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Pathogenicity of neutralization escape mutants of mouse hepatitis virus: correlation with T- and B-cell depletions

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SUMMARY

Viral pathogenicity is a result of an imbalance between viral replication and the host's immune defences. When the virus is lymphotropic, understanding the pathogenic process of the viral disease becomes complicated because virus/lymphocyte interactions can alter the cell's integrity and subsequently induce immunodeficiency. The immune system plays an important role in the outcome of acute disease induced by the mouse hepatitis virus type 3 (MHV3). The use of attenuated escape mutants provides a tool to study the role of viral properties involved in its pathogenicity. We selected MHV3 mutants by virtue of their resistance to neutralization by monoclonal antibodies (mAb), in order to study their pathogenic properties. We reported that two MHV3 escape mutants were attenuated in their pathogenic properties according to inoculation site and with regard to survival time and ability to deplete T- and B-cell subpopulations in the spleen, thymus and bone marrow of susceptible Balb/c mice. The highly attenuated CL12 mutant could not induce depletion in T or B cells following intraperitoneal (i.p.) or intranasal (i.n.) inoculations, at three days postinfection. The less attenuated 51.6 mutant, however, maintained the ability to deplete T and B cells following i.p. inoculation, as described with the pathogenic MHV3. In contrast, no depletion of T cells following i.n. inoculation was induced with this mutant, although B lineage cells decreased. The use of such mutants enabled us to examine the role of each compartment of the immune system, since the highly attenuated CL12 mutant induced no immunodeficiency, as defined by immune cell depletion, whereas the less attenuated 51.6 mutant maintained its ability to decrease only the B-cell compartment after i.n. inoculation. Results are discussed with regard to the virus/lymphocyte interactions during the pathogenic process.

Key-words: MHV3, T lymphocyte, B lymphocyte Neutralization; Pathogenicity, Escape mutants, Inoculation route, Lymphocytic populations.

INTRODUCTION

Viral pathogenicity results from an imbalance between viral replication and the host's immune defences (Sissons and Borysiewicz, 1985). Cellular resistance of mice to infection caused by a particular virus may indicate the absence of a specific genetically controlled viral permissivity factor in target cells (Brinton and Nathanson, 1981) or host factors, including the integrity of

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cellular and humoral immune defences. Helper and cytotoxic T lymphocytes are involved in the viral elimination process and are dependent upon the thymus for normal T lymphopoiesis (Borysiewicz and Sissons, 1986; Raff and Owen, 1971). Specific antiviral antibodies play an important role in viral elimination, promoting antibody-mediated dependent cell cytotoxicity, complement-mediated cell lysis and neutralization of infectious viral particles (Sissons and Oldstone, 1980; Sissons *et al.*, 1982). Efficient humoral immune response to viral infection depends on normal B lymphopoiesis, which takes place in the bone marrow (Osmond and Nossal, 1974). When the virus is lymphotropic, understanding the pathogenic process of the viral disease is more complicated, as virus/lymphocyte interactions may alter cell integrity and induce subsequent immunodeficiency.

Mouse hepatitis virus type 3 (MHV3), a hepatotropic strain, is a coronavirus. Its inoculation into mice is usually followed by a generalized infection characterized by acute hepatic necrosis, killing the animal within a couple of days (Piazza *et al.*, 1965). The susceptibility of inbred mice to MHV3 hepatic disease is dependent on genetically controlled host factors (Le Prevost *et al.*, 1975a; Levy-Leblond *et al.*, 1979; Levy *et al.*, 1982). Resistant A/J mice undergo a subclinical infection, whereas other strains, such as BALB/c or C57BL/6 are fully susceptible to fulminant hepatitis. Resistant A/J, however, become fully susceptible to acute disease following immunosuppressive treatments (Dupuy *et al.*, 1975; Leray *et al.*, 1982) or intracerebral inoculation (Lamontagne *et al.*, 1989a). (C57BL/6 × A/J)F_1 or C3H mice, however, can survive the acute phase of the disease but develop humoral and cellular immunodeficiency, leading to paralysis and death (Le Prevost *et al.*, 1975b; Leray *et al.*, 1982). No difference in virus titres was observed in the target organs of the resistant and susceptible mouse strains (Lamontagne *et al.*, 1989a) despite different histological lesions and *in vitro* replication. Resistance to MHV3 infection is under the control of *H2*- or non-*H2*-related genes and expressed as haemopoietic cells and mature splenic or thymic cells (Levy-Leblond *et al.*, 1979; Dupuy *et al.*, 1984). Histopathological studies have revealed that lymphoid organs such as the thymus and spleen showed injuries or atrophy in susceptible C57BL/6 mice infected with pathogenic L2-MHV3 strain (Lamontagne *et al.*, 1989a)). Splenic T or B cells were depleted in L2-MHV3-infected susceptible mice (Lamontagne *et al.*, 1985, 1989a).

In addition, all thymocyte subpopulations decreased in thymus from infected susceptible mice (Lamontagne and Jolicœur, 1991). Bone marrow pre-B and mature B cells also decreased in bone marrow, but not the terminal deoxytransferase$^+$ (Tdt$^+$) or pro-B cells (Jolicœur and Lamontagne, 1989).

The susceptibility of BALB/c mice is associated with impaired early activation of the immune system, demonstrated by the failure of lymphocyte proliferation resulting from the decrease in IL2 production (Dindzans *et al.*, 1987).

Genetically determined sensitivity to MHV3 infection is correlated with viral replication in the macrophages (Lamontagne *et al.*, 1989b; McNaughton and Patterson, 1980), pre-B and B-lymphocytes (Jolicœur and Lamontagne, 1989), and in the stromal thymic cells and their complexed thymocytes (Lamontagne and Jolicœur,

BSA = bovine serum albumin.
cμ$^+$ = cytoplasmic μ chain.
ELISA = enzyme-linked immunosorbent assay.
FCS = fœtal calf serum.
FITC = fluorescein isothiocyanate (conjugated).
i.c. = intracerebral(ly).
i.n. = intranasal(ly).
i.p. = intraperitoneal(ly).
mAb = monoclonal antibody.
MHV3 = mouse hepatitis virus type 3.
PBS = phosphate-buffered saline.
PE = peritoneal exudate.
PFU = plaque-forming unit.
p.i. = postinfection.
sμ$^+$ = surface μ chain.
TRITC = tetramethyl rhodamine isothiocyanate (conjugated).

