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Influences of nutrition on immunity and susceptibility to mouse hepatitis virus type 2

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Summary. Resistance to mouse hepatitis virus (MHV) in C3H mice is a genetic trait which appears 3–4 weeks after birth. However, when these animals were weaned on a low protein diet (8% casein), they remained susceptible to MHV-2 infection until they reached 8–9 weeks of age. During this period, the protein-restricted C3H mice were as susceptible to MHV-2 as the genetically susceptible congenic C3Hss strain. The delay in the emergence of resistance in the protein-restricted mice could be corrected by injecting these animals with spleen cells from 6-week-old C3H mice. Thymocytes from normal C3H mice, and splenocytes and thymocytes from protein-restricted C3H mice, were not protective. However, spleen cells from the protein-restricted mice were more responsive to phytohaemagglutinin, lipopolysaccharide and concanavalin A than spleen cells from normal C3H. The enhanced lymphoproliferative response in spleen cells from protein-restricted mice was abrogated by the addition of plastic-adherent cells obtained from normal C3H spleens. Spleen cells from protein-restricted and from genetically susceptible C3Hss mice also possessed more spontaneous cytotoxicity against MHV-infected 3T3 fibroblasts.

Abbreviations: Con A, concanavalin A; LP, low protein diet; LPS, bacterial lipopolysaccharide; MHV-2, mouse hepatitis virus type 2; NP, normal protein diet; PHA, phytohaemagglutinin; $TCID_{50}$, 50% tissue culture infectious dose.

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INTRODUCTION

It has been known for some time that protein calorie malnutrition can increase susceptibility to infection and can also cause immune dysfunction (Beisel, 1979; Gross & Newberne, 1980). The mechanism of the immune dysfunction and the way in which it affects a host's resistance to infectious disease are, however, not clearly understood. Generally, undernutrition-induced susceptibility to infection has been studied separately from undernutrition-induced changes in immune function, making it difficult to establish cause and effect relationships. In addition, while classical studies have stressed the role of protein calorie malnutrition in causing immunosuppression, results of several recent studies have suggested that some immune parameters are actually enhanced by protein undernutrition (Cooper, Good & Mariani, 1974; Malave, Naneth & Pocino, 1980; Petro, Chien & Wilson, 1982).

The purpose of the work reported here was to take advantage of a well-defined animal model under study in our laboratory to investigate the relation between dietary protein restriction-induced susceptibility to infection and changes in the immune response caused by such dietary restriction. In this model resistance of the host (C3H mice) to the infectious agent, mouse hepatitis virus type 2 (MHV-2) is controlled by a single gene or a closely linked group of genes (Weiser, Vellisto & Bang, 1976) originally found in mice of the Princeton strain (PRI). Through repetitive backcross-

ing between MHV-2 resistant C3H and the susceptible PRI mice an MHV-2 susceptible strain (C3Hss) congenic and histocompatible with the C3H strain was obtained by Weiser *et al.* (1976). Bang, Bang & Foard (1981) have also demonstrated that the genetically resistant C3H mice, if weaned on a low protein diet, become susceptible to MHV-2. This paper describes our attempts to determine whether changes in immune function induced by dietary protein restriction are involved in the increase in susceptibility to MHV-2 of the genetically resistant C3H mice.

MATERIALS AND METHODS

Animals and diets

ALL mice in this study were from our inbred colonies. The MHV-2 resistant C3H strain is a Heston subline obtained from Dr H. B. Andervont in 1958 by Dr Frederik Bang. The MHV-2 susceptible Princeton (PRI) strain was also obtained by Dr Bang from Dr John Nelson of the Rockfeller Institute. The C3Hss strain is an MHV-2 susceptible recombinant strain which bears the PRI gene for susceptibility to MHV-2 on the C3H background (Weiser *et al.*, 1976). All strains were maintained by brother-sister mating in our laboratory. The MHV-2 resistant C3H mice were weaned at 21 days of age, either on a low protein diet (8% high nitrogen casein) or normal protein diets (Purina lab chow or 27% casein). The composition of the casein diets has been described previously by Malave *et al.* (1980). All animals were housed in plastic cages and provided food in pellet form and water *ad libitum*.

