SUSCEPTIBILITY TO MOUSE HEPATITIS VIRUS STRAIN 3 IN BALB/cJ MICE: FAILURE OF IMMUNE CELL PROLIFERATION AND INTERLEUKIN 2 PRODUCTION

Dindzans, V.J., Zimmerman, B., Sherker, A. and Levy, G.A.

Mount Sinai Hospital and Hospital for Sick Children, University of Toronto, Toronto Ontario, Canada

INTRODUCTION

Susceptibility of inbred mice to the Coronavirus, mouse hepatitis virus strain 3 (MHV-3), is dependent on host factors which are under strict genetic control (1). Fully susceptible BALB/cJ mice die with fulminant hepatitis when inoculated with 1-5 plaque forming units (PFU) of MHV-3, while fully resistant A/J mice develop no evidence of disease even when infected with 5×10^7 PFU of MHV-3. Differences in viral replication both in-vivo and in-vitro do not account for strain-dependent differences in resistance to MHV-3, and we have suggested that variation in susceptibility/resistance of inbred mice reflects defects in the host immune response to the virus (2,3).

The interactions between a virus and the cellular elements of the host immune system during viral infection are complex (4). They include uptake of the virus by macrophages and genetically restricted cooperation with antigen-specific T and B lymphocytes resulting in activation of the immune system. Full activation of the immune system is dependent on secretion of cytokines by both macrophages/monocytes (interleukin 1, IL-1) and T lymphocytes (IL-2, IL-3) as well as enhanced expression of the IL-2 receptor on activated T lymphocytes (5). The interleukins facilitate the proliferative expansion of virus-sensitized T and B lymphocyte clones. Subsequently these cells differentiate into virus-specific effector cells (cytotoxic T lymphocytes, helper/suppressor T lymphocytes and antibody secreting plasma cells) in the host immune response.

In this study, we have characterized the primary and secondary cellular immune responses to MHV-3 in vitro, in an attempt to determine factors which might account for susceptibility/resistance in fully susceptible BALB/cJ and fully resistant A/J mice.

M. M. C. Lai et al. (eds.), *Coronaviruses*

METHODS

MHV-3 INFECTION

BALB/cJ and A/J mice (Jackson Laboratories, ME; 6-8 weeks old) were primed by injection of 5×10^6 plaque forming units (PFU) of UV irradiated MHV-3 (UV MHV-3) in complete Freund's adjuvant intramuscularly (500 ul/mouse). After 3 weeks, the mice were boosted with 5×10^6 PFU of UV MHV-3 injected intraperitoneally (IP) and they were sacrificed 10-14 days later. A second group of A/J mice was primed as outlined above, except that 2 IP infections of live MHV-3 (1×10^4 PFU) were used. Spleens were removed and splenic mononuclear cells (SMNC) were isolated by centrifugation over Ficoll-Hypaque.

SMNC were washed and resuspended in RPMI 1640 medium (supplemented with 10% fetal calf serum (FCS) 4mM glutamine, 5×10^{-5} M mercaptoethanol, 15 mM HEPES, 100 U/ml penicillin and 100 ug/ml streptomycin at pH 7.25) at a concentration of 2×10^6 cells/ml.

CELLULAR PROLIFERATION

Splenic mononuclear cells (2×10^6/ml) from MHV primed and unprimed mice were cultured in 96 well flat bottomed microtest culture plates in complete HEPES buffered RPMI 1640 (200 ul/well) and cultured with 1.0×10^6 PFU of MHV-3, UV MHV-3 or the vehicle for the MHV-3 stock preparation. For the final 24 hours of culture, 1 uCi of tritiated thymidine (^{3}H-TdR, specific activity: 25 Ci/mmol) was added to each well. Cultures were harvested onto glass fibre filters and ^{3}H-TdR incorporation measured.

IL-1 PRODUCTION

Peritoneal exudate cells (PEC) from A/J and BALB/cJ mice were obtained 3 days after IP injection of 2 ml of Brewer thioglcollate, washed and resuspended in complete HEPES buffered RPMI 1640. PEC were adjusted to 5×10^6 cells/ml and incubated with MHV-3 or UV MHV-3 at a multiplicity of infection of 2 for 1 hour at 37°C. The cells were washed and finally adjusted to 1×10^6/ml for culture. As a positive control, PEC were incubated with 10 ug/ml of E. coli 0111:B4 butanol extracted lipopolysaccharide (LPS). Supernatants were collected and stored at -20°C until use.

