INFECTION AND IMMUNITY, June 1979, p. 770-773
0019-9567/79/06-0770/04$02.00/0

Vol. 24, No. 3

Effect of Protein Deficiency on Suppressor Cells

MANOOCHEHR KHORSHIDI AND NAHID MOHAGHEGHPOUR*

Department of Pathobiology, School of Public Health and Institute of Public Health Research, University of Teheran, Teheran, Iran

Received for publication 1 September 1978

The effects of moderate protein deficiency on the in vitro response of spleen cells to phytohemagglutinin in A/Jax mice were studied. The response of spleen cells from protein-deficient mice to phytohemagglutinin was found to be enhanced as compared with that of cells from control animals. Since inadequate development or function of suppressor cells in the protein-deficient mice offered a possible explanation for the enhanced lymphoproliferative activity, cocultures of spleen cells from protein-deficient and control animals were tested for their responses to phytohemagglutinin. Suppression of [^{3}H]thymidine incorporation was detected in cocultures of 25% mitomycin-treated spleen cells from control animals and 75% spleen cells from protein-deficient mice. The suppressor (regulator) elements in control spleens were found to reside in the adherent cell population.

Several reports have indicated that animals subjected to moderate protein deficiency show enhanced cellular immune responses, as measured by resistance to viral infection, graft-versus-host reaction, allograft rejection, phytohemagglutinin (PHA) responsiveness, and development of specific cytotoxicity against tumor cells (1-3, 6, 7). Similarly, in vitro responses of lymphocytes to antigens (8) or mitogen (11) have been found to be enhanced in some malnourished children. Enhanced cellular immune responses in moderately protein-deficient (PD) rats was shown to be due to the reduced level of blocking antibody (7). On the other hand, Cooper and co-workers (2) have attributed the enhancement of cellular immune responses in mice subjected to chronic moderate protein deficiency to an increased output of thymic hormone (thymin). Since inadequate development or function of suppressor cells in the PD animals offered a possible explanation for the enhanced lymphoproliferative activity, cocultures of spleen cells from moderately PD and control mice were tested for their response to PHA.

(This work was taken in part from studies submitted to the University of Teheran by M.K. in partial fulfillment of the requirements for the M.S. degree in Immunobiology.)

MATERIALS AND METHODS

Animals. One hundred fifty A/Jax male and female mice, the original breeding pairs of which were purchased from Yeda Research and Development Co. Ltd., Weizmann Institute of Science, Rehovot, Israel, in 1975, were used in this study. Animals bred in our animal facilities were separated from their mothers 21 days after birth and distributed into two dietary groups: PD and control. At this age the mean weight of the mice was 11.2 ± 0.2 g. Groups of four to five mice of the same sex were housed in autoclavable plastic cages fitted with stainless-steel covers, holding nipple-type water bottles.

Diets. The PD groups were given an ad libitum diet containing 6% casein (enzymatic casein hydrolysate; Sigma Chemical Co., St. Louis, Mo.), whereas the control group received a 22% casein diet (Table 1). The diets were isocaloric, prepared fresh every week, and stored at 4°C, according to the method described by Fernandes et al. (3).

Serum samples. Blood samples were obtained from the retroorbital venous plexus at the time of sacrifice. The serum separated from each blood sample was stored at −20°C.

Rabbit anti-mouse thymocytes serum. Antithymocyte serum used in this investigation was obtained from Modabber, School of Public Health, University of Teheran. This antiserum was prepared by intramuscular injection of 4×10^7 thymocytes from BALB/c mice into a rabbit according to the method described by Cerny (personal communication). Ten days later the rabbit was bled, and the separated serum was absorbed with bone marrow cells from A/Jax mice before use.

Serum protein studies. Total serum proteins were determined by using the biuret method. The levels of serum albumin and immunoglobulins were measured by cellulose acetate electrophoresis.