Virus

Mouse hepatitis virus type 2 (MHV-2) was originally obtained from Dr John Nelson at the Rockfeller Institute and subsequently maintained by successive liver passages in PRI mice. Virus stocks were prepared as 10% w/v liver suspensions in bicarbonate-free Hanks' balanced salt solution (Gibco, Grand Island, NY) supplemented with 2% fetal calf serum (FCS) and stored at $-70°$. Virus was titrated *in vitro* on macrophage monolayers prepared from thioglycollate-elicited C3Hs peritoneal exudate cells by the method described by Weiser *et al.* (1976). Virus titre is given as the 50% tissue culture infectious dose ($TCID_{50}$) per ml.

Determination of susceptibility to MHV-2

Groups of C3H and C3Hss mice were challenged i.p. with 1000 $TCID_{50}$ doses of MHV-2 i.p. The C3Hss mice were fed standard laboratory diets. In order to test the effects of low protein diet on susceptibility of mice to MHV-2, the genetically resistant C3H mice were weaned at 21 days of age and fed either the low protein (LP) diet or the normal protein (NP) diet.

Spleen and thymus cell adoptive transfer

Spleen and thymus cell suspensions were obtained from 6-week-old NP or LP mice as previously described (Smith, Esa & Stiff, 1982) and suspended in HBSS. Protein-restricted mice (LP) were then injected i.p. with 3×10^7 spleen or thymus cells from either NP or LP mice. Recipient mice were then challenged 20–30 min later with 10^3 $TCID_{50}$ doses of MHV-2.

Lymphocyte stimulation by mitogens

Stimulation of NP and LP spleen cells by non-specific mitogens was determined as previously described by Malave *et al.* (1980). Three mitogens were tested. These were concanavalin A (Con A, Miles Laboratories, Elkhart, IN), *Eschericia coli* lipopolysaccharide (LPS, Difco, Detroit, MI) and phytohaemagglutinin (PHA, Welcome Reagents Division, Research Triangle Park, NC).

Preparation of adherent spleen cells

Spleen cells, $1{\cdot}5 \times 10^8$ from LP or NP mice, were incubated in RPMI-1640 with glutamine (Gibco), 5% FCS, 200 U/ml penicillin and 100 μg/ml streptomycin in a 90-mm glass petri-dish at 37° and in 5% CO_2 atmosphere for 90 min. After incubation, non-adherent cells in the supernatant fluid were removed by rinsing the cultures three times in warm medium. Ten ml of medium were added to the remaining adherent cells. The cultures were vigorously agitated by repeated pipetting and then scraped off with a rubber policeman to detach the remaining adherent cells. Adherent cells were washed three times and their viability determined by the trypan-blue exclusion test.

Spontaneous splenic cytotoxicity

In order to asses the level of spontaneous cytotoxic activity in spleen cells from NP, LP, C3Hss and PRI mice, the short-term ^{51}chromium release assay was used as described by Welsh *et al.* (1983), using YAC-1 lymphoma cells, MHV A59-infected or normal 3T3 fibroblasts as target cells.

Statistical analysis

The results were analysed by the Student's *t*-test.

RESULTS

Effects of protein restriction

Mice weaned on the low protein casein diet failed to gain weight for the first week on the diet and quickly fell behind their age-matched controls in total body weight. As has previously been observed (Gerbase-Delima *et al.*, 1975; Bell, Halzell & Price, 1976; Malave *et al.*, 1980), protein restriction preferentially affected lymphoid organ weight and cellular content because the reduction in these parameters was proportionally greater than the reduction in total body weight (data not shown). As shown in Table 1, 6-week-old C3H mice maintained on low protein diet for 3 weeks were 95% susceptible to 1000 $TCID_{50}$ of MHV-2 injected i.p. In contrast, only 4% of C3H mice fed the normal protein diets were susceptible to the same dose of MHV-2. Susceptibility to MHV-2, as measured by mortality rates in the protein-restricted but genetically resistant C3H mice, was similar to that obtained in the genetically susceptible congenic C3Hss mice. In both cases, death occurred 2–3 days after i.p. challenge, and similar histological lesions consisting of massive hepatic necrosis and lymphoid depletion were observed. The virulence of the virus in C3H NP or C3Hss mice was also similar, as the 50% lethal i.p. dose, determined by the method of Reed & Meunch (1938), in both cases was approximately 1 $TCID_{50}$.