IL-1 BIOASSAY

IL-1 was assayed using the technique described by Mizel et al. (6). Thymocytes from young C3H/HeJ mice (5.0×10^5/ml) were cultured for 72 hours in 96 well, flat-bottomed microtest plates in RPMI 1640 (supplemented with 10% FCS, 4mM glutamine, 5×10^{-5}M mercaptoethanol, 100 U/ml penicillin and 100 ug/ml streptomycin), with concanavalin A (Con A, 2.5 ug/ml) and serial 2 fold dilutions of the sample to be analyzed. ^{3}H-TdR (1 uCi) was added for the final 4 hours of culture. For each sample, net CPM was calculated by the formula:

NET CPM = CPM (thymocytes + Con A + supernatant) - CPM (thymocytes + Con A).

IL-2 PRODUCTION

Spleen cells from MHV-3 primed or unprimed mice were adjusted to 2 x 10^6 cells per ml and cultured with 5 x 10^5 PFU/ml of MHV-3 or UV MHV-3 in HEPES buffered complete medium. The supernatants were collected and stored at -20^{o}C until assayed for IL-2 activity.

IL-2 BIOASSAY

Supernatants were assayed for IL-2 activity as described by Gillis et al. (7). IL-2 dependent cells (HT-2), provided by Gordon Mills (Hospital for Sick Children, Toronto, Canada), were washed and adjusted to 1 x 10^5 cells per ml. 1 x 10^4 cells in 100 ul of complete RPMI medium 1640 was added to flat bottomed microtest wells containing 0.1 ml of serial dilutions of the supernatant to be titrated. Cultures were incubated for 24 hours, with ^{3}H-TdR (1 uCi) added for the final 6 hours of culture. One unit of IL-2 activity was defined as the amount of IL-2 containing supernatant that produced 30% of the maximal proliferative response observed with a reference IL-2 sample obtained from the supernatant of the IL-2 producing cell line MLA-144.

DETECTION OF IL-2 RECEPTOR BY FLUORESCENCE ACTIVATED CELL SORTING (FACS).

Rat anti-mouse IL-2 receptor antibody was kindly provided by T. Malek. Its production and characterization has been described elsewhere. SMNC (2 x 10^6/ml) from UV MHV-3 primed A/J and BALB/cJ mice were incubated with MHV-3 (1 x 10^6 PFU/ml) in secondary culture. At various times, 1 x 10^6 SMNC were incubated with a saturating amount of anti IL-2 receptor antibody at 40^{o}C for 30 minutes, washed twice and then incubated with fluorescein isothiocyanate-conjugated F(ab)'2 anti-rat IgG at 4^{o}C for 30 minutes. Control staining was performed with normal BALB/cJ mouse serum instead of anti IL-2 receptor antibody. Washed and resuspended cells were observed under a fluorescence microscope or subjected to flow cytofluorometric analysis using a FACS analyzer.

RESULTS AND DISCUSSION

The results of our experiments indicate that the in vitro cellular immune response to MHV-3 is profoundly impaired in fully susceptible BALB/cJ mice. The proliferative activity of SMNC stimulated with MHV-3 and UV MHV-3 reflects activation of immune cells (predominantly T and B lymphocytes) in response to the virus (8,9). Therefore, the proliferative response to MHV-3 may be considered a measure of the overall immune response to the virus. Incubation of SMNC from resistant A/J mice with live MHV-3 in primary culture resulted in a peak of tritiated thymidine uptake from days 7-9, whereas no proliferative response to UV MHV-3 was seen. (Fig. 1). In secondary culture, using A/J mice primed with UV MHV-3, stimulation with live MHV-3 resulted in an augmented and earlier (day 3-7) peak of proliferation while incubation with UV MHV-3 generated no response (Fig. 2). The most vigorous proliferative response was obtained when SMNC, obtained from

A/J mice primed with live MHV-3, were incubated in secondary culture (Fig. 3).

