ranging from 5 to 50 μg in 0·1 ml, were used to induce skin reactions and 12·5 μg in 0·1 ml PBS was found to be the amount of PPD needed to induce maximum skin reactivity. Therefore, in experiments described hereafter, twice this concentration of PPD, i.e. 25 μg in 0·1 ml PBS was used.

Histological examination of the skin reaction was carried out and found to be in accordance with the classical delayed cutaneous reaction to tuberculin as described by Flax & Waksman (1962) and, therefore, considered to be a parameter of cellular immunity in our studies.

Immune haemolysis activity of serum (CH50) was used as indicator of the complement system. The measurement of CH50 followed Mayer's method (Mayer, 1961). The measurement of CH50 in rat serum, however, required a different optimal temperature compared with other mammals. Therefore, the complement titre was measured by 50% haemolysis of sensitized sheep erythrocytes in the gelatin veronal buffer (GVB) with Ca^{2+}, Mg^{2+} and an ionic strength of 0·147 at 20° for 60 min (Sakamoto, 1975). After centrifugation supernatants were separated and oxidized haemoglobins were measured spectrophotometrically at 541 nm.

RESULTS

Experiment 1

Sixty animals were divided into three groups fed 18%, 5%, and 0·5% protein diets three times a week for 8 weeks. The 18% protein group was considered the control group and 5% or 0·5% protein groups were considered protein-calorie malnourished. Seven animals from each group were killed at the end of 4 and 8 weeks and examined. Due to difficulties in obtaining serum samples in the 0·5% protein group, CH50 was measured in only five rats in this group. Three animals from each group were used as a non-tubercle bacilli sensitized control group. The rats were sensitized on the twelth day before PPD injected at the end of both 4 and 8 weeks.

Experiment 2

For study of rehabilitation of malnutrition, fifty animals were divided into two groups. The control group of ten rats were fed the 18% protein diet for 14 weeks. Five of these rats were killed at the end of 8 weeks and five at the end of 14 weeks. Another forty rats were fed the 0·5 protein diet for 8 weeks and fifteen were killed and examined. After that, the diet was changed to the 18% protein diet fed to the remaining twenty-five rats for 6 weeks after the diet change. The food was given twice a week for this entire experiment. Ten rats were killed for the first week examination during the rehabilitation experiment and five rats were killed at the end of 2, 4 and 6 weeks. The state of nutritional recovery was estimated by measuring the body weight increase and haematological observations during the nutritional rehabilitation period. In Experiment 2, all the rats were sensitized with tubercle bacilli in the 2 weeks before diet transfer. Therefore, the rats in the experimental (0·5% protein) group were in a malnourished state when sensitized.

Nutrient intake and body weight change

Nutrient intake in the 18% and 5% protein groups became constant after the third and fifth week respectively, and thereafter the rats were fed during the remainder of the experiment at the following levels: those at the 18% level were fed at 20 g/day/rat and, those at the 5% level at 15 g/day/rat. The rats fed at the 0·5% level consumed only about 7·5 g/day/rat.

On the other hand, the energy intake in the three groups at the nutritional plateau stages (6 weeks or

longer) is 83, 62, and 31 cal respectively. One might consider the 5% fed rats as mild to moderately protein energy deficient, while the 0·5 fed rats were markedly protein energy deficient, but particularly protein deficient.

In Experiment 2, the average intake of diet was 18·4 g/day/rat in the animals fed 18% protein and 6·2 g/day/rat in the 0·5% protein group. After the dietary change at the end of 8 weeks from 0·5% to 18% protein for nutritional rehabilitation, the animals showed a remarkable increase in food intake. The average intake was 13·5 g/day/rat. Therefore, energy and protein intake, calculated from food intake, increased remarkably during rehabilitation, from 26 cal/day and 0·03 g protein/day to 56 cal and 2·4 g protein/day respectively.

As shown in Fig. 1, the body weight of the rats fed the 18% protein in Experiment 1 continued to gain normally. On the other hand the rats fed 5% protein showed a growth failure, and the 0·5% protein group showed not only a growth failure but a loss from their initial body weight.