Ontogeny of MHV resistance in NP and LP mice

Neonatal mice from the resistant C3H strain are susceptible to MHV and acquire resistance at about 3 weeks of age (Levy-LeBlond & Dupuy, 1977). Experiments were, therefore, performed to see the effect of post-weaning protein restriction on the ontogeny of resistance to MHV-2. Groups of NP or LP mice ranging in age from 2 to 10 weeks were challenged.

Table 1. Effect of low protein diet on susceptibility to MHV-2

Strain	Type of diet	No. of mice	% mortality
C3H	Low protein (LP)	22	95
C3H	Normal protein (NP)	25	4
C3HSS	Normal protein (NP)	12	100

All mice were 6 weeks old and had been on their respective diets for at least 18 days before challenge with 1000 $TCID_{50}$ MHV-2 intraperitoneally. Death occurred 2–3 days after challenge.

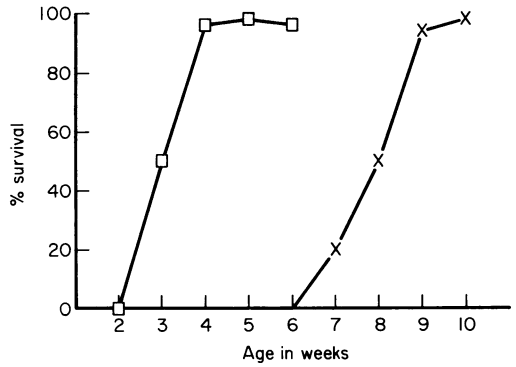

Figure 1. Differences in the ontogeny of resistance to MHV-2 in C3H mice on normal or low protein diets. Mice were challenged i.p with 10^3 $TCID_{50}$ of MHV-2 at the ages indicated: (□) normal protein; (X) low protein. Protein restriction results in a delay in the ontogeny of resistance.

Figure 1 shows the results of this experiment. NP mice were found to be fully susceptible to the virus at 2 and 3 weeks after birth. At 4 weeks of age, four in six mice did not survive challenge with MHV-2. However, at 5 weeks of age and beyond, all C3H mice fed normal diets were resistant to MHV-2 (seven animals per group). In contrast, LP mice remained susceptible to infection until they reached 7 weeks of age, and did not become as resistant as NP mice until they were 9 weeks old.

Transfer of resistance to protein-restricted mice with non-immune cells from normal mice

Previous work has shown that protein restriction induces considerable depletion of lymphoid cells in spleen and thymus (Bell *et al.*, 1976; Malave *et al.*, 1980). In order to determine if the susceptibility of the protein-restricted mice was related to this lymphoid hypoplasia, experiments were performed to determine if infusion of these mice with syngeneic lymphoid cells from NP or LP mice would confer protection against a virus infection. Six-week-old LP mice received 3×10^7 spleen or thymus cells from age-matched NP or LP mice i.p., and were challenged with 1000 $TCID_{50}$ of MHC-2 i.p. 20–30 min later. The results are shown in Table 2. Significant protection was afforded by spleen cells from NP mice. However, spleen cells from LP mice and thymus cells from both NP and LP mice failed to confer protection to LP mice, as the mortality rate of mice receiving these cells was similar to that of animals injected with buffer alone.

Table 2. Protective effect of normal C3H lymphoid cells on protein-restricted syngeneic mice

Type of cells transferred	No. of mice	% mortality
Spleen C3H (NP)	18	23
Thymus C3H (NP)	9	88
Spleen C3H (LP)	3	100
Thymus C3H (LP)	3	100
None	12	92

3×10^7 cells from 6-week-old C3H mice were injected i.p. into recipient protein-restricted C3H mice 20–30 min before challenge with 1000 $TCID_{50}$ MHV-2.