1991). We reported elsewhere that T-cell depletion results from nonproductive but lytic viral replication in the lymphoid cells in contact with infected thymic stromal cells, whereas pre-B and B cells support productive and lytic viral replication (Jolicœur and Lamontagne, 1989; Lamontagne and Jolicœur, 1991).

A useful tool in the study of the role of viral properties in pathogenicity is the attenuated escape mutants. Several viral genes, including those coding for the nucleocapsid (Fleming *et al.*, 1983), the peplomeric protein (S) (Makino *et al.*, 1987; Talbot *et al.*, 1984) or the polymerase (Lai *et al.*, 1981), have been implicated as determinants of MHV virulence. The S protein is central to cellular tropism and MHV virulence. It is responsible for attaching the virus to the cell receptor, so that a point mutation in the S gene could modify viral pathogenicity and its target cells. We selected MHV3 mutants by virtue of their resistance to neutralization by anti-S monoclonal antibodies (mAb), in order to study their pathogenic properties. We show that the pathogenic properties of two MHV3 escape mutants were attenuated with regard to survival time (depending on inoculation site) and ability to deplete T- and B-cell subpopulations in the spleen, thymus and bone marrow of susceptible BALb/c mice.

MATERIAL AND METHODS

Mice

BALB/c mice were purchased from Centre d'élevage des animaux de laboratoires (CNRS, Orléans, France) or Jackson Laboratories (Bar Harbor, ME). The animals were tested either upon arrival or before use for the presence of anti-MHV antibodies by ELISA using an MHV3 preparation as antigen. During experiments, the animals were housed in a sterile atmosphere (Forma Scientific, Marietta, OH).

Viruses

Pathogenic MHV3 is a subcloned virus passaged into L2 cells. Mutants MHV3-51.6 and MHV3-CL12 were selected from pathogenic MHV3 virus in the presence of S protein-specific A51 and A37 mAb, respectively. The mAb were chosen to select escape mutants retaining partial pathogenic property. These mAb were able to neutralize MHV3 and to slightly inhibit cellular fusion, but did not protect the sensitive mice against MHV3 infection (Martin *et al.*, 1990). All viruses were cultured in L2 cells.

In vivo pathogenicity

The mortality and virus titres in organs were determined after 100 plaque-forming unit (PFU) intraperitoneal (i.p.) or intranasal (i.n.) injections. Immunological analyses were performed on mice receiving 1000 PFU. Groups of 10 mice were used for mortality study and for measurement of viral titres in target organs; groups of three mice were used for histopathology and immunological studies.

Viral titration

The infected brain, liver, kidney, and lungs were collected and minced in a culture medium. The fragments were frozen at –70°C, then thawed-frozen three times and centrifuged, and the supernatants were then used as viral suspensions. Virus titration was performed by the plaque-forming method onto L2 cells. Less than 10 PFU/g was considered as a negative result.

Cells

L2 cells, a continuous mouse fibroblast cell line (obtained from Dr. R. Anderson, University of Calgary, Manitoba, Canada), were grown in Eagle's MEM with glutamine (5 mM), 5% FCS (fœtal calf serum), and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml) (GIBCO Laboratories, Grand Island, NY). L2 cells were used for propagating, cloning, and viral titrating. Peritoneal exudate (PE) cells were obtained after washing the peritoneal cavity with 8 ml of RPMI-1640 containing 1% heparin (Allen & Hanbury, Toronto, Ontario, Canada). PE cells were seeded in microplates (5×10^5 cells/well) and cultured at 37°C under 5% CO_2 for 24 h before infection. Spleens were aseptically removed and then minced in RPMI-1640 supplemented with 10% FCS, glutamine and antibiotics. Thymuses were aseptically collected and teased apart using needles in RPMI-1640 medium and 20% FCS as previously described (Lamontagne *et al.*, 1989a).

For *in vitro* studies, spleen or thymic lymphocytes were purified by depositing cell suspensions on Lymphoprep gradients (Cedarlane, Hornby, Ontario, Canada) and centrifuged, and the cell frac-

tions were then collected, washed, and electronically counted (Coulter counter, Coulter Electronics, Hialeah, FL). Bone marrow cells were obtained by flushing the femoral shafts four times with 1 ml cold RPMI-1640 medium. Large particles were removed by sedimentation on a cushion of 1 ml of FCS for 5 min. The cell suspension was then centrifuged, resuspended in 1 ml of RPMI medium, and electronically counted (Coulter Counter, model ZM, Coulter Electronics, Hialeah, FL).

Immunofluorescent labelling

Splenic T cells ($CD4^+CD8^-$, $CD4^+CD8^+$) were labelled with the following mAb: fluorescein isothiocyanate (FITC) -conjugated anti-CD4 (mAb RM 4-5, Cedarlane, Hornby, Ontario, Canada) and phycoerythrin-conjugated anti-CD8a (mAb 53-6.7, Cedarlane). A PE-conjugated polyclonal goat anti-mouse μ chain (Cedarlane) was used for labelling of splenic B cells. Single-cell suspensions were prepared from pooled organ homogenates from each group of three mice, and 1×10^6 cells were incubated with an optimal dilution of mAb for 30 min at 4°C, washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), and fixed in PBS pH 7.2 containing 1% formaldehyde, then stored at 4°C (Starkey *et al.*, 1988). For flow cytometry analysis, using a "FACSCAN" flow cytometer (Becton-Dickinson, Mountain View, CA), cells were gated according to forward and side angle light scatter. Gates were adjusted to include the discrete mononuclear population. Five thousand cells were analysed per sample. Thymocytes were similary labelled, and $CD4^+CD8^+$, $CD4^+CD8^-$ or $CD4^-CD8^+$ subpopulations were recorded.

Double labelling of 14.8 and μ chains

Labelling of bone marrow $14.8^+\mu^-$ (pro-B) or $14.8^+\mu^+$ cells was performed, as described by Park and Osmond (1987). Briefly, bone marrow cells were incubated with rat mAb 14.8 at 4°C for 30 min, washed, incubated for 30 min with FITC anti-rat IgG (Cappel Biomedical, Malvern, PA) in humidified atmosphere, and washed by centrifugation through FCS gradients. Cells were then cytocentrifuged and methanol-acetic acid fixed, and μ chains were labelled using tetramethyl rhodamine isothiocyanate (TRITC) -conjugated anti-μ chains (Cappel Biochemical) for 30 min. The cell spots were washed and kept overnight in PBS.