Cell suspension. Spleens were aseptically removed from one to five PD or control mice after cervical dislocation. The content of each spleen was teased out with forceps in RPMI 1640 medium (Microbiological Associates, Bethesda, Md.), and the cell suspension was passed through sterile gauze. Cells were then sedimented by centrifugation at 400 × *g* for 10 min at 4°C, resuspended in the medium, and washed three times. Viable cells were counted by the trypan blue exclusion technique with 0.1% solution of the dye, and by this criterion 90 to 95% of cells were viable. The

TABLE 1. *Composition of diets*

Ingredients[a]	PD		Control	
	g	kcal	g	kcal
Casein[b]	6	24	22	88
Dextrose[c]	41	164	33	132
Cornstarch	41	164	33	132
Corn oil	5	45	5	45
Salt mixture[b]	4		4	
Vitamins and minerals[b]	20		20	
Agar[d]	1		1	
Water	100		100	

[a] Agar was dissolved in hot water to which all ingredients except the vitamins were added and mixed in a blender. Vitamins were added when the temperature of the mixture reached 60°C.

[b] From Nutritional Biochemicals, Cleveland, Ohio.

[c] From Mallinckrodt Chemicals, St. Louis, Mo.

[d] From Difco Laboratories, Detroit, Mich.

cell concentration was then adjusted to the required number of cells for each experiment.

Enumeration of thymus-derived lymphocytes. In each experiment enumerating thymus-derived lymphocytes (T cells), 6×10^5 spleen cells were allowed to incubate for 30 min at 37°C in 0.2 ml of RPMI 1640 containing 10% antithymocyte serum. After this period of incubation the cells were washed once with RPMI 1640, and then 0.4 ml of a 1:10 dilution of guinea pig complement (Flow Laboratories, Rockville, Md.) was added to each tube. After a second 30-min incubation, the cells were washed once with RPMI 1640 and then exposed to 0.2 ml of trypan blue (3 parts of 1% trypan blue plus 7 parts of 2% ethylenediaminetetraacetate). After 5 min of incubation at 37°C, the contents of the tubes were examined microscopically and the percentage of dead cells was determined.

As controls, spleen cells were also treated with normal rabbit serum and complement. The cytotoxic index was calculated according to the following formula: cytotoxic index = [(percentage of dead cells in antithymocyte serum − percentage of dead cells in normal rabbit serum)/(100 − percentage of dead cells in normal rabbit serum)] × 100.

Separation of NA cells from spleens of control mice. Four million spleen cells in 1 ml of RPMI 1640 were incubated stationary for 2 h at 37°C in Falcon petri dishes (35 by 10 mm) in a CO_2 incubator. Nonadherent (NA) cells were harvested by gently shaking the dishes back and forth, aspirating the supernatant fluid, and collecting the cells into tubes. To facilitate removal of the adherent (A) cells, fresh medium was added to each petri dish and the plates were then incubated on ice. After 1 h of incubation, the A cells were removed with a rubber policeman. The A and NA cells were then washed twice with the medium and treated with mitomycin C.

To identify each cell population with regard to macrophage content, A and NA cells were stained with Wright-Giemsa, acid phosphatase, tartrate-resistant acid phosphatase, chloroacetate esterase, naphthol esterase, nonspecific esterase, and fluoride-resistant esterase (10, 13, 14). By such cytochemical criteria, the macrophage content of NA cells was considerably lower (less than 5 to 10%) than that of the A-cell population.

Mitomycin treatment. One milliliter of spleen cells (4×10^6 ml) was incubated with 0.2 ml of mitomycin C (Sigma Chemical Co.) containing 0.5 mg/ml at 37°C for 30 min. The cells were then washed three times with the medium and adjusted to contain 5×10^5 cells per ml.