Mitogenesis of LP and NP spleen cells

Several workers have found evidence for the enhancement of mitogenesis and various other immunological functions in mice subjected to restricted protein intake (Cooper *et al.*, 1974; Malave *et al.*, 1980; Petro *et al.*, 1982). In order to determine the effects of protein restriction on lymphocyte proliferation in our system and to search for the aetiology of the reported enhancement of this function, we examined the response of NP and LP spleen cells to PHA, Con A and LPS. As summarized in Table 3 and Fig. 2, spleen cells from protein-restricted mice responded better to all three mitogens than spleen cells from mice fed a normal protein diet. At a PHA concentration of 1 μg/ml, protein restriction enhanced [^{3}H]thymidine uptake by approximately 75% (Table 3). Similar results were obtained for Con A and LPS (Fig. 2).

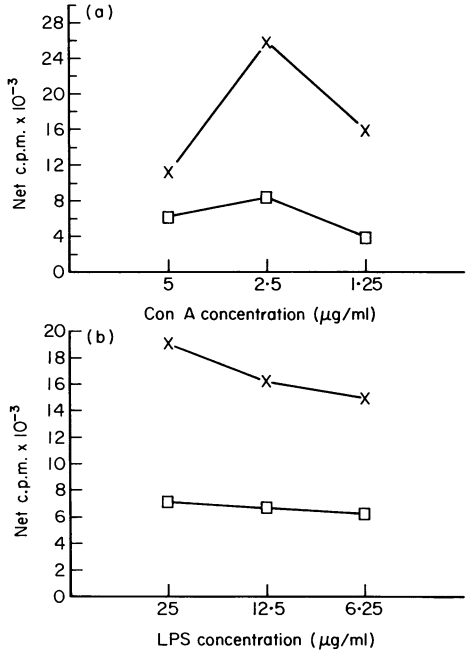

Figure 2. Effects of protein restriction on mitogen stimulation of spleen cells. $2{\cdot}5 \times 10^5$ spleen cells from protein-restricted or normal mice were stimulated with (a) Con A or (b) LPS. After 60 hr incubation at 37° in 5% CO_2, [^{3}H]thymidine was added. Incubation was continued for another 12 hr and then [^{3}H]thymidine uptake was measured. Values represent mean net c.p.m. $\times 10^{-3}$ of triplicate cultures: (□) normal protein; (X) low protein. Mean c.p.m. ± 1 SD obtained in unstimulated spleen cells from protein-restricted mice was 3091 ± 1145, and that of spleen cells from normal mice was 2951 ± 487.

Table 3. PHA-induced proliferative responses of spleen cells from protein restricted and normal C3H mice*

	PHA concentration (μg/ml)			
	0	0·5	1·0	2·0
Low protein				
Counts/minute†	3091 ± 1145	6683 ± 1375	$65{,}030 \pm 5563$	$45{,}970 \pm 5628$
Stimulation index	–	2·0	21·0	14·8
Normal protein				
Counts/minute	2951 ± 487	$2532 \pm 559{\cdot}6$	$37{,}960 \pm 6643$	$13{,}134 \pm 2761$
Stimulation index	–	0	12·8	4·4

* Spleen cells were obtained from 6-week-old C3H mice, maintained since weaning either on low or normal protein diets.

† $2{\cdot}5 \times 10^5$ spleen cells were cultured in RPMI-1640 + 5% FCS for 60 hr with mitogen. [^{3}H]thymidine was then added and the culture continued for another 12 hr. Results are expressed as mean counts per minute (c.p.m. ± 1 SD, or as the stimulation index calculated as mean c.p.m. test: c.p.m. control (no PHA).