Double labelling of μ chains

Cytoplasmic μ $(c\mu)^+$, surface μ $(s\mu)^-$ (pre-B) and $c\mu^+s\mu^+$ (B) cells were similarly labelled (Park and Osmond, 1987). Bone marrow samples (4×10^6 cells) were incubated for 30 min on ice with an optimal dilution of FITC anti-μ chains (Cappel Biochemical) for sυ labelling. The cells were washed twice by centrifugation through FCS at 200 *g* at 4°C for 7 min. Cells were resuspended in 1 ml of PBS supplemented with 5% BSA. Bone marrow was cytocentrifuged and fixed as above. Cytoplasmic μ chains were labelled with an optimal dilution of TRITC anti-μ directly on the cell spots, then incubated for 30 min at room temperature in a humidified chamber, then washed four times and kept overnight in PBS.

Slide mounting

Slides were mounted on a medium containing 90% glycerol (Fisher Scientific Co.) in PBS pH 8.0, and 0.1% p-phenylenediamine (Fisher Scientific Co.). A fluorescence microscope equipped with a mercury lamp and phase contrast optics was used. Slides were examined for $14.8^+\mu^-$, $14.8^+\mu^+$, $c\mu^+s\mu^-$, and $c\mu^+s\mu^+$ B-lineage cell populations. The diameter of at least 100 cells at each subpopulation was measured using calibrated micrometer scale. The percentage of labelled cells was determined by counting a total of 1000 cells. The absolute numbers were calculated by the percentage of positive labelled cells and the total bone marrow count.

Statistical analysis

Statistical analysis was performed using Student's *t* test.

RESULTS

Pathogenicity of MHV3 escape mutants in BALB/c mice

We studied the pathogenicity of MHV3 escape mutants by i.p. or i.n. inoculation of BALB/c mice with 100 PFU of pathogenic L2-MHV3 or mutant viruses. As shown in figure 1, mortality occurred in all groups of mice i.p. (A) infected with L2-MHV3 or MHV3-51.6 or MHV3-CL12 mutant viruses. The time of death, however, was delayed in MHV3-51.6 or MHV3-CL12-infected mice. The MHV3-CL12 mutant was nonpathogenic by the i.n. inocula-

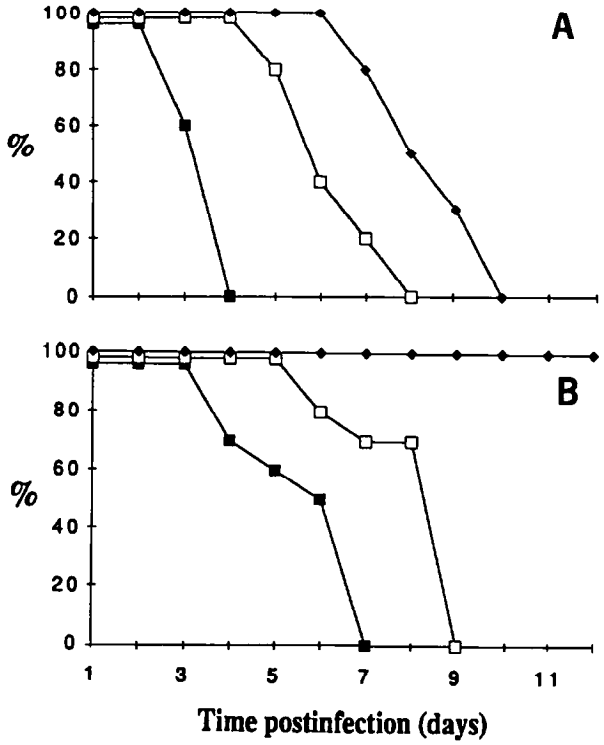

Fig. 1. Percentage of surviving BALB/c mice infected with pathogenic or mutant viruses.

L2-MHV3 (■), MHV3-51.6 mutant (□) and MHV3-CL12 mutant (◆) viruses were injected intraperitoneally (A) or intranasally (B). Groups of 10 mice were infected with 100 PFU of virus.

tion route (B). The MHV3-51.6 mutant virus expressed a lower virulence both i.p. and i.n. than did L2-MHV3. These results indicate that the MHV3-CL12 mutant expresses a more greatly modified pathogenic property than the MHV3-51.6 mutant, particularly following i.n. inoculation.

Viral replication of MHV3 or its attenuated mutants in i.p. or i.n. infected BALB/c mice

To study the mechanism involved in the pathogenic process of attenuated mutants, the levels of viral replication in various organs were evaluated. Groups of BALB/c mice were i.p. or i.n. infected with pathogenic L2-MHV3 or attenuated mutants, and virus titres in the liver, kidneys, brain and lungs were recorded at various times postinfection (p.i.) (fig. 2). Infectious viral particles were found in all organs of pathogenic L2-MHV3-infected mice as early as two or three days p.i. No significant difference in virus titres, however, was observed between i.n. or i.p. infected mice, except in the lungs (fig. 2C) ($p < 0.001$), where a higher virus titre was produced following i.n. inoculation. In contrast, viral replication was delayed in all organs from mice i.p. infected with the MHV3-51.6 mutant (six days). There was a longer delay of six to twelve days in viral replication in all organs of MHV3-CL12 i.n. infected mice, although virus titres appeared sooner in the lungs and brain (fig. 2B and C). Gross examination showed thymus atrophy in pathogenic L2-MHV3-infected mice but not in MHV3-CL12- and to a lesser extent, in MHV3-51.6-infected mice. These results suggest a correlation between viral pathogenicity, delay in viral replication, and thymic atrophy.