At the end of 8 weeks of experiment, the body weight of the two low protein groups showed significant differences ($P<0·01$) compared to the control group on the 18% protein diet. The increments of weight gain are shown also in Fig. 1. The feed efficiency during rehabilitation was greatest in the early stage. Body weight gain was still increasing significantly even at the end of 6 weeks of nutritional rehabilitation.

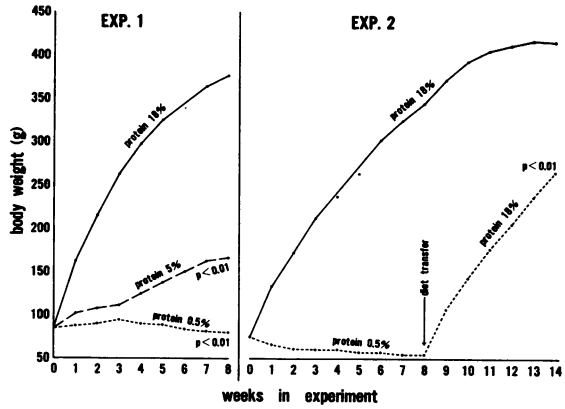

Figure 1. Body weight curve of the rats fed by 18% and 5% and 0·5% protein diet during the course of malnutrition and nutritional rehabilitation. ●——●, Fed an 18% protein diet for 8 weeks in Expt. 1 and for 14 weeks in Expt. 2 (control group) ($\Delta=+4·88$ g/day, Expt. 1) ($\Delta=+4·10$ g/day, Expt. 2); ●— —●, fed by 5% protein diet for 8 weeks (malnourished group) ($\Delta=+1·29$ g/day, Expt. 1); ●- - - -●, fed by 0·5% protein in diet for 8 weeks in Expt. 1 ($\Delta=-0·18$ g/day, Expt. 1) ($\Delta=-0·20$ g/day, Expt. 2). Diet was changed to 18% protein diet for six weeks in Expt. 2 (nutritional rehabilitation group) ($\Delta=+4·7$ g/day). Δ, Average body weight increase per rat.

Haematological observations

As shown in Table 2, the number of erythrocytes, leucocytes, haemoglobin, and haematocrit were reduced remarkably in the 5% protein groups at the end of 8 weeks of Experiments 1 and 2. These values in the 0·5% protein group were significantly lower ($P<0·01$) than that in the 18% protein group. During rehabilitation, the number of erythrocytes recovered very rapidly. By the end of 2 weeks of rehabilitation, the erythrocyte count of the experimental (0·5% protein) group was the same level as that of the control group. The leucocyte number showed a more gradual increase and at the end of 4 weeks the value had reached the level of the control group or higher. Haematocrit also showed a rapid recovery, returning to normal in 2 weeks, while the haemoglobin required 4 weeks for rehabilitation.

In the 0·5% protein group in Experiment 2, there were significantly fewer ($P<0·01$) granulocytes and lymphocytes as compared with the control group and the number of lymphocytes was only half the number of the 18% protein group.

In the 0·5% protein group, six out of ten rats showed an anaemia with the appearance of many erythroblasts. During nutritional rehabilitation, the number of lymphocytes and granulocytes recovered in parallel with the recovery of the total number of leucocytes.

Biochemical assays of serum

By the eighth week in the 5% protein group, total protein, albumin, and globulin were significantly lower as compared with the 18% protein group. In the 0·5% protein group, total protein, albumin, and globulin were also remarkably low.

Two weeks after the diet transfer to the 18% protein diet, creatinine became high, suggesting possible acceleration of protein metabolism and creatinine synthesis. After 4 and 6 weeks, creatinine value was again reduced below normal. Total protein, globulin and albumin recovered to normal after 2 weeks of nutritional rehabilitation.

