MOUSE HEPATITIS VIRUS 3 PATHOGENICITY AND B AND T LYMPHOTROPISMS

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INTRODUCTION

Viral pathogenicity may be regulated by a host defense mechanism during virus-cell interaction at the immune system level. When the virus is lymphotropic, understanding the pathogenic process of the viral disease is complicated by the virus-lymphocyte interaction which may alter the integrity of the cell and induce a subsequent immunodeficiency.

Mouse hepatitis virus type 3 (MHV3), a hepatolytic virus, is a member of the coronavirus group. Its inoculation in mice induces an acute infection characterized by an hepatic necrosis, killing the animal within a couple of days (1). The outcome of the MHV3 infection varies according to the mouse strain: resistant A/J mice support a subclinical infection, while the other strains, including C57BL/6 mice, are fully susceptible to acute disease. The immune system plays an important role in the outcome of acute disease induced by MHV3. Resistant A/J mice become fully susceptible to accute hepatitis after immunosuppressive treatments (2,3). The genetically-determined resistance of A/J mice develop as the cell populations mature, as non-adherent spleen cells, T lymphocytes, and a third population present in bone marrow (4,5). Efficient cellular and humoral immune responses to viral infection depend on normal B or T lymphopoiesis which take place in bone marrow or thymus (6,7). The specific anti-viral humoral and cellular immune responses act as mechanisms in viral elimination, promoting cellular and antibody-mediated dependent cell cytotoxicity or neutralization of infectious viral particles (8-10).

Lymphoid cells are believed to be the target host for MHV3 replication (11) and at the same time, the effectors of the elimination of the virus. Humoral and cellular immunodeficiencies previously observed in MHV3 chronically-infected (12) related to the loss of non-adherent cells in spleen and thymus despite the stimulation of bone marrow precursor cells (13). No information is available on the mechanism which causes cell depletions in lymphoid organs and subsequently develops into humoral and cellular immunodeficiency, and the role of MHV3 lymphotropism in the pathogenic process of acute disease.

To evaluate the role of B and T lymphotropisms in the MHV3 pathogenicity, B and T lymphoid subpopulations have been studied in vivo in pathogenic MHV3 (L2-MHV3) and its non-pathogenic variant, derived from a chronically

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infected YAC cell line (YAC-MHV3), acutely-infected susceptible C57BL/6 or resistant A/J mice or in vitro in purified T and B cell subpopulations.

METHODS

Mice: A/J and C57BL/6 mouse strains were purchased from the Jackson Laboratories (Bar Harbor, ME). Before being used, the animals were tested for the presence of anti-MHV antibodies, by ELISA by using MHV3 preparation as Ag. During experiments the animals were housed in a sterile atmosphere (Forma Scientific, Marietta, OH).

Viruses: Pathogenic MHV3 was a cloned substrain in L2 cells (L2-MHV3) as previously described (14). YAC-MHV3 variant was a cloned virus derived from persistently infected lymphoid YAC cells (15). Viruses were passaged in L2 cells before use and their pathogenic properties were verified regularly.

Cells: L2 cells, a continous mouse fibroblast cell line, were grown in Eagle's MEM with glutamine (2mM), 5% FCS (GIBCO Laboratories, Grand Island, NY), and antibiotics. L2 cells were used for propagation, cloning and titration of viruses. The thymic cells were obtained by teasing of the thymuses apart in HBSS with 10% FCS at room temperature by using needles. The bone marrow cells were collected from femurs. Femoral shafts were flushed four times with 1 ml. cold Eagle's MEM supplemented with 10% FCS. Large particles were removed by sedimentation on a cushion of 1 ml. of FCS for 5 minutes. The cell suspension was then centrifugized and resuspended in 1 ml. of Eagle's MEM with 10% FCS. Cell preparations were electronically counted (Coulter Counter, Coulter Electronics, Hialeah, FL) and cell viability was assayed by the trypan blue exclusion test. B Cell purification was done by panning according to the Wysocki and Sato's method (16) with some modifications. Before purification, adherent monocytes were removed by incubating the cell suspension (supplemented with 20% FCS) in a petri dish for 1 h at 37°C with 5% CO_2. After incubation, the dish was washed thoroughly with HBSS. The non-adherent cell suspension (10^7/ml) was pipetted into an anti-IgM coated plastic dish and incubated twice at room temperature for 30 min. Non-adherent cells were sucked off from the dish and the remaining adherent cell layer was washed four times with Ca-Mg-free PBS and then removed with a policeman. Immunofluorescence study revealed that the final cell population contained around 94% of sIg+cells.