Splenic lymphoid T and B cells from i.p. or i.n. infected BALB/c mice with MHV3 or its attenuated mutants

We have previously demonstrated a correlation between the pathogenicity of MHV3 infection and depletion of splenic T and B cells in susceptible C57BL/6 mice (Lamontagne *et al.*, 1989a). mAb escape mutants may be attenuated because they have lost the ability to induce lymphoid depletion in the spleen, favouring the development of an inflammatory response in infected organs. To verify this hypothesis, the percentages and absolute numbers of splenic $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets were recorded in groups of mice i.p. or i.n. infected with either pathogenic L2-MHV3 or the attenuated mutants. Depletion of splenic $CD4^+CD8^-$ cells was higher in i.p. or i.n. L2-MHV3-infected mice (fig. 3) ($p < 0.001$), whereas there was no decrease, in absolute numbers, in those of mice i.n. infected with attenuated mutants. Similarly, the splenic $CD4^-CD8^+$ subset was depleted in i.p. or i.n. L2-MHV3-infected mice ($p < 0.01$) but remained at normal levels in mice i.n. infec-

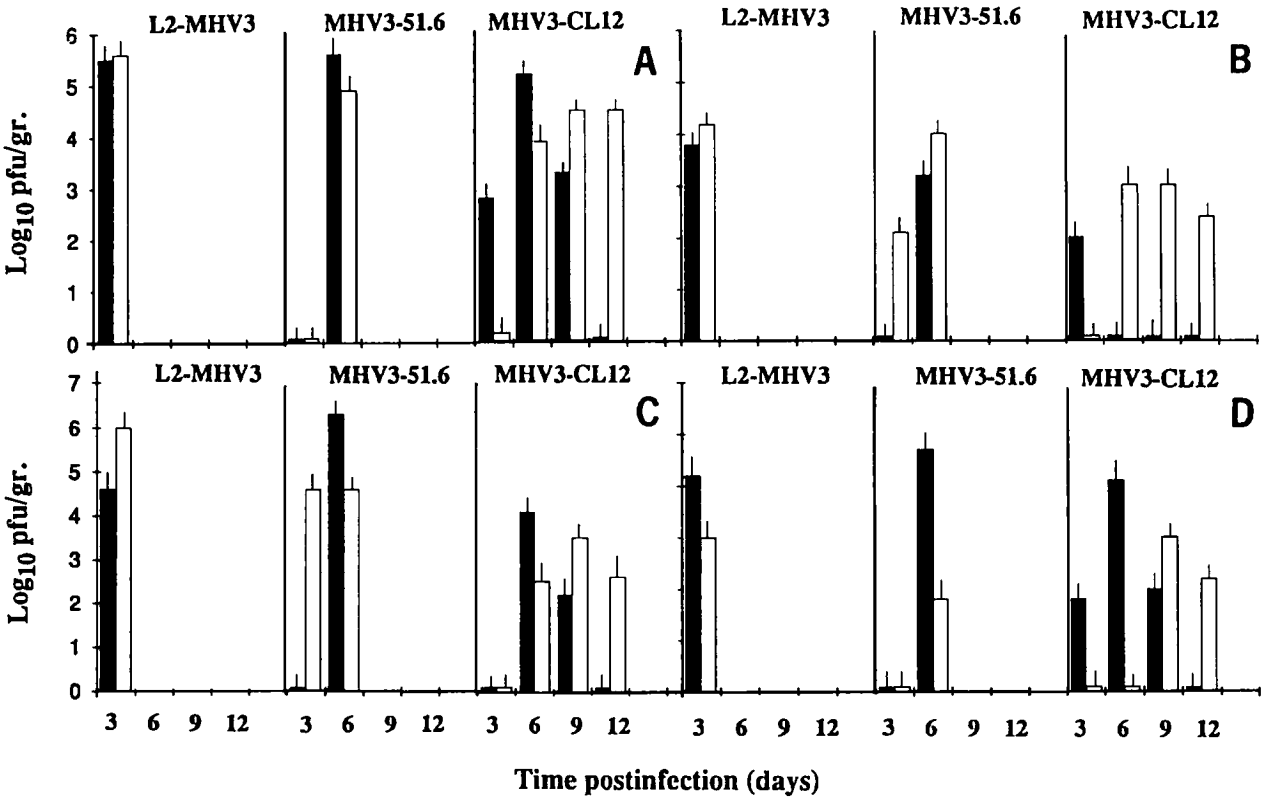

Fig. 2. Virus titres in liver (A), brain (B), lungs (C) and kidneys (D) in infected BALB/c mice with pathogenic L2-MHV3 and with MHV3-51.6 or MHV3-CL12 mutant viruses at various p.i. times following i.p. (■), or i.n. (□) inoculations.

Groups of 10 mice were infected with 100 PFU of virus.

ted with attenuated mutants, at three or six days p.i. The level of splenic B lymphocytes drastically decreased, in absolute numbers, in MHV3-51.6 or L2-MHV3 i.p. infected mice, but slightly increased in i.n. infected mice three days p.i. These cells were not affected during i.p. or i.n. infection with the attenuated MHV3-CL12 mutant (table I). At six days after i.n. inoculation, however, B cells strongly decreased in MHV3-CL12 or MHV3-51.6-infected mice. Thus, the low pathogenicity of attenuated mutants mays be correlated with their inability to induce substantial depletion in splenic T or B lymphocytes, according to the inoculation site. Splenic T and B cells were affected during MHV3-51.6 mutant infection, whereas only the T-cell subpopulations were slightly affected in MHV3-CL12-infected mice.

Thymocyte subpopulations of i.p. or i.n. infected BALB/c mice with MHV3 or its attenuated mutants

Depletion of splenic T cells may reflect the inability of the thymus to produce mature T-cell subpopulations during a viral infection. To verify this hypothesis, we analysed the thymocyte subsets in BALB/c mice i.p. or i.n. infected with 100PFU of L2-MHV3 or the attenuated mutants (fig. 4). The virus dose used did not significantly affect the survival rate of mice or the virus titres in various organs, despite the fact that mice generally died a few hours sooner. The thymuses were strongly depleted after i.p. infection with L2-MHV3 or MHV3-51.6, but not during MHV3-CL12 infection. No major differences in the percentages of the thymocyte subpopulations were

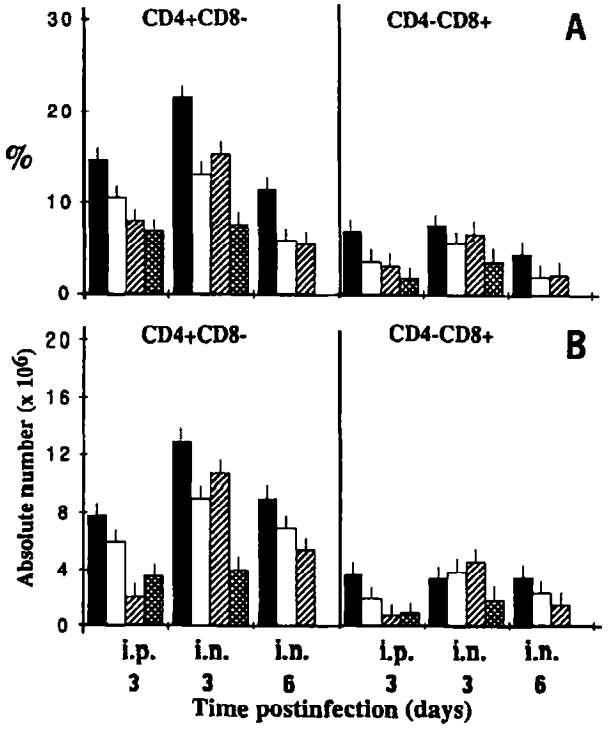

Fig. 3. Percentage (A) and absolute number (B), at 3 or 6 days p.i., of $CD4^+CD8^-$ or $CD4^-CD8^+$ cells in spleen of mock-infected (■) or pathogenic L2-MHV3 (cross-hatched), MHV3-51.6 mutant (□) or MHV3-CL12 mutant (hatched) virus-infected BALB/c mice following i.p. or i.n. inoculations.