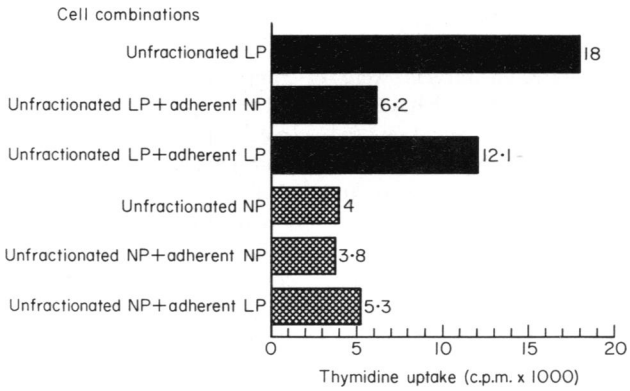

Figure 3. Modulation of mitogenesis by plastic-adherent spleen cells from protein-restricted or normal C3H mice. $3{\cdot}75 \times 10^5$ unfractionated NP or LP cells were combined with $1{\cdot}25 \times 10^5$ plastic-adherent cells obtained from spleen cells of NP or LP mice. 2·5 μg/ml Con A was added to triplicate cultures and [^{3}H]thymidine uptake was determined. Value represent mean net c.p.m. $\times 10^{-3} \pm 1$ SD.

Three concentrations of each mitogen were tested, and in all cases LP spleen cells responded significantly better than NP spleen cells ($P < 0{\cdot}01$).

Malave & Pocino (1981) have presented data suggesting that protein restriction in mice alters an immunoregulatory function of spleen adherent cells. In order to explore how protein restriction affects the accessory role of adherent cells, experiments were performed in which adherent cells obtained from NP spleens were combined with unfractionated LP or NP spleen cells, and adherent cells obtained from LP spleens were similarly added to NP or LP spleen cells. When these cell combinations were stimulated with 2·5 μg/ml Con A, it was found that NP adherent cells significantly suppressed the proliferation of spleen cells from protein-restricted mice. In contrast, LP-adherent cells, when added to unfractionated LP or NP cells, did not significantly affect their proliferation (Fig. 3).

Effect of protein restriction on spontaneous cytotoxicity

We were interested in determining whether resistance to MHV-2 was associated with an increased level of spontaneous splenic cytotoxicity, and whether dietary protein restriction influenced spleen cell cytotoxicity. In initial experiments, we attempted to determine whether genetically MHV-resistant, MHV-susceptible, and protein-restricted (MHV-susceptible) mice differed in their ability to lyse targets sensitive to typical natural killer (NK) cells. To do this, short-term chromium release experiments were performed using LP, NP and C3Hss spleen cells as effector cells, and ^{51}chromium-labelled YAC-1 lymphoma cells as targets. The results from these experiments are summarized in the first section of Table 4. Spleen cells from MHV-susceptible and MHV-resistant mice did not differ in their levels of non-immune cytotoxic activity against YAC-1 target cells. Moreover, protein restriction did not influence this activity, since mice fed 8% casein diet since weaning did not differ from mice fed protein-sufficient diets in the capacity to lyse YAC-1 cells.

Several observations reported by Welsh *et al.* (1983) have indicated that splenic cytotoxicity exhibited by

Table 4. Non-immune splenic cytotoxicity against YAC-1 and MHV-infected 3T3 fibroblasts*

Effector spleen cells	Targets†	% specific release 100:1	50:1	25:1
C3H (NP)	YAC	21·1	18·9	16·5
C3H (LP)	YAC	24·2	15·4	9·1
C3Hss	YAC	19·3	12·1	10·0
C3H (NP)	MHV-fibroblasts	17·1	8·5	6·4
C3H (LP)	MHV-fibroblasts	32·2	29·0	26·2
C3Hss	MHV-fibroblasts	27·9	14·6	3·7

* Spleen cells were added to ^{51}Cr-labelled YAC-1, uninfected or MHV-infected fibroblasts at 100:1, 50:1, and 25:1 effector to target ratios.

† Uninfected fibroblasts were not lysed and are not included in the table.

murine strains against MHV-infected targets was different from typical NK activity. Unlike NK cells, cells effective against MHV-infected targets were Ia-positive, plastic-adherent, contained the light chain of gammaglobulin, and did not have sialo Gm_1 (a newly discovered surface marker for NK cells). We therefore decided to explore whether MHV-resistant and MHV-susceptible strains differed in the capacity to lyse MHV-infected targets, and also whether protein restriction influenced this non-immune splenic, virus-directed cytotoxicity. 3T3 fibroblasts, either uninfected or infected with MHV-A59 virus, were used as targets.