Histopathological examination: Groups of mice from strains, inoculated i.p. of $10^4 TCID_{50}$ with L2-MHV3 or YAC-MHV3 were necropsied at 72 h p.i. The spleen were collected, fixed in Perfix (Fisher Scientific, Montréal, Qué, Can.) and processed for routine light microscopic examination (hematoxylin-eosine stain).

Virus titration and virus-producing cell assay. Viral suspensions were serially diluted in 10-fold steps and tested in L2 cells cultured in 96-well microtiter plates. CPE characterized by syncytia formation and cell lysis were recorded at 72 h.p.i. and virus titers expressed as $TCID_{50}$. The titrations were made in triplicate. Single cell suspensions of B lymphocytes or thymic cells, prepared as above, were deposited on a gradient of Lymphoprep (Cedarlane, Hornby, Ont. Canada). The suspensions were collected in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, antibiotics and glutamine. Cell preparations consisted of extensive washings (at least three times) to remove free virus and count the cells electronically. The cells were diluted at a concentration of 10^4 cells/ml and measured for virus-producing cell assay by 10-fold step dilutions until 100 cells/ml; thus by 2-fold step in RPMI 1640 medium. Virus production by each cell dilution was tested in L2 cells as described for viral titration. The percentage of

virus-producing cells were calculated by the ratio between the number of cells in the last dilution causing 50% of CPE and the initial concentration.

Immunofluorescence analysis. Double labeling of u chains (pre-B and B cells) was done as per Park and Osmond (17). Briefly described, bone marrow samples (100ul of 4 x 10^7 nucleated cells/ml suspension) were incubated for 30 min on ice an optimal dilution of FITC anti-u chains (Cappel Biomedical, Malvern, PA) for surface (su) labeling. The cells were washed twice by centrifugation through FCS at 200g at 4°C for 7 min. Cells were resuspended in PBS with EDTA (2.7mM) supplemented with 5% BSA. Bone marrow cells were cytocentrifugized at 1000 rpm for 5 min. (Cytospin; Shandon Southern Instrument Inc. Sewickly, PA) and fixed in precooled acetic acid-methanol (5% v/v) for 12 min on ice and then washed 4-5 times in cooled PBS. Cytoplasmic u (cu) chains were labelled with an optimal dilution of TRITC anti-u directly on the cell spots, then incubated 30 min at room temperature in a humidified chamber, then washed 4 times and mounted on a medium containing 90% glycerol in PBS and 0.1% p-phenylenediamine.

Double labeling of CD4 and CD8 thymic cells (4 x 10^7 cells/mL) were incubated for 30 min on ice with rat-FITC anti-CD4 (Dimension Laboratories, Missisauga Ont. Canada). The cells were washed twice by centrifugation through FCS at 200 g at 4°C for 7 min. Cells were then resuspended in RPMI 1640 medium and, incubated with anti-CD8 (Dimension Laboratories, Missisauga, Ont. Canada) for 30 min on ice. Cells were washed and re-incubated with mouse TRITC anti-IgG (Cappel Biomedical, Malvern, PA, USA) for an additional 30 min on ice. Cells were resuspended in PBS with EDTA supplemented with 5% BSA.

Percentage of labelled cells was determined by counting a total of 1000 cells. The absolute numbers were calculated by the percentage of positive labeled cells and the total bone marrow count. A diameter of at least 100 cells at each subpopulation was measured using a calibrated micrometer scale.

RESULTS

Microscopic evaluation of spleen in resistant A/J or susceptible C57BL/6 mice

To determine the role of the immune system in genetic resistance or in viral nonpathogenicity, microscopic evaluation of spleen of pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice have been studied. A splenic atrophy characterized by disappearance of lymphoid follicles has been noted in L2-MHV3 infected A/J and C57BL/6 mice. The lesions observed were more severe in C57BL/6 mice than in A/J mice. The YAC-MHV3 variant also produced lesions in spleen of C57BL/6 mice, but were less severe.