Groups of three mice were infected with 1000 PFU of virus. Mock-infected mice received a similar volume of PBS.

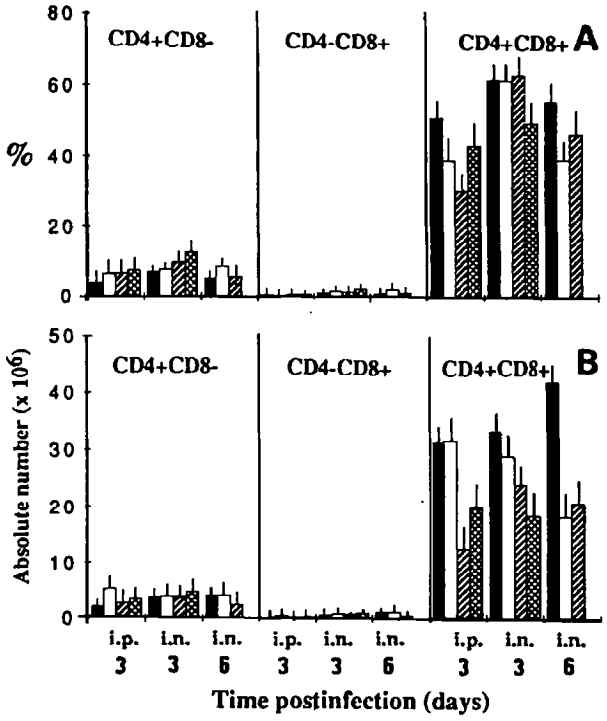

Fig. 4. Percentage (A) and absolute number (B), at 3 or 6 days p.i., of $CD4^+CD8^-$, $CD4^-CD8^+$ or $CD4^+CD8^+$ cells in thymus of mock-infected (■) or pathogenic L2-MHV3 (cross-hatched), MHV3-51.6 mutant (□) or MHV3-CL12 mutant (hatched) virus-infected BALB/c mice following i.p. or i.n. inoculations.

Groups of three mice were infected with 1000 PFU of virus. Mock-infected mice received a similar volume of PBS.

observed in mice infected with the viruses (fig. 4A). The levels of $CD4^+CD8^+$ cells, however, as measured by the absolute number, were markedly decreased during L2-MHV3 or MHV3-51.6 infections ($p < 0.001$) but not in mice infected with the MHV3-CL12 mutant, at three days

Table I. Percentages and absolute numbers of splenic B lymphocytes in BALB/c mice i.p. infected with pathogenic L2-MHV3 or MHV3-51.6 or MHV3-CL12 attenuated mutants, at 3 and 6 days p.i.

Time p.i. (days)	Inoculation site[a]	Uninfected[b]	Viruses[b] MHVB3-CL12	MHV3-51.6	L2-MHV3
3	i.p.	10.9 ± 0.1	10.0 ± 0.1	7.9 ± 0.1(*)	13.2 ± 0.2(*)
		(7.17 ± 0.07)	(7.43 ± 0.08)	(2.75 ± 0.03)(*)	(2.95 ± 0.03)(*)
3	i.n.	10.9 ± 0.1	12.6 ± 0.2	13.5 ± 0.2(*)	16.3 ± 0.3(*)
		(7.17 ± 0.07)	(7.91 ± 0.08)	(10.61 ± 0.11)(*)	(14.9 ± 0.15)(*)
6	i.n.	11.3 ± 0.1	4.6 ± 0.1(*)	2.9 ± 0.2(*)	NA
		(5.80 ± 0.06)	(3.58 ± 0.05)(*)	(1.41 ± 0.09)(*)	NA

[a] Groups of three mice were i.p. or i.n. infected with 1000 PFU of virus.
[b] Percentage (absolute number × 10^5).
NA = not applicable.
(*) $p < 0.001$.

p.i. (fig. 4B). The level of $CD4^+CD8^+$ cell depletion was lower in mice infected with the attenuated mutants, but the depletion was higher in MHV3-51.6 than in MHV3-CL12-infected mice. In contrast, this thymocyte subset was strongly depleted after six days in mice i.n. infected with the attenuated mutants ($p < 0.001$). No significant decrease in the $CD4^+CD8^-$ or $CD4^-CD8^+$ subpopulations was observed. Thymic depletion thus results principally from the decrease in immature $CD4^+CD8^+$ cells in infected mice.

Bone marrow B-cell subpopulations of BALB/c mice i.p. or i.n. infected with MHV3 or its attenuated mutants

The splenic-B-cell decrease observed in L2-MHV3 or MHV3-51.6 mutant-infected BALB/c mice may reflect the inability of the bone marrow to produce mature B cells during the viral infection, as previously observed in infected C57BL/6 mice (Jolicœur and Lamontagne, 1989). To verify this hypothesis, we analysed the percentage and absolute numbers of pro-B ($14.8^+\mu^-$), pre-B ($c\mu^+s\mu^-$) and mature B ($c\mu^+s\mu^+$) in the bone marrow of BALB/c mice i.p. or i.n. infected with L2-MHV3 or MHV3-51.6 or MHV3-CL12 mutant viruses (fig. 5). Percentages of pro-B cells increased in all infected mice, whereas absolute numbers were not affected in L2-MHV3- or 51.6-infected mice. Pro-B cells increased, however, in i.p. or i.n. CL12-infected mice. Similarly, the percentage of pre-B cells was not strongly affected in all groups of infected mice (fig. 5A). In addition, the percentage of B cells remained at normal levels in i.p. or i.n. infected mice. The percentage of B lineage cells corresponds to the ratio of these cells to other lineage cells in the bone marrow, and reflects not only the variations in B lineage cells, but also in the other haemopoietic compartments. The absolute number, thus, enables us to analyse the B lineage cells independently of modifications occurring in the other bone marrow cell compartments. The absolute number of pre-B cells decreased in mice i.p. or i.n. infected with L2-MHV3 and MHV3-51.6 viruses at three days p.i. ($P < 0.001$) but not in MHV3-CL12-infected (fig. 5B). B cells were depleted only in mice i.p.