Spleen cell culture. Spleen cells (5×10^5/ml) were cultured in triplicate in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (Difco Laboratories, Detroit, Mich.), 1% glutamine (Difco Laboratories), 100 IU of penicillin, and 100 μg of streptomycin. The cells were incubated with a previously determined optimal concentration of PHA-P (Difco Laboratories) at 37°C in a humid atmosphere containing 5% CO_2 for 72 h. Sixteen hours before termination of the cultures, 1 μCi of tritiated thymidine (Radiochemical Centre, Amersham, England) of 21.5 Ci/mmol was added to each culture tube. Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer (model 3330; Packard Instrument Co., Inc., Rockville, Md.), and the mean counts per minute of each triplicate sample was used in calculating the stimulation index according to the following formula: stimulation index = mean counts per minute of cultures in the presence of PHA/mean counts per minute of cultures in the absence of PHA.

Coculture experiments. Mitomycin C-treated spleen cells from normal and PD mice were assayed for suppressive activity by cocultivation with PD spleen cells.

Presentation of data. All experiments were repeated three to five times. Representative data from these experiments are cited.

RESULTS

Growth of mice on the two diets is summarized in Table 2. The average weight of animals kept on a 6% casein diet (PD group) was significantly lower than that of the controls, which received a 22% casein diet. By 3 months of age, both male and female mice on the control diet had a mean weight gain of approximately 13 g; however, the average weight gained by the mice on the low-protein diet was only about 5 g.

Total serum protein concentrations were considerably lower in the PD animals than in the control mice ($P < 0.05$) (Table 3). The serum albumin level in the PD mice was similarly lower than in the control group ($P < 0.01$). The mean spleen weight was also significantly reduced in the PD animals ($P < 0.01$); however, the percentage of thymus-derived lymphocytes was comparable in both groups (Table 2).

Cellular immunity measured by lymphoproliferative response of spleen cells to PHA was not affected by moderate protein deficiency. On the contrary, significant enhancement of the reactivity to PHA was noted in the spleen cells of animals fed the 6% casein diet for 3 months (Table 4). Although the spontaneous proliferations of PD spleen cells were significantly lower than that of the controls, e.g., 68 versus 363 cpm,

TABLE 2. *Effect of diet on body and spleen weight and percentages of thymus-derived lymphocytes in PD and control mice*

Dietary group[a]	Mean wt ± standard deviation		T cells (%)
	Body (g)	Spleen (mg)	
PD	16.4 ± 2.7[b]	33 ± 11[b]	35 ± 14
Control	24.4 ± 2.5	70 ± 19	36 ± 10

[a] Each group consisted of 45 to 55 animals.
[b] Significant difference between PD and control mice at $P < 0.01$ by Student's t test.

TABLE 3. *Effect of diet on serum protein concentration*

Dietary group[a]	Serum protein concn (g/100 ml)		
	Total proteins	Albumin	Immunoglobulin
PD	4.7 ± 0.7[b]	2.4 ± 0.3[c]	1.0 ± 0.3
Control	5.3 ± 0.2	3.0 ± 0.1	1.2 ± 0.2

[a] Each group consisted of 45 to 55 animals.
[b] Significant difference between PD and control mice at $P < 0.05$.
[c] Significant difference between PD and control mice at $P < 0.01$ by Student's t test.

TABLE 4. *Effect of protein deficiency on the PHA response of mouse spleen cells*

Cells in culture[a]	Mean count ± standard deviation ($\times 10^{-3}$)[b]	Stimulation index
75% CS + 25% CS_m	17.5 ± 1.4	30
75% PDS + 25% PDS_m	44.2 ± 2.8	123
75% PDS + 25% CS_m	23.7 ± 5.7	95

[a] Pooled spleen cells from four to six mice. Abbreviations: CS, normal spleen cells; CS_m, mitomycin-treated CS; PDS, spleen cells from PD mice; PDS_m, mitomycin-treated PDS.
[b] Response of PDS + PDS_m was significantly different from that of CS + CS_m ($P < 0.01$); response of PDS + PDS_m was different from PDS + CS_m at $P < 0.05$.

the mitogen reactivity of PD cells was at least 180% of that of the controls when stimulated with 1.5 μg of PHA (data not shown). Enhanced mitogen reactivity was noted when animals were tested 28 days after the beginning of the diet.