Pre-B and B cell subpopulations in bone marrow from L2-MHV3 or YAC-MHV3 infected C57BL/6 or A/J mice

We have recently observed a decrease in the number of splenic B lymphocytes in susceptible C57BL/6 mice infected with the pathogenic L2-MHV3 virus (19). This decrease may reflect the virus-induced cell lysis or the inability of animal to compensate the loss of splenic lymphoid cells. Lymphopoiesis disorders during the maturation process of B cell subpopulations in the bone marrow may explain the absence of splenic B cells. To verify this hypothesis, we have evaluated the absolute number of the B (cu+su+) and pre-B (cu+su-) cell subpopulations in the bone marrow. Groups of three susceptible C57BL/6 or resistant A/J mice have been infected intraperitoneally (i.p.) with 10^4 $TCID_{50}$ of pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 viruses. Groups of uninfected mice received i.p. with similar

Table 1. Absolute number of mature B cells in bone marrow from pathogenic L2-MHV3 or non-pathogenic YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice.

Mice	Virus	Time postinfection (days)			
		0	1	3	7
A/J	L2-MHV3	2.2±0.2[a]	2.7±0.3	3.0±0.2	2.3±0.3
	YAC-MHV3	2.2±0.2	2.3±0.3	2.2±0.2	2.1±0.2
C57BL/6	L2-MHV3	2.1±0.2	3.0±0.3	0.3±0.5	n.a.[b]
	YAC-MHV3	2.1±0.2	2.4±0.3	2.3±0.2	2.2±0.2

a: x 10^6 cells
b: n.a. not applicable

volume of PBS. Absolute number of B cells rapidly decreased in the bone marrow of C57BL/6 mice infected with L2-MHV3 until the death of mice (Table 1). Slight increases have been noted at days 1 and 3 in L2-MHV3 resistant A/J mice but returned to normal values at day 7. No significant changes has been detected in both strain mice infected with YAC-MHV3 virus.

Similarly, absolute number of pre-B cells decreased in the bone marrow of C57BL/6 mice infected with L2-MHV3 only (Table 2). These results indicate that depletion of bone marrow pre-B and B cells is responsible for the maintenance of low number of splenic B cells. In addition, the B lineage cell disorder in bone marrow correlates with viral pathogenicity or genetically-determined sensitivity to MHV3 viral infection.

Diminished number of B cell subpopulations in the bone marrow during the viral infection is perhaps caused by a blockade of cell mitosis or a viral-induced cell lysis. To verify this hypothesis, size distribution of the

Table 2. Absolute number of pre-B (cu+ su-) cells in bone marrow from pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice.

Mice	Virus	Time postinfection (days)			
		0	1	3	7
A/J	L2-MHV3	0.8±0.2[a]	0.9±0.1	0.8±0.1	0.8±0.1
	YAC-MHV3	0.8±0.1	0.8±0.1	0.8±0.2	0.8±0.2
C57BL/6	L2-MHV3	0.8±0.1	0.9±0.1	0.5±0.2	n.a.[b]
	YAC-MHV3	0.8±0.2	0.8±0.2	0.8±0.1	0.8±0.1