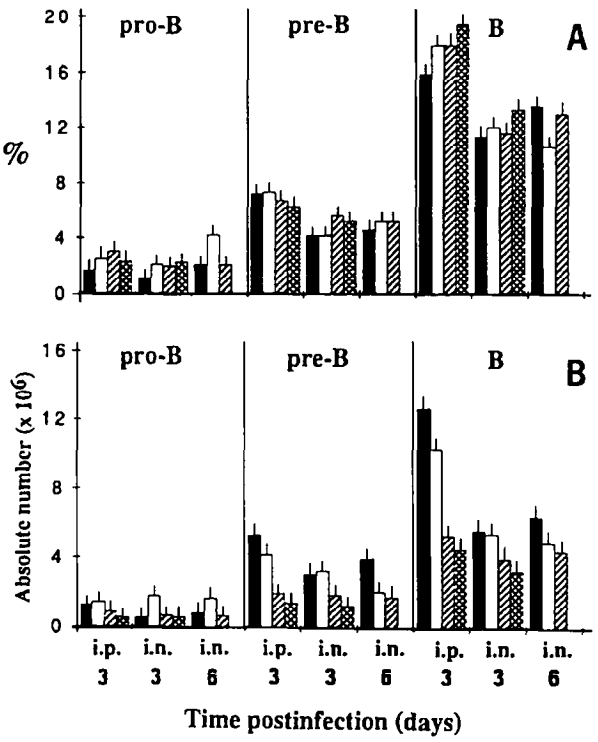

Fig. 5. Percentage (A) and absolute number (B), at 3 or 6 days p.i., of pro-B, pre-B or B cells in bone marrow from mock-infected (■) or pathogenic L2-MHV3 (cross-hatched), MHV3-51.6 mutant (□) or MHV3-CL12 mutant (hatched) virus-infected BALB/c mice following i.p. or i.n. inoculations.

Groups of three mice were infected with 1000 PFU of virus. Mock-infected mice received a similar volume of PBS.

infected with L2-MHV3 or MHV3-51.6 viruses ($p < 0.001$). Such a depletion was less evident in mice i.n. infected.

These results suggest that pre-B and B cells were strongly depleted during L2-MHV3 or 51.6 viral infections, in contrast to those seen with the MHV3-CL12 virus. In addition, other bone marrow cells were similarly depleted (results not shown).

DISCUSSION

This work demonstrates a relationship between the pathogenic properties of attenuated MHV3 mutants and the depletion of T- or B-lineage cells

in the spleen, thymus and bone marrow. Viral pathogenicity reflects the ability of a virus to induce clinical disease or death in mice. To study the mechanisms of viral pathogenicity, two approaches may be undertaken: analysing the replication of a virulent virus in various target cells from resistant or susceptible mouse strains or studying the alterations of the biological properties of attenuated viral mutants in a susceptible mouse strain. We have previously worked on the first approach using pathogenic L2-MHV3 in resistant A/J or susceptible C57Bl/6 mice and have demonstrated a correlation between viral pathogenicity and the depletion of cells in lymphoid organs, resulting from either productive or abortive viral replication (Jolicœur and Lamontagne, 1989; Lamontagne *et al.*, 1989a; Lamontagne and Jolicœur, 1991). The use of neutralization escape mutants highlights the role of T- or B-cell depletions in the pathogenic process during acute hepatitis. Pathogenic L2-MHV3 induces the depletion of splenic, thymic and bone marrow T- or B-lineage cells following both i.p. or i.n. inoculations. Similar decreases in the B-lineage cells were seen in mice infected with the less attenuated MHV3-51.6 mutant, whereas splenic T and thymocyte subpopulations were less affected. The highly attenuated MHV3-CL12 mutant did not induce B- or T-lineage cell depletion, at least before six days p.i. The liver is the main target organ of an MHV3 infection and the privileged site for its replication (Le Prevost *et al.*, 1975a). High titres of pathogenic L2-MHV3 occurred rapidly in the liver after i.p. inoculation, whereas MHV3-51.6 or MHV3-CL12 attenuated mutants manifested a delay in the occurrence of viral titres in i.p. or i.n. infected mice. The histopathological examinations revealed some dramatic effects in i.p. pathogenic L2-MHV3-infected mice, particularly in the liver, where main hepatocellular necrosis in the absence of inflammatory cells was evidenced. Lesions were less extensive in i.n. infected mice, with fewer inflammatory cells present. Moderate liver necrosis foci with inflammatory cells, however, were seen in both MHV3-51.6 and MHV3-CL12-infected mice after i.p. or i.n. inoculations (results not shown). The delay in the production of virus titres is thus associated with less extensive histopathological lesions in the liver and with low or no transaminase activity (measuring the level of liver necrosis) found in the serum of such mice, unlike those observed in L2-MHV3-infected mice (results not shown). In addition, viral replication was more delayed in MHV3-CL12 mutant after i.n. inoculation. Thus, viral pathogenicity correlates more with the time of appearance of virus titres in target organs rather than with the level of viral titres in the liver, brain, lungs or kidneys. Such a correlation has already been observed in resistant A/J or susceptible C57BL/6 mice in spite of scarce lesions in the liver of resistant mice (Lamontagne and Jolicœur, 1991). The delay in viral replication may be explained by the inability of the attenuated mutants to replicate in primary target cells or perhaps an inefficient spreading of infection to other organs.

PE cells are the first target cells of an MHV3 infection by i.p. inoculation. Preliminary works show that the MHV3-CL12 mutant replicates very poorly in PE cells in contrast with MHV3-51.6 or L2-MHV3 viral replication, suggesting that macrophages can normally play their antiviral roles following i.p. infection with an MHV3-CL12 mutant. The change of role of macrophages and lymphoid cells from a barrier to infection to being the host cell targets for viral replication is further strengthened by the fact that genetically determined resistance of A/J mice to i.p. MHV3 infection can be abrogated after intracerebral (i.c.) inoculation (Lamontagne *et al.*, 1989a). Interestingly, more than 60% of MHV3-CL12-infected mice survived up to 10 days p.i. following i.c. inoculation (results not shown) in contrast to those infected with MHV3-51.6 or pathogenic L2-MHV3, suggesting that the MHV3-CL12 mutant can be highly attenuated and thus unable to replicate in some unidentified brain target cells. Following i.n. inoculation, MHV strains initially replicate in the nasal epithelium rather than in the macrophages, and then appear in the olfactory pathways within the brain (Barthold and Smith, 1983; Goto *et al.*, 1977). This way, the virus can persist for more than 20 days in the posterior brain, whereas it is eliminated within 10 days from the nose (Barthold, 1988). No information is available on the viral persistency mechanism in the brain following i.n. inoculation.