Table 2. Haematological observations of rats in malnutrition and nutritional rehabilitation§

	Weeks	Prot. % in diet	Erythrocyte† ($\times 10^4$)	Leucocyte† ($\times 10^2$)	Haematological observations (Number¶ (%)) Rod-shaped nuclear	Segmented nuclear	Eosinophils	Monocytes	Lymphocytes	Haemoglobin† (g/dl)	Haematocrit† (%)
Expt 1‡		18	592 ± 25·5	115 ± 7·0						15·9 ± 0·4	45·4 ± 0·9
	4	5	621 ± 39·1	96 ± 9·0						14·2 ± 0·6*	39·9 ± 0·7**
		0·5	522 ± 39·7	48 ± 7·0**						14·9 ± 0·4	34·4 ± 3·5**
		18	593 ± 17·4	117 ± 9·0						14·7 ± 0·4	46·2 ± 1·1
	8	5	770 ± 25·4*	88 ± 8·1**						15·4 ± 0·4	49·0 ± 1·2
		0·5	565 ± 37·4	58 ± 3·6**						10·7 ± 0·4**	32·0 ± 0·7**
	8††	18	642 ± 31·6	91 ± 14·0	—	—	—	—	—	15·6 ± 0·8	52·2 ± 1·3
	14††	18	588 ± 15·1	111 ± 12·5	4·9 ± 1·05 (4·8 ± 1·10)	53·9 ± 9·52 (47·2 ± 3·46)	0 (0)	0 (0)	52·4 ± 4·43 (48·0 ± 3·18)	13·9 ± 0·7	39·0 ± 1·5
	8	0·5	303 ± 35·0**	26 ± 11·0**	0·1 ± 0·09** (0·6 ± 0·40)	44·3 ± 0·75** (23·0 ± 5·77)	0 (0)	0 (0)	14·9 ± 4·03** (66·4 ± 7·74)	6·1 ± 0·2**	18·3 ± 1·2**
Diet transfer 0·5% to 18% protein diet											
Expt 2‡	10(2)‡‡	18	574 ± 13·8	66 ± 7.5**	2·6 ± 0·89** (3·6 ± 0·98)	38·9 ± 8·25** (56·2 ± 7·70)	0·3 ± 0·16 (0·4 ± 1·64)	1·3 ± 0·29 (2·0 ± 6·33)	17·8 ± 4·88** (37·8 ± 8·29)	12·8 ± 0·6*	38·0 ± 1·0**
	12(4)‡‡	18	539 ± 34·1*	137 ± 11·0*	5·1 ± 1·02 (3·6 ± 0·51)	70·8 ± 6·82 (52·4 ± 5·12)	0 (0)	1·0 ± 0·73 (0·9 ± 0·58)	60·0 ± 10·43 (43·2 ± 5·25)	13·8 ± 0·4	38·8 ± 0·5**
	14(6)‡‡	18	635 ± 15·0	134 ± 7·7**	4·4 ± 0·93 (3·2 ± 0·58)	47·3 ± 11·65 34·0 ± 6·61	0 (0)	0 (0)	82·9 ± 7·27 (62·8 ± 6·89)	14·1 ± 0·6	42·2 ± 0·4*

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$§§.

† Erythrocytes, leucocytes, haemoglobin and haematocrit were measured by Automatic Blood Cell Counter (MEK 1100, Nihon-Kohden).

‡ Data based on ten rats in all groups in Expt 1 and five rats in Expt 2.

§ Blood taken from vena portae.

¶ Haematological observations in Expt 1 were not observed.

†† Control group.

‡‡ Figures in parentheses are weeks of nutritional rehabilitation.

§§ Significance was compared with 18% protein as control in Expt 1, and 18% protein in the same week in 8 and 14 week. In 10 and 12 week data were compared with 18% protein in 8 week.

Tuberculin skin reactivity

Tuberculin skin reactivity of the rats at different protein levels and in the course of nutritional rehabilitation is shown in Fig. 2. At the end of 4 weeks the rats fed with 18% protein showed positive reactivities with induration and erythema showing an average diameter of 12·4 ± 1·39 mm. The rats fed the 5% and 0·5% protein diets showed lower intensities showing 7·7 ± 1·03 mm and 6·2 ± 1·5 mm in average diameter of reactivity. Therefore, after 4 weeks feeding with low protein diets, tuberculin reactivity of the rats showed lower reactivity but did not disappear completely.