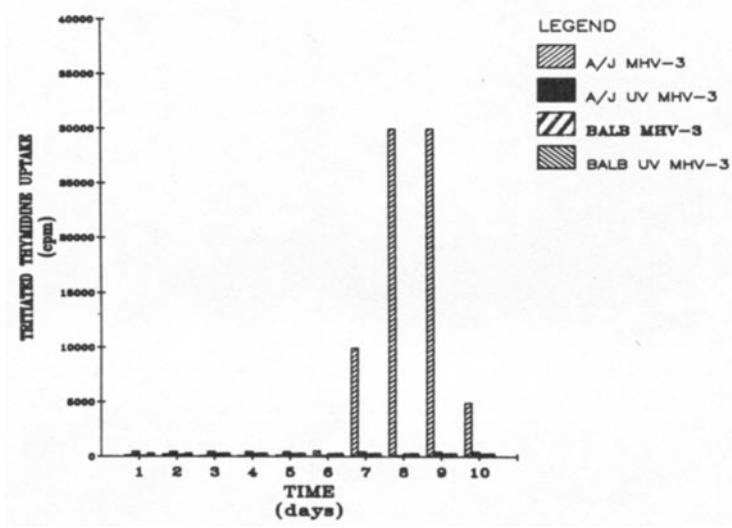

Fig. 1 Time course for the proliferative response of naive SMNC from A/J and BALB/cJ mice in primary culture with MHV-3 and UV MHV-3.

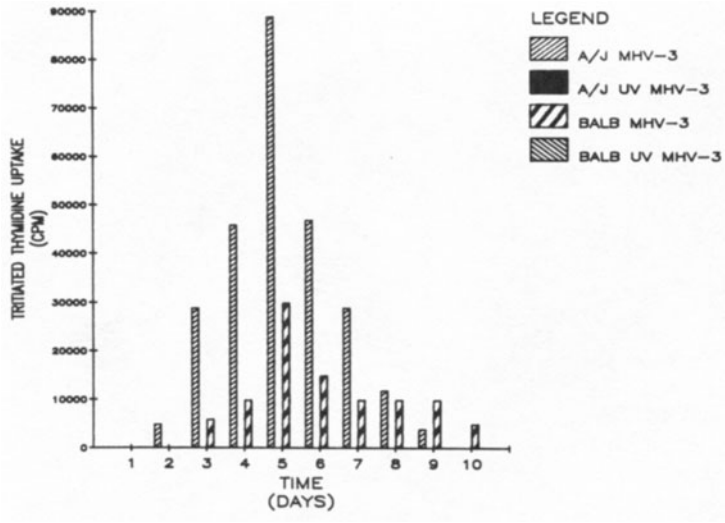

Fig.2 Time course for the proliferative response of SMNC from A/J and BALB/cJ mice primed with UV MHV-3 in secondary culture with MHV-3 and UV MHV-3.

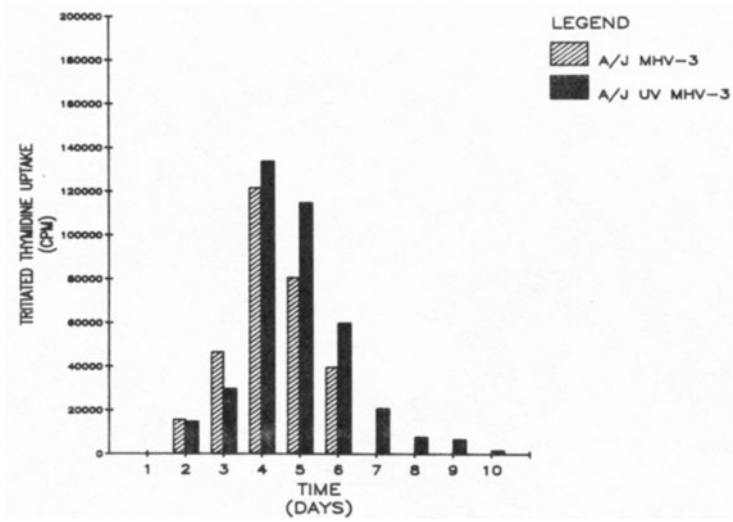

Fig.3 Time course for the proliferative response of SMNC from A/J mice primed with live MHV-3 in secondary culture with MHV-3 and UV MHV-3.