The results of these experiments, also depicted in Table 4, show that spleen cells from C3Hss, LP and NP mice could all lyse infected fibroblasts. Surprisingly, cells from NP mice, which are the least susceptible to this virus, had the lowest splenic cytotoxicity. Moreover, when mice of this same strain were fed the low protein diet, their splenic cytotoxicity against MHV-infected targets was increased significantly ($P < 0{\cdot}01$). Fibroblasts not infected with MHV were not lysed by any of the spleen cells (data not shown).

DISCUSSION

Our data indicate that protein malnutrition converts genetically MHV-resistant C3H mice to MHV-susceptible, and confirm earlier work by Ruebner & Bramhall (1960) and Bang *et al.* (1981). The effect of diet restriction on host resistance was, however, only transient, and completely disappeared after about 8 weeks of age. Since neonatal C3H mice are never resistant to this virus infection, and become resistant at about the third week of age, it would appear that post-weaning protein restriction only delays the development of adequate host resistance. Similar delays in the ontogeny of resistance mechanisms due to post-weaning dietary restriction have been reported by Dubos & Schaedler (1958).

The data obtained in the present study also suggest that the increased susceptibility of C3H mice to MHV-2 infection was due to an immunological alteration caused by dietary protein restriction. It appears that the diet-induced hypoplasia was partly responsible for the increased susceptibility of undernourished mice to MHV-2. It was possible to protect protein-restricted mice from the lethal effects of MHV-2 infection by injection with unfractionated non-immune spleen cells from age-matched C3H mice. Unfractionated spleen cells from protein-restricted mice, when given to protein-restricted mice in numbers sufficient to compensate for the hypoplasia found in these mice, did not protect the recipient mice from MHV-2 infection. This result may indicate that spleen cells from protein-restricted mice were either functionally aberrant or were deficient in one or more cell types. Furthermore, the fact that thymus cells, unlike spleen cells, were not protective, suggests that the protein-deficient, MHV-susceptible mice lacked more mature lymphocytes. Our results would seem to support the contention that undernutrition causes a relative increase in the number of immature lymphocytes (Chandra, 1979a, b; Heresi & Chandra, 1980).

In order to evaluate further the immunocompetence of the protein-restricted mice, we examined the mitogen-induced lymphocyte proliferation responses in our mice and in age-matched controls. It is clear from the data that lymphocytes from protein-deficient mice respond better than cells from normal mice to PHA, Con A and LPS. These data confirm and extend observations reported earlier by Cooper *et al.* (1974), Malave *et al.* (1980) and Petro *et al.* (1982). These experimental results are at variance with all the results obtained in undernourished human subjects. It is quite possible that this discrepancy is due to the multiple deficiencies involved in human malnutrition where deficiencies in vitamins and trace elements (particularly zinc) are often present (Good, Fernandez & West, 1979).

There is little information concerning disturbance in immune regulation in protein restriction. Malave & Pocino (1981) found evidence for loss of a suppressor cell function in mice fed a low protein diet. On the other hand, Chandra (1977) reported evidence for increased suppression in malnourished children. The results reported above indicate that protein restriction alters a regulatory function of plastic adherent spleen cells, and that this is wholly or partly responsible for the enhanced proliferative responses exhibited by lymphocytes of protein-restricted animals. It would appear from this study that, as previously demonstrated by Koros *et al.* (1971) and Malave *et al.* (1980), undernutrition causes a loss of suppressor cell function.

The role of non-immune cytotoxicity in protection against MHV infection is a subject of current study. Data from several laboratories (Schindler, Engler & Kirchner, 1982; Sorensen *et al.*, 1982) suggest that natural cytotoxicity is not important in resistance of mice to MHV, while other data indicate that this form

of cytotoxicity plays a protective role (Bukowski *et al.*, 1983). In this study, increased proliferative and cytotoxic responses were inversely related to MHV resistance (whether resistance was genetically acquired or induced by protein restriction). Therefore, our data suggest that enhanced proliferative and cytotoxic responses may not be beneficial in MHV infection.

Results from this study indicate that protein restriction, when encountered early during the weaning period, retards the development of adequate resistance mechanisms. While the delay in this case was short, it was significant. A similar delay in the ontogeny of host resistance may underlie the high mortality rates often found in weanling children growing up in developing countries.

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