Inflammatory foci were observed in the liver of attenuated virus-infected mice in contrast to pathogenic L2-MHV3-infected mice, suggesting that the virus mutants cannot replicate in the liver cells or could have lost their ability to thwart the natural or immune cell responses. We have previously demonstrated that the 51.6 mutant does not replicate in endothelial cells, and the CL12 mutant, in Kuppfer cells and hepatocytes (Martin *et al.*, 1990).

On the other hand, there is a correlation between L2-MHV3 pathogenicity and atrophy of the spleen or thymus, indicating that immune cells may instead play an important role in the pathogenic process of an acute disease (Lamontagne *et al.*, 1989a). Furthermore, the involution of the thymus or spleen has been reported in mice after i.c. infection with lethal doses of the neurotropic MHV-JHM strain, but not following i.p. inoculation (Knobler *et al.*, 1988), suggesting that the primary target cell, depending on the inoculation site, plays an important role in the pathogenicity of replicating virus or in the spreading of infection.

The level of attenuation of MHV3-51.6 or -CL12 mutants correlates with their ability to deplete splenic T or B cells. The highly attenuated MHV3-CL12 mutant cannot induce rapid depletion of T or B cells following i.p. or i.n. inoculations. The less attenuated MHV3-51.6 mutant, however, maintained its ability to deplete T and B cells following i.p. inoculation, as described with the L2-MHV3, except that it cannot deplete T cells following i.n. inoculation. The use of these mutants enables us to examine the role of each compartment of the immune system, considering that the highly attenuated MHV3-CL12 mutant does not induce immunodeficiency, as defined by immune cell depletions, whereas the less attenuated MHV3-51.6 mutant maintains its ability to induce a decrease only in the B-cell compartment, after i.n. inoculation.

The mechanisms involved in the differences between the pathogenic properties of neutralization escape MHV3 mutants are not yet elucidated. We have previously demonstrated that the major difference between the nonpathogenic YAC-MHV3 virus and pathogenic L2-MHV3 is the inability of YAC-MHV3 to replicate in thymic stromal cells or in B lymphocytes (Jolicœur and Lamontagne, 1989; Lamontagne and Jolicœur, 1991). Preliminary results indicate that CL12 mutant cannot induce B-cell lysis *in vitro* in contrast with MHV3-51.6 or L2-MHV3 viruses. A correlation between viral pathogenicity and *in vitro* viral replication in various target cells has frequently been reported to explain a genetic resistance to the various MHV strains (Boyle *et al.*, 1987; Knobler *et al.*, 1984; Lamontagne *et al.*, 1989b; McNaughton *et al.*, 1980). MHV pathogenicity is principally carried by genes coding for the RNA polymerase and the S viral glycoprotein responsible for the attachment of the virus to a cell-surface receptor (Fleming *et al.*, 1986; Lai *et al.*, 1981; Talbot *et al.*, 1984). On the other hand, immunodominant sites responsible for the binding of neutralizing antibodies are located on the S glycoprotein, particularly on the S2 subunit. Other domains are also identified near the proteolytic cleavage site and along the globular section of the S1 subunit (Daniel *et al.*, 1993; Routledge *et al.*, 1991). In contrast, virus-mediated fusion activity is located at a site different from the neutralization epitopes, as shown by the binding sites of several mAb (Collins *et al.*, 1982; Routledge *et al.*, 1991). MAb used for the selection of attenuated MHV3 mutants were able to neutralize L2-MHV3 at high dilutions and to slightly inhibit cellular fusion, but did not protect sensitive mice against pathogenic L2-MHV3 infection (Martin *et al.*, 1990), suggesting that these viruses are modified at a site included in a neutralization epitope and located near the fusion site, probably on the S2 portion of the viral glycoprotein (Daniel *et al.*, 1993).

Neutralization-resistant variants of MHV-JHM may be generated by large deletions or by point mutations (Gallagher *et al.*, 1990; Routledge *et al.*, 1991). No difference in the size of the mRNA coding for the S protein nor in the size of the S protein was detected between MHV3-CL12 or -51.6 mutants and pathogenic L2-MHV3 virus, suggesting that these neutralization-resistant variants express a point mutation in a neutralization site, inducing a conformational change (Daniel *et al.*, 1993; Martin *et al.*, 1990).

Point mutations in the S protein cannot totally explain the pathogenic properties of the virus,

since some conformational changes in the S protein may affect the viral tropism for one target cell without altering the tropism for other target cells (Dalziel *et al.*, 1986; Fleming *et al.*, 1986). Viral pathogenicity is a complex property modulated by the viral tropism for several target cells. On the other hand, the study of viral pathogenicity cannot be dissociated from the immune response mechanisms in the various target organs. Integrity of the immune system is essential for mounting an efficient response and eliminating viral production by the various infected cells. The correlations observed in this work, between the pathogenicity of attenuated CL12 and 51.6 mutants, shed light on the roles of T or B lymphocytes as other target cells for viral replication and the consequences on virus-induced immunodeficiency. Viral replication in immune cells may also be responsible for the decrease in the number of cells involved in the natural resistance to viral infection *i.e.* macrophages or NK cells, and in a second stage, T and B lymphocytes responsible for mounting efficient cellular and humoral immune responses. Effective clearance of viral infection in the brain requires $CD4^+CD8^-$ and $CD4^-CD8^+$ lymphocytes and also some unidentified bone marrow cells (Williamson and Stohlman, 1990). In addition, as has been demonstrated elsewhere, hepatitis does not result from lytic viral replication in hepatocytes but rather is correlated directly with spontaneous, T-lymphocyte-instructed expression of a procoagulant monokine that exhibits prothrombin-cleaving activity. This observation suggests that MHV3 induces cellular injury through the activation of the coagulation cascade and microcirculation damage (Levy and Abecassis, 1989). Vigorous procoagulant activity responses were observed in splenic mononuclear cells from susceptible C57BL/6 or BALB/c mice, whereas no activity was detected in cells from resistant A/J mice (Dindzans *et al.*, 1985). These results indicate that MHV3 tropism for macrophages or lymphoid cell subpopulations may alter their production of various cytokines involved in inflammatory responses and in the pathogenic process.