Incubation with MHV-3 or UV MHV-3 resulted in a peak of proliferation from day 2-6, suggesting that infection with live virus is the most effective method to prime the immune system. The ineffectiveness of UV MHV-3 (relative to MHV-3) as a stimulant in the in vitro cellular immune response may simply reflect the small amounts of viral protein antigens present in the viral preparation. However, differences in immune processing of a live virus as compared to inactivated viral antigens may also contribute to this phenomenon. In contrast, SMNC from fully susceptible BALB/cJ mice, were completely unresponsive to MHV-3 or UV MHV-3 in primary culture (Fig. 1). In secondary culture, using SMNC from BALB/cJ mice primed with UV MHV-3, no proliferative response was seen in 3 out of 4 experiments. However, a small peak of ^{3}H-TdR uptake was seen in one experiment when UV MHV-3 primed SMNC were cultured with MHV-3 (Fig. 2).

In order to assess the activation of T lymphocytes during the in vitro immune response to MHV-3, supernatants from the SMNC cultures were analyzed for the presence of the T lymphocyte lymphokine, IL-2. In both primary and secondary cultures of SMNC from resistant A/J mice, proliferative activity was associated with enhanced secretion of IL-2 early in the proliferative response. (Table I). In SMNC cultures from fully susceptible BALB/cJ mice no increase in IL-2 activity was detected. (Table 1). Despite the occurence of proliferation in one secondary culture with MHV-3 (Fig. 2) there was no associated increase in IL-2 activity. This suggests that the enhanced DNA synthesis may have occured in cell populations other than T cells (8,9). The fact that SMNC from both strains responded equally to stimulation with the T cell mitogen, Con A (data not shown) indicates that the pathways for IL-2 secretion are present in both strains.

TABLE I

PRODUCTION OF INTERLEUKIN 2 DURING THE IMMUNE RESPONSE TO MHV-3 IN VITRO

IN VITRO CULTURE	STIMULUS	INTERLEUKIN 2 ACTIVITY (U/ML)	
		BALB/cJ	A/J
PRIMARY	LIVE MHV-3	0	0.4
	UV MHV-3	0	0
SECONDARY[A]	LIVE MHV-3	0	0.8
	UV-MHV-3	0	0
SECONDARY[B]	LIVE MHV-3	-	4
	UV-MHV-3	-	1.6

A Mice were vaccinated with 5 X 10^6 PFU of UV MHV-3 in complete Freund's adjuvant and sacrificed 14 days after a second injection of UV MHV-3.

B Mice were infected with 1 X 10^4 PFU of live MHV-3 twice and 14 days later spleens were harvested.

Expression of the receptor for IL-2 has been shown to be enhanced in activated T cells (10) and necessary for the T cell response to IL-2 stimulation. We used FACS to analyze the expression of the IL-2 receptor in SMNC after stimulation with MHV-3 (Fig. 4). In resistant A/J mice there was an increased number of T lymphocytes expressing a high density of IL-2 receptors, while no increase in the expression of IL-2 receptors was detected in susceptible BALB/cJ mice.