At the end of 8 weeks, the rats fed the 18% protein showed positive reactivity showing 14·5 ± 0·88 mm in average diameter of erythema and induration. Rats in

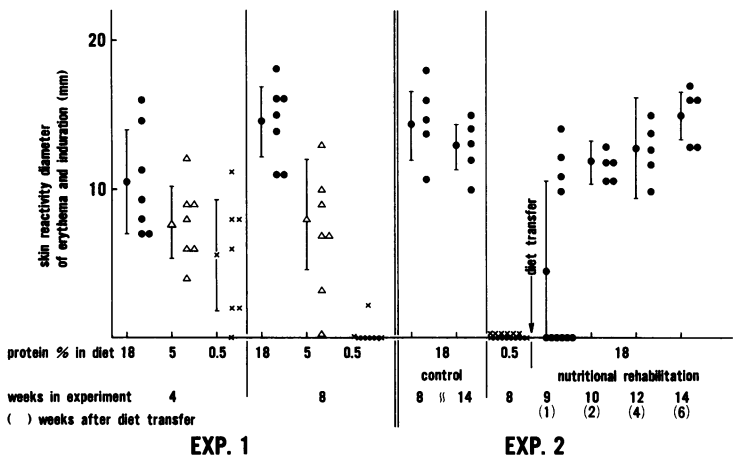

Figure 2. Changes of tuberculin skin reactivity in the rats during the course of malnutrition and nutritional rehabilitation. •, Fed by 18% protein diet; Δ, fed by 5% protein diet; ×, fed by 0·5% protein diet. Bars represent ± one standard deviation from the mean. The rats were tested more than 12 days after sensitization with 0·15 mg of heat-killed tubercle bacilli (Aoyama B strain) in 0·05 ml of liquid paraffin injected into the footpad of rats. Erythema and induration were read 24 h after intradermal injection of 25 μg of purified protein derivative of tuberculin (PPD) in 0·1 ml of phosphate-buffered saline (PBS, pH 7·36).

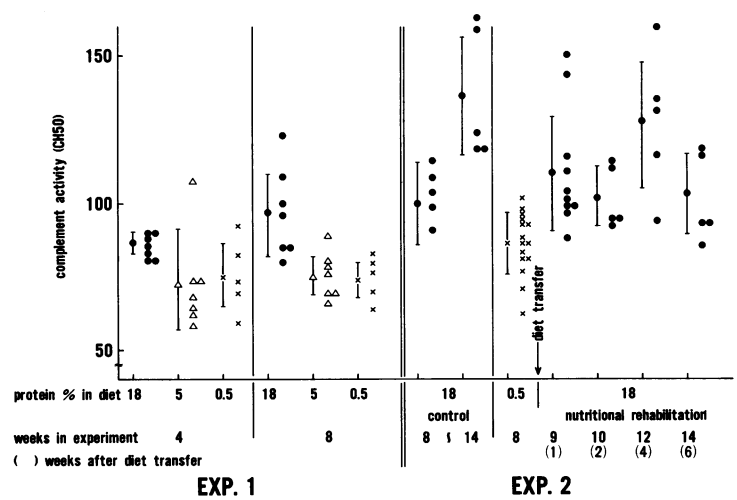

Figure 3. Changes of complement activity in the rats during the course of malnutrition and nutritional rehabilitation. •, 18% protein diet; Δ, 5% protein diet; ×, 0·5% protein diet. Bars represent ± one standard deviation from the mean. Complement titre was measured more than 13 days after sensitization of heat-killed tubercle bacilli.

the 5% protein group showed weaker reactivity at the same level as those of 4 weeks. Except one weakly reactive rat, all the rats fed 0·5% protein showed negative skin reactivity.

One week after nutritional rehabilitation to 18% protein, tuberculin reactivity of four out of ten rats recovered very rapidly. Two weeks after onset of rehabilitation tuberculin reactivity of all rats showed erythema and induration of $11{\cdot}4 \pm 0{\cdot}33$ mm in diameter. After 4 and 6 weeks of nutritional rehabilitation, tuberculin reactivity showed $12{\cdot}5 \pm 0{\cdot}74$ mm and $12{\cdot}8 \pm 0{\cdot}87$ mm respectively showing the level as at 2 weeks and the same as the control.