a: x 10^6 cells
b: n.a. not applicable

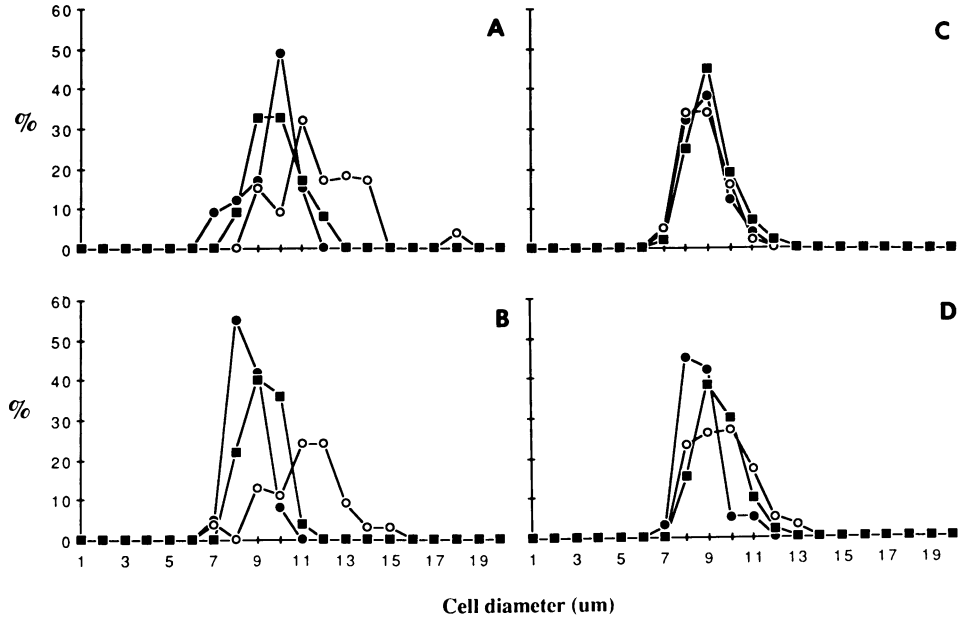

Fig. 1. Size distribution of bone marrow pre-B (cu+su-) (A,C) and B (cu+su+) (B,D) cell subpopulations from uninfected (●), pathogenic L2-MHV3 (O) or non-pathogenic YAC-MHV3 (■) infected susceptible C57BL/6 (A,B) or resistant A/J (C,D) mice. Groups of three susceptible C57BL/6 or resistant A/J mice have been infected with 10^4 $TCID_{50}$ of pathogenic L2-MHV3 or non-pathogenic YAC-MHV3. Groups of uninfected mice received i.p. a similar volume of PBS. Bone marrow cells were stained with a FITC anti -u chain for su labeling, cytocentrifugized, fixed in acetic acid/methanol solution, and labeled with a TRITC anti-u (cu). Diameter of at least 100 cells of each subpopulation was measured using a calibrated micrometer scale (standard deviation < 3%).

different B cell subpopulations were analyzed at 48 h.p.i. in bone marrow of L2-MHV3 or YAC-MHV3 infected C57BL/6 or A/J mice (Fig. 1). Size distribution of B cell subpopulations in the bone marrow from uninfected mice varied from 7 to 12 um. Normal-sized pre-B and B cells decreased in L2-MHV3 infected C57BL/6 mice, contrary to the A/J mice or YAC-MHV3 infected mice. In addition, an abnormally larger-sized cell subpopulation, with a cell diameter varying from 12 to 17 um, occurred in L2-MHV3 infected C57BL/6 mice.

In vitro infection of purified bone marrow mature B cells

To determine the permissivity role of B cells to MHV3 viral replication, upon cell depletion accompanied by cell enlargement, we performed in vitro L2-MHV3 or YAC-MHV3 viral infection (1 m.o.i.) in mature B cells (cu+su+) collected from uninfected C57BL/6 and A/J mice and purified by a panning method using anti-u chains antibodies. Cellularity, percentage of virus-producing cells and viral titer were recorded at 48 h.p.i. (Table 3).

The number of L2-MHV3 infected B cells from C57BL/6 mice greatly decreased whereas B cells from A/J mice were slightly affected. Majority of L2-MHV3 infected B cells from C57BL/6 mice were productively infected (>80%) and virus titer in the supernatant reached 4.1+0.3 $TCID_{50}$/ml. Few B cells from A/J mice were also successfully L2-MHV3 infected and lower

Table 3. Cellularity, percentage of virus producing cells, and viral titers in in vitro pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 infected purified bone marrow mature B (cu+su+) cells from resistant A/J or susceptible C57BL/6 mice.

Mouse strain	Virus	Cellularity (10^5)	Virus producing cells (%)	Viral titers ($TCID_{50}$/ml)
A/J	Uninfected	3.0±0.2	n.a.[a]	n.a.
	L2-MHV3	2.4±0.1	0.02±0.02	2.8+0.3
	YAC-MHV3	2.6±0.3	<0.005	>1.6
C57BL/6	Uninfected	4.0±0.2	n.a.	n.a.
	L2-MHV3	1.4±0.1	>80	4.1±0.3
	YAC-MHV3	3.8±0.2	<0.005	<1.6

a: n.a. not applicable

virus titers in supernatants detected. No cell depletion or viral replication were found in YAC-MHV3 infected cells. Similar results were obtained with pre-B cells (results not shown). These results demonstrate that lytic viral infection occurs in B lineage cell subpopulations and is responsible for cell depletions as observed in vivo in L2-MHV3 infected C57BL/6.