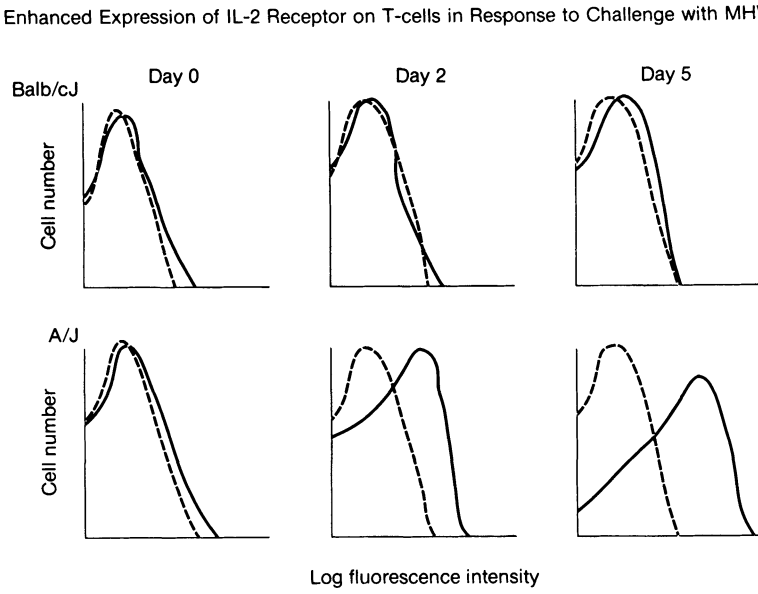

Fig.4 Fluorescence activated cell sorting (FACS) of SMNC during MHV-3 infection. Primed SMNC were incubated with rat anti-mouse IL-2 receptor antibody (.....) or anti-fluorescein conjugated anti-mouse IgG and analyzed by FACS.

In order to assess the response of macrophages during in vitro culture with MHV-3, supernatants from cultures of PEC were analyzed for the monokine, IL-1. High titers of IL-1 were detected in the supernatants of PEC from both resistant A/J mice and susceptible BALB/cJ mice (Table 2).

TABLE II

PRODUCTION OF INTERLEUKIN 1 DURING MHV-3 STIMULATION IN VITRO

STIMULUS[A]	INTERLEUKIN 1 ACTIVITY[B] (U/ML)			
	BALB/cJ		A/J	
	HOURS AFTER STIMULATION			
	8	12	8	12
LPS	7	17	19	11
LIVE MHV-3	6.4	9	4	0
UV MHV-3	0	0	0	0

A Peritoneal macrophages were stimulated with 10 ug/ml of lipolysaccharide (LPS) (E. Coli 0111:B4), live MHV-3 at a multiplicity of infection of 2 or 1 X 10^6 PFU of UV MHV-3. Supernatants were collected and assayed for interleukin 1 activity.

B Interleukin 1 activity was determined using thymocytes from young C3H/HeJ mice as described in the materials and methods.

PEC from both strains responded equally when cultured with lipopolysacharide, a nonspecific IL-1 stimulant, with titers approximately two-fold greater than those obtained in response to MHV-3. Incubation of PEC with UV MHV-3 resulted in no augmentation of IL-1, again suggesting that pathways in the immune responses to live virus and inactivated viral antigens may be different. Despite secretion of IL-1, foci of syncitia appeared at 5-7 hours and the cytopathic effect was complete by 12 hours in monolayers of MHV-3 infected PEC from both strains.

In an attempt to restore the proliferative response of BALB/cJ SMNC, recombinant IL-1 and IL-2 were added to the in vitro cultures. However, both recombinant IL-1 and IL-2 failed to reconstitute the proliferative response to MHV-3 (Table III).

Although the mechanisms of resistance to MHV-3 infection are not known, it is clear that the host immune system plays an important role (3). Experimental ablation of immune cell populations by X irradiation (11), anti lymphocyte serum (11) or infection with frog virus-3 (12) rendered resistant A/J mice susceptible. Furthermore, reconstitution of susceptible neonatal A/J mice with adult immune cells required at least T lymphocytes and adherent cells (13).

Our results are consistent with the results of a previous study in which delayed cutaneous hypersensitivity (DTH) to MHV-3 (a marker of cell mediated immunity) correlated with resistance in A/J mice while susceptible DBA mice did not

respond (14). In contrast, DTH to MHV-JHM, while demonstrating genetic variation in inbred mice, did not correlate with susceptibility to disease (15). This suggests that the mechanisms for pathogenesis of disease by MHV-3 and MHV-JHM may be different.

TABLE III

FAILURE OF IL-1 AND IL-2 TO RECONSTITUTE THE PROLIFERATIVE RESPONSE TO MHV-3 IN SPLENIC MONONUCLEAR CELLS FROM BALB/cJ MICE

RECOMBINANT (UNITS ADDED)		PROLIFERATIVE RESPONSE (CPM)	
IL-1	IL-2	MHV-3	CON A
-	-	150	342,000
10	-	210	362,000
-	10	165	410,000
10	10	147	376,500

Our data indicate that susceptibility to MHV-3 in BALB/cJ mice is associated with an impaired activation of the immune system as demonstrated by the failure of proliferation during in vitro culture. Further analysis indicated an absence of both IL-2 secretion and the generation of IL-2 receptors, which suggests that T lymphocytes were not activated by stimulation with MHV-3.