Complement level (CH50) in sera

Complement level in the sera is shown in Fig. 3. The pattern of changes of complement level in various nutritional states shows different patterns from the tuberculin skin reactivity.

At the end of 4 and 8 weeks, complement titre of the 5% and 0·5% protein groups showed the same tendency to decrease below the 18% protein group but the differences are not significant. The activity of the complement system was maintained even in the malnourished rats. This shows remarkable contrast to the tuberculin skin reactivity which disappeared in the malnourished rats.

In the nutritional rehabilitation experiment, the malnourished rats, after 8 weeks of feeding of the 0·5% protein diet, CH50 showed slightly lower titre; but, again, the decrease was not significant. One week after nutritional rehabilitation, however, all the rats showed normal levels of CH50 and two of ten rats showed significantly high CH50 level. It should be noted that these two rats showed negative tuberculin skin reactivity at the same time. At 2, 4, and 6 weeks of nutritional rehabilitation, all rats showed normal or elevated CH50 values. There was no significant difference in complement titre between non-sensitized rats and rats sensitized with tubercle bacilli.

DISCUSSION

A state of malnutrition can be induced by feeding Sprague–Dawley male rats with 5 or 0·5% protein diets with fairly high reproducibility as described in Experiments 1 and 2 in this paper. The degree of malnutrition may be defined by changes in body weight and further confirmed by haematological observation and biochemical findings in the sera.

In the group of malnourished rats, tuberculin skin reactivity was reduced after 4 weeks in rats fed the 0·5% or 5% protein diet. The same tendency persisted in rats fed the 5% protein diet for 8 weeks. Tuberculin reactivity disappeared completely in all rats after 8 weeks feeding of the 0·5% protein diet.

On the other hand, serum complement titres showed slightly lower levels in all the groups on low protein feeding after 4 weeks or 8 weeks as compared with the group on the 18% protein diet, but the difference was not significant. Even after 8 weeks' feeding of the 0·5 protein diet, the level of serum complement was maintained while tuberculin skin reactivity disappeared in all the rats of this group.

From these findings, this form of experimental malnutrition is one useful way to induce depressed cellular immunity while maintaining the level of complement activity in serum. This characteristic pattern was also observed in the course of ageing as well as in the tumour-bearing host (Nishioka, Kawamura, Hirayama, Kawashima & Kogure, 1976) and the complement system is considered to be more resistant than cellular immunity in such cases.

By changing the 0·5% protein diet to an 18% protein diet, partial nutritional rehabilitation was experimentally achieved in the rats. The rehabilitation state was confirmed as measured by the food intake and body weight increase, and by recovery to normal state in biochemical parameters in serum and haematological picture.

As for tuberculin reactivity, in Experiment 2 all the rats fed for 8 weeks on the 0·5% protein diet showed entirely negative reactions, confirming the first experiment. After 1 week of nutritional rehabilitation, six out of ten rats remained negative but the other four rats showed a positive reaction. The former six rats did not recover but the latter four rats recovered to control level in haematological observations and haemoglobin in sera. After 2 weeks all the rats recovered tuberculin skin reactivity.

As for CH50, all the rats showed normal or higher value even after one week of nutritional rehabilitation. The findings show that rat complement activity determined by CH50 is preserved in severe rat protein-calorie malnutrition as compared with the remarkable reduction in tuberculin reactivity.

These findings suggest that nutritional deprivation may affect various components of the immune system to a variable extent, cell-mediated immunity being

influenced the most. This can be explained as the complement system compensates for the deficient state of the cell-mediated immunity in the malnourished host and the maintained complement system acts to keep the host defence mechanism even when cell-mediated immunity is impaired. Also, earlier recovery of the complement level to a normal or higher level as compared with that of the tuberculin reaction in rehabilitated malnourished rats is an important observation.