Since the number of T cells in the spleens of PD mice was unchanged and yet reactivity to PHA was significantly higher than that in control mice, it seemed possible that cells involved in the regulation of PHA response were affected by moderate protein deficiency. To examine this possibility, PD spleen cells were cocultured with spleen cells from control mice. In initial experiments, increasing numbers of mitomycin-treated spleen cells from control mice were added to cultures containing decreasing numbers of PD spleen cells. A typical dose-response curve was obtained, with suppression of [^{3}H]thymidine incorporation occurring at 0.75×10^5 (15%) mitomycin-treated control spleen cells. Cocultures of 25% spleen cells from control animals and 75% PD spleen cells resulted in significant depressions of mitogen response in the PD cultures ($P < 0.01$) (Table 4). In these experiments the number of cells in the control cultures (spleen cells from PD or control mice alone) was kept constant at 5×10^5 by addition of 1.25×10^5 corresponding mitomycin-treated spleen cells per tube. Response of 5×10^5 mitomycin-treated PD or control spleen cells to PHA was minimal, (e.g., 185 cpm).

To identify the cell population(s) which exerts suppressive activity on the PHA response of spleen cells, mitomycin-treated A or NA cells from control animals were cocultured with spleen cells from PD mice. Whereas the presence of 25% control mitomycin-treated A cells significantly suppressed the proliferative response of 3.7×10^5 PD cells ($P < 0.01$), the presence of 1.25×10^5 control mitomycin-treated NA cells in the mixture had no effect on the PHA response (Table 5). Mitomycin-treated A cells from PD mice, similarly, lacked the capacity to suppress the proliferative response of PD cells. In a representative experiment, [^{3}H]thymidine incorporation in the presence of 25% mitomycin-treated A cells was 85% of that of the control cultures (20.7×10^{-3} versus 25.9×10^{-3} cpm).

DISCUSSION

The results of the present study demonstrate that the in vitro responses of spleen cells from PD mice to PHA, a T-cell mitogen, are enhanced compared with the reactions of cells from control

TABLE 5. *PHA responses of spleen cells from PD mice incubated with control mitomycin-treated A and NA spleen cells*

Cells in culture[a]	Mean count ± standard deviation ($\times 10^{-3}$)[b]	Stimulation index
75% PDS + 25% PDS_m	36.2 ± 2.4	51
75% PDS + 25% A_m	9.4 ± 0.3	29
75% PDS + 25% NA_m	35.2 ± 3.2	68

[a] Pooled spleen cells from four to six mice. Abbreviations: PDS, spleen cells from PD mice; PDS_m, mitomycin-treated PDS; A_m, mitomycin-treated A cells from control mice; NA_m, mitomycin-treated NA cells from control mice.
[b] Response of PDS + PDS_m was significantly different from that of PDS + A_m ($P < 0.01$); response of PDS + PDS_m was not significantly different from that of PDS + NA_m.

animals. The augmented PHA response of spleen cells from PD mice was found to be due to impairment of the regulatory mechanism for the following reasons. Considerable suppression in the reactivity of PD spleen cells was noted when these cells were cocultured with mitomycin-treated spleen cells from control mice. Suppression could not be attributed to tissue culture artifacts, since cell concentration was kept constant by addition of mitomycin-treated cells to control cultures (cells from PD or control mice alone). Carry-over or leakage of mitomycin from control spleen cells is unlikely to be responsible for the observed suppression of the PHA response, since not every cell population (e.g., mitomycin-treated NA cells) had inhibitory activity.