To determine if the viral replication generates the abnormal-sized cells, size distribution of in vitro infected mature B cells was analyzed. Majority (>95%) of L2-MHV3 infected B cells from C57BL/6 mice expressed abnormal increase in cell diameters (11 to 19 um) with a simultaneous decrease in the percentage of normal sized cells. Normal-sized distribution of B cells was observed in L2-MHV3 infected A/J or in YAC-MHV3 infected cells. These results demonstrate that L2-MHV3 viral replication induces in vitro the formation of abnormally large lymphocytes as detected in the bone marrow of in vivo infected C57BL/6 mice.

T cell subpopulations in thymus from pathogenic L2-MHV3 or non-pathogenic YAC-MHV3 infected susceptible C57BL/6 or resistant A/J mice

We have recently demonstrated that viral pathogenicity or susceptibility to MHV3 infection correlated with viral replication in vivo in thymus and in vitro in isolated thymocytes (20). To evaluate the immune disorders induced in thymus by the viral infection, the thymic T cell subpopulations have been analyzed in L2-MHV3 or YAC-MHV3 infected ($10^4 TCID_{50}$ i.p.) C57BL/6 or A/J mice at 48 hrs p.i.. As shown in Table 4, CD4+CD8+, CD4+CD8- and CD4-CD8+ thymic cell subpopulations decreased in pathogenic L2-MHV3 infected susceptible C57BL/6 mice whereas no significant depletions of these subpopulations were detected in resistant A/J mice or in both strain mice infected with YAC-MHV3. In addition, the ratio CD4/CD8, representing the functional level of the immune system, also decreased in L2-MHV3 infected C57BL/6 mice, indicating that the diseased mice not only express an immunodeficient state, they are also immunosuppressed (Table 5).

Table 4. Percentage of CD4+CD8+, CD4+CD8-, and CD4-CD8+ cells from the thymus of pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice.

Mouse strain	Virus	Thymic cell subpopulations (x 10^6)		
		CD4+CD8+	CD4+CD8-	CD4-CD8+
A/J	Uninfected	57±5	12±1	6.2±0.5
	L2-MHV3	62±5	12±2	7.0±0.5
	YAC-MHV3	60±3	12±1	7.0±0.6
C57BL/6	Uninfected	60±3	11±1	6.0±0.3
	L2-MHV3	45±4	6.0±0.3	4.1±0.2
	YAC-MHV3	66±5	11±0.4	6.1±0.3

These results indicate that T immunodeficiency occurs in acutely-infected mice and relates to depletion of all thymic lymphoid cell subpopulations. In addition, the T lineage cell disorder in thymus correlates with viral pathogenicity or genetically-determined sensitivity to MHV3 viral infection.

In vivo viral replication in thymocytes from L2-MHV3 or YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice

The loss of thymic T cell subpopulations in diseased mice may be related to a cytolytic MHV3 infection. To verify this hypothesis, viral replication was evaluated by the percentage of thymocytes expressing intracellular viral proteins, detectable by an indirect immunofluorescent assay, or of thymocytes producing infectious virions, in L2-MHV3 or YAC-MHV3 infected A/J or C57BL/6 mice at 48h. p.i.(Tableau 6). Interestingly, the majority of thymic cells from L2-MHV3 infected C57BL/6 mice expressed intracellular viral proteins, however, a nominal amount of cells produced infectious viruses. This interaction suggests that an abortive viral replication, blocked at the level of virion assembly, occured in T cells.

Table 5. CD4/CD8 ratio of thymic cells in pathogenic L2-MHV3 or nonpathogenic YAC-MHV3 infected resistant A/J or susceptible C57BL/6 mice.

Mouse strain	Virus	CD4/CD8 ratio
A/J	Uninfected	1.72±0.10
	L2-MHV3	1.60±0.09
	YAC-MHV3	1.63±0.17
C57BL/6	Uninfected	1.83±0.15
	L2-MHV3	1.08±0.12
	YAC-MHV3	1.63±0.20

Tableau 6. L2-MHV3 or YAC-MHV3 viral replication in thymocytes from *in vivo* infected A/J or C57BL/6 mice.