ACKNOWLEDGMENTS

The authors are indebted to Professor Emeritus N. Shimazono and Professor S. Margen for preparation of the manuscript and for their valuable comments.

The collaboration and technical assistance of Ms Ishii, and Ms Kobayashi in the Wayo Women's University, and Ms Madarame and Ms Kitamura in the Tokyo Metropolitan Institute of Medical Science are gratefully acknowledged.

This study was partly supported by a Grant in Immunology, Ministry of Education Science and Culture of Japan (No. 310606), Ministry of Health and Welfare (No. 53–12) and the U.S.–Japan Cooperative Program on Malnutrition.

REFERENCES

ARKIN H. & COLTON R.R. (1961) *Tables for Statisticians.* Barnes & Nobles, New York.

CHANDRA R.K. (1975a) Antibody formation in first and second generation offspring of nutritionally deprived rats. *Science,* **190,** 289.

CHANDRA R.K. (1975b) Serum complement and immunoconglutinin in malnutrition. *Archs Dis. Childh.* **50,** 225.

COOPER W.C., GOOD R.A. & MARIANI T. (1974) Effects of protein insufficiency on immune responsiveness. *Am. J. clin. Nutr.* **27,** 647.

DENNIS K.L., DUDRICK S.J. & ABDOU N.I. (1974) The effect of dietary protein depletion on immunocompetence: the importance of nutritional depletion prior to immunologic induction. *Ann. Surg.* **179,** 168.

EDELMAN R., SUSKIND R., OLSON R.E. & SHIRISHINHA S. (1973) Mechanisms of defective delayed cutaneous hypersensitivity in children with protein-calorie malnutrition. *Lancet,* **1,** 506.

FERNANDES G., YUNIS E.J. & GOOD R.A. (1976) Influence of protein restriction on immune function in NZB mice. *J. Immunol.* **116,** 3.

FLAX M.H. & WAKSMAN B.H. (1962) Delayed cutaneous reactions in the rat. *J. Immunol.* **89,** 496.

FOLCH J., LEE M. & SLOANESTANLEY G.H. (1956) A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226,** 497.

GOOD R.A., FERNENDES G., YUNIS E.J., COOPER W.C., JOSE D.C., KRAMER T.R. & HANSEN M.A. (1976) Nutrition deficiency, immunological function and disease. *Am. J. Path.* **84,** 599.

LEFFORD M.J. (1974) The measurement of tuberculin hypersensitivity in rats. *Int. Archs Allergy,* **47,** 570.

MAYER M.M. (1961) *Experimental immunochemistry,* 2nd edn. Thomas, Illinois.

MUNSON D., FRANCO D., ARBERTER A., VELEZ H. & VITALE J.J. (1974) Serum level of immunoglobulins, cell-mediated immunity and phagocytosis in protein-calorie malnutrition. *Am. J. clin. Nutr.* **27,** 625.

MURRAY M.J. & MURRAY A.B. (1977) Starvation supression and refeeding activation of infection. *Lancet,* **1,** 123.

NATIONAL ACADEMY OF SCIENCES (1968) *Nutrient Requirement of Laboratory Animals.* National Research Council. Publication 990.

NISHIOKA K., KAWAMURA K., HIRAYAMA T., KAWASHIMA K. & KOGURE M. (1976) The complement system in tumor immunity. Significance of elevated level of complement in tumor bearing hosts. *Ann. N.Y. Acad. Sci.* **276,** 303.

PHILBRICK D.J. & HILL D.C. (1974) Development of malnutrition in rats. *Am. J. clin. Nutr.* **27,** 813.

SAKAMOTO M. (1975) Study on rat complement. I. Immune adherence and immune hemolysis activity of rat serum. *Japan J. exp. Med.* **45,** 183.

SMYTHE P.M., SCHONLAND M., BRERETON-STILES G.G., COOVADIA H. M. & LOENING W.E.K. (1971) Thymolymphatic deficiency and depression of cell mediated immunity in protein-calorie malnutrition. *Lancet,* **2,** 939.

WEIBEL E.R. (1963) *Morphometry of the Human Lung.* Springer-Verlag, Berlin.