A cells in the spleens of control mice exerted the suppressive activity. Addition of mitomycin-treated A cells to cultures containing PD spleen cells resulted in a marked suppression of [^{3}H]thymidine incorporation. As anticipated, control NA spleen cell populations were unable to alter the PHA response of PD spleen cells. T cells did not appear to exert the observed suppressive effect because, in a preliminary experiment, T-cell-depleted spleen cells from control mice were capable of reducing the PHA response of PD spleen cells by 59%. Considering our observation regarding the inability of A cells from PD mice to suppress the in vitro response of PD spleens cells to PHA, one can assume that the enhanced lymphoproliferative response of PD spleen cells simply reflects inadequate development or function of suppressor cells in the PD mice. Suppression of lymphocyte responsiveness to PHA by A cells or macrophages has been reported in other systems (4, 5, 9, 12). Even though the proportion of macrophages in A-cell populations estimated by cytochemical criteria was considerably higher than that found in NA-cell populations, the identity of suppressor A cells with macrophages remains to be established.

ACKNOWLEDGMENTS

This work was initiated in response to a discussion with F. Z. Modabber, and we are grateful to him for continued interest. We also thank Elenor Bet-Sayyad for excellent secretarial assistance.

This investigation was supported in part by funds from the School of Public Health and Institute of Public Health Research, University of Teheran, and the Research Council of the Ministry of Science and Higher Education, Iran.

LITERATURE CITED

1. **Bell, R. G., and L. A. Hazell,** 1975. Influence of dietary protein restriction on immune competence. J. Exp. Med. **141**:127-137.
2. **Cooper, W. C., R. A. Good, and T. Marian.** 1974. Effects of protein insufficiency on immune responses. Am. J. Clin. Nutr. **27**:647-664.
3. **Fernandes, G., E. J. Yunis, and R. A. Good.** 1976. Influence of protein restriction on immune functions in NZB mice. J. Immunol. **116**:782-790.
4. **Folch, H., M. Yoshinaga, and B. H. Waksman.** 1973. Regulation of lymphocyte response to phytohemagglutinin. J. Immunol. **110**:835-839.
5. **Glaser, M., H. Kirchener, and R. B. Herberman.** 1975. Inhibition *in vitro* lymphoproliferative responses to tumor-associated antigens by suppressor cells from rats bearing progressively growing Gross leukemia virus-induced tumors. Int. J. Cancer **16**:384-393.
6. **Jose, D. G., W. C. Cooper, and R. A. Good.** 1971. How protein deficiency enhances cellular immunity. J. Am. Med. Assoc. **218**:1428-1429.
7. **Jose, D. G., and R. A. Good.** 1971. Absence of enhancing antibody in cell-mediated immunity to tumor heterografts in protein deficient rats. Nature (London) **231**: 323-325.
8. **Jose, D. G., J. S. Welch, and R. L. Doherty.** 1970. Humoral and cellular immune responses to streptococci and other antigens in Australian aboriginal school children. Aust. Paediatr. J. **6**:192-201.
9. **Kirchner, H., A. V. Muchmore, T. M. Chusad, H. T. Holden and R. B. Herberman.** 1975. Inhibition of proliferation of lymphoma cells and T lymphocytes by suppressor cells from spleens of tumor bearing mice. J. Immunol. **114**:206-210.
10. **Li, C. Y., K. W. Lam, and L. T. Yam.** 1973. Esterases in human leukocytes. J. Histochem. Cytochem. **21**:1-12.
11. **Rafii, M., S. Hashemi, J. Nahani, and N. Mohaghegh-pour.** 1977. Immune responses in malnourished children. Clin. Immunol. Immunopathol. **8**:1-6.
12. **Veit, B. C., and J. D. Feldman.** 1976. Altered lymphocyte functions in rats bearing syngeneic moloney sarcoma tumors. J. Immunol. **117**:655-660.
13. **Yam, L. T., C. Y. Li, and W. H. Crosby.** 1971. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. **55**:283-290.
14. **Yam, L. T., M. Tavassoli, and P. Jacobs.** 1975. Differential characterization of the reticulum cell in lymphoreticular neoplasms. Am. J. Clin. Pathol. **64**:171-179.