Mouse strain	Virus	Intracellular viral proteins expressing cells (%)	Virus producing cells (%)
A/J	L2-MHV3	< 1	< 0.002
	YAC-MHV3	< 1	< 0.002
C57BL/6	L2-MHV3	81+4	0.08+0.04
	YAC-MHV3	< 1	< 0.002

No viral replication was detected in thymic T cells from L2-MHV3 infected A/J mice or YAC-MHV3 infected mice.

DISCUSSION

We have demonstrated, in this work, that the B and T lymphocytes are the target cells *in vivo* and *in vitro* for the pathogenic L2-MHV3 viral replication. The specific virus-lymphocyte interactions correlate with viral pathogenicity. *In vivo* results reveal that bone marrow and thymus to be the other target organs for MHV3 virus infection. These results on bone marrow B lymphocytes extend the previous works of Piazza et al. (1) which demonstrated that MHV3 replication accompanied by decrease in number of lymphocytes, monocytes and polymorphonuclear cells occurred in acutely-infected mice. In addition, thymic atrophy, previously observed in MHV3 infected C57BL/6 mice (2,3), results in loss of the thymic cells in the first days of viral infection. On the other hand, the absence of detectable hematopoietic disorders in the bone marrow and cell depletions in the thymus of L2-MHV3 infected resistant A/J mice or in nonpathogenic YAC-MHV3 infected mice confirm the integral role played by the primary lymphoid organs in inducing a resistance to acute disease (2,18). Analysis of B lymphocyte subpopulations in the bone marrow revealed depletions of pre-B and B lymphocytes in L2-MHV3 infected susceptible C57BL/6 mice only. Abnormal forms of B lineage cells were also observed in the bone marrow of diseased mice. These large mononucleated cells may have resulted from an impairment of cell metabolism following viral infection and leading to cell lysis. In determining the number of splenic B lymphocytes in L2-MHV3 susceptible C57BL/6 mice, we found that the cell depletion (18) reconciles our previous findings on the correlation between impairment of B cells (12) and the loss of cellularity in the lymphoid organs (13). The mechanism against MHV3 viral infection is controlled under H-2 or non-H-2 related genes (20) and expressed as hemopoietic cells, mature splenic cell or thymic cells and macrophages (5,19).

The *in vitro* results of B lymphocytes thus correlate and explain the *in vivo* observations. L2-MHV3 infection of bone marrow purified B cells from C57BL/6 revealed that these cells are productively infected, expressed abnormal morphology and lead to subsequent cell lysis. The permissivity of T lymphocytes to MHV3 replication, however, remains unclear. No viral replication can be detected *in vitro* in purified T cells from thymocytes preparation, despite the high percentage of viral protein expressing cells in thymus from diseased mice. Preliminary result on *in vitro* viral replication in enriched thymic epithelial cell preparation indicates that viral infection primarly occurs in thymic epithelial cells, also present in the thymocyte preparation. The infection is then transmitted to T lymphocytes

by a yet unknown mechanism. Thus the lack of lymphoid repopulation in the spleen following an MHV3 infection explains the depletion of mature B and T cells into bone marrow and thymus.

As the result of a viral permissivity of B and T lymphocytes, two immunological consequences occur. First, B and T lymphocytes partially act as a natural barriers to MHV3 replication in producing no or low level of infectious virions in extracellular media, and second, in inducing B and T lymphocyte cell lysis and subsequent cellular and humoral immunodeficiencies. As previously demonstrated, MHV3 viral replication is restricted in splenic or thymic lymphoid cells in resistant A/J mice and delayed in susceptible C57BL/6 mice (19). Such immunodeficiencies is perhaps responsible for the inefficient viral elimination process. Virus titers continue to increase until death in C57BL/6 mice whereas they decrease in A/J mice despite similar virus titers observed in various organs in susceptible C57BL/6 or resistant A/J mice infected with L2-MHV3 or YAC-MHV3 for the first 72 h.p.i. (18). In addition, virus-induced cell lysis of lymphocytes was ineffective in the viral elimination process. This situation has been previously observed in (C57BL/6 x A/J)F1 mice surviving an acute disease (21).

Further work will be performed to determine the mechanism involved in T cell depletion during the acute disease and the occurrence of immunodeficiency in chronically-infected mice.

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