Since exogenous IL-2 did not reconstitute the proliferative response, inability of T lymphocytes to recognize MHV-3 may be the primary defect in the immune response to MHV-3. Although macrophages from the susceptible BALB/cJ mice secreted high titers of IL-1 in response to MHV-3, the adequacy of antigen presentation to T lymphocytes was not assessed by our experiments. Antigen presentation is known to involve an association between the antigen and Ia histocompatibility antigen leading to recognition through a specific T cell receptor. Presentation to some T cell subsets also seems to require a membrane - membrane interaction between presenting cells and the T cell. Therefore, failure of immune recognition of MHV-3 by T lymphocytes may be the result of a macrophage defect (inadequate antigen presentation) and/or a lymphocyte defect (inability to recognize and/or respond to antigens of MHV-3). Further experiments are underway to distinguish these possibilities.

REFERENCES

1. Wege, H., S. Sidell and V. ter Meulen. 1982. The biology and pathogenesis of coronaviruses. Cuir. Top. Microbiol. Immunol. 99:131.

2. MacNaughton, M.R. and S. Patterson. 1980. Mouse hepatitis virus strain 3 infection of C57, A/Sn and A/J strain mice and their macrophages. Arch. Virol. 66:71.

3. Dindzans, V., P. MacPhee, L. S. Fung, J.L. Leibowitz and G.A. Levy. 1985. The immune response to mouse hepatitis virus: Expression of monocyte procoagulant activity and plasminogen activator during infection in vivo. J. Immunol. 135:4189.

4. Zinkernogel, R.M. and H. Hengartner. 1985. On the role of viruses in the evolution of immune responses. Br. Med. Bull. 41:92.

5. Marx, J.L. 1983. Chemical signals in the immune system. Science 221:1362.

6. Mizel, S.B., J.J. Oppenheimer and D.L. Rosenstreich. 1978. Characterization of lymphocyte to activating factor produced by the macrophage cell line, P388D I. Enhancement of LAF production by activated T lymphocyte. J. Immunol. 120:1497.

7. Gillis, S., M.M. Fern, W. Ou and K.A. Smith. 1978. T cell growth factor: Parameters of production and a microassay for activity. J. Immunol. 120:2027.

8. Osborne, D.P., Jr. and D.H. Katz. 1973. Antigen-induced deoxyribonucleic acid synthesis in mouse lymphocyte activation by hemocyanin and dinitrophenyl carrier conjugates. J. Immunol. 111:1164.

9. Osborne, D.P., Jr. and D.H. Katz. 1973. Antigen-induced deoxyribonucleic acid synthesis in mouse lymphocytes. II. Analysis of cell populations required for and responding to antigen stimulation in vitro. J. Immunol. 111:1176.

10. Smith, K.A. 1984. Interleukin 2. Ann. Rev. Immunol. 2:319.

11. Dupuy, J.M., E. Levey-Leblond and C. Le Provest. 1975. Immunopathology of mouse hepatitis virus type 3 infection. II. Effect of immunosuppression in resistant mice. J. Immunol. 114:226.

12. Pereira, C.A., A.M. Steffan and A. Kirn. 1984. Kupffer and endothelial liver cell image renders A/J mice susceptible to mouse hepatitis virus type 3. Virus Res. 1:557.

13. Levy-Leblond, E. and J.M. Dupuy. 1977. Neonatal susceptibility to MHV-3 infection in mice. I. Transfer of resistance. J. Immunol. 118:1219.

14. Le Provost, C., E. Levy-Leblond, J.L. Virelizier and J.M. Dupuy. 1975. Immunopathology of mouse hepatitis virus type 3 infection. I. Role of humoral and cell mediated immunity in resistance mechanisms. J. Immunol. 114:221.

15. Kyuwa, S., K. Fujiwara and K. Yamanouchi. 1984. Genetic control of delayed type hypersensitivity to mouse hepatitis virus in mice. Japan, J. Exp. Med. 54:217.