Further work is in progress to determine the permissiveness of various T- and B-cell subpopulations for replication of the attenuated MHV3 mutants and to identify the role of primary cell targets in the outcome of infection, depending on inoculation site.

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Pouvoir pathogène de mutants d'échappement à la neutralisation du virus de l'hépatite murine: corrélation avec la perte des lymphocytes T et B

La pathogénicité virale résulte d'un équilibre entre la replication virale et les mécanismes de défense immunitaire de l'hôte. Le processus pathogénique peut être compliqué par le fait qu'un virus lymphotrope puisse altérer l'intégrité lymphocytaire et induire subséquemment une immunodéficience. L'évolution de la maladie induite chez la souris par un virus lymphotrope, le virus de l'hépatite murine de type 3 (MHV3), dépend de l'intégrité du système immunitaire. L'utilisation de mutants d'échappement est un moyen par lequel il est possible de déterminer l'importance du lymphotropisme dans le processus pathogénique. Nous avons sélectionné des virus MHV3 mutants en fonction de leur résistance à la neutralisation par des anticorps monoclonaux, et nous avons analysé leurs propriétés pathogéniques. Nous avons montré que l'atténuation des mutants obtenus était en relation avec leur capacité d'induire une diminution des sous-populations de lymphocytes T et B dans la rate, la moelle osseuse et le thymus de souris BALB/c. Le mutant MHV3-CL12, fortement atténué, n'induit aucune diminution des lymphocytes, après trois jours d'infection à la suite d'une inoculation intrapéritonéale (i.p.) ou intranasale (i.n.). Le mutant MHV3-51.6, partiellement atténué, a gardé la capacité d'induire une perte de lymphocytes T et B après une inoculation i.p., et celle des lymphocytes B seulement après une inoculation i.n. L'utilisation de tels mutants permet de disséquer le rôle de différentes populations lymphocytaires au cours du processus pathogénique induit par le virus MHV3.

Mots-clés: MHV3, Lymphocyte T, Lymphocyte B, Neutralisation; Pouvoir pathogène, Mutants d'échappement, Voie d'inoculation, Populations lymphocytaires.

References

Barthold, S.W. (1988), Olfactory neural pathway in mouse hepatitis virus nasoencephalitis. *Acta Neuropathol.*, 76, 502-506.

Barthold, S.W. & Smith, A.L. (1983), Mouse hepatitis virus S in weanling Swiss mice following intranasal inoculation. *Lab. Anim. Sci.*, 33, 355-360.

Borysiewicz, L.K. & Sissons, J.P.G. (1986), Immune response to virus-infected cells. *Clin. Immunol. Allergy*, 6, 159-187.

Boyle, J.F., Weismiller, D.G. & Holmes, K.V. (1987), Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues. *J. Virol.*, 61, 185-189.

Brinton, M.A. & Nathanson, N. (1981), Genetic determinants of virus susceptibility: epidemiologic implications of murine models. *Epidemiol. Rev.*, 3, 115-154.

Collins, A.R., Knobler, R.L., Powell, H. & Buchmeier, M.J. (1982), Monoclonal antibodies to murine hepatitis virus 4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. *Virology*, 119, 358-371.

Dalziel, R.G., Lampert, P.W., Talbot, P.J. & Buchmeier, M.J. (1986), Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. *J. Virol.*, 59, 463-471.

Daniel, C., Anderson, R., Buchmeier, M.J., Fleming, J.O., Spaan, W.J.M., Wege, H. & Talbot, P.J. (1993), Identification of an immunodominant linear neutralization domain on the S2 portion of the murine coronavirus spike glycoprotein and evidence that it forms part of a complex tridimensional structure. *J. Virol.*, 67, 1185-1191.

Dindzans, V.J., MacPhee, P.J., Fung, L.S., Leibowitz, J.L. & Levy, G.A. (1985), The immune response to mouse hepatitis virus: expression of monocyte procoagulant activity and plasminogen activator during infection *in vivo*. *J. Immunol.*, 135, 4189-4195.

Dindzans, V.J., Zimmerman, B., Sherker, A. & Levy, G.A. (1987), Susceptibility to mouse hepatitis virus strain 3 in BALB/cJ mice: failure of immune cell proliferation and interleukin 2 production. *Adv. Exp. Med. Biol.*, 218, 411-420.

Dupuy, J.M., Levy-Leblond, E. & Le Prevost, C. (1975), Immunopathology of mouse hepatitis virus type 3 infection. — II. Effect of immunosuppression in resistant mice. *J. Immunol.*, 114, 226-230.

Dupuy, J.M., Dupuy, C., Decarie, D. (1984), Genetically determined resistance to mouse hepatitis virus 3 is expressed in haematopoietic donor cells in radiation chimeras. *J. Immunol.*, 133, 1609-1613.

Fleming, J.O., Stohlman, S.A., Harmon, R.C., Lai, M.M.C., Frelinger, J.A. & Weiner, L.P. (1983), Antigenic relationship of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus. *Virology*, 131, 296-307.

Fleming, J.O., Trousdale, M.D., El-Zaatari, F.A., Stohlman, S.A. & Weiner, L.P. (1986) Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. *J. Virol.*, 58, 869-875.

Gallagher, T.M., Parker, S.E. & Buchmeier, M.J. (1990), Neutralization-resistant variants of a neurotropic coronavirus are generated by deletions within the amino-terminal half of the spike glycoprotein. *J. Virol.*, 64, 731-737.

Goto, N., Hirano, N., Aiuchi, M., Hayashi, T. & Fujiwara, K. (1977), Nasoencephalopathy of mice infected intranasally with a mouse hepatitis virus, JHM strain. *Jpn. J. Exp. Med.*, 47, 59-70.

Jolicœur, P. & Lamontagne, L. (1989), Mouse hepatitis virus 3 pathogenicity expressed by a lytic viral infection in bone marrow $14.8^{+}\mu^{+}$ B lymphocyte subpopulations. *J. Immunol.*, 143, 3722-3730.

Knobler, R.L., Tunison, L.A. & Oldstone, M.B.A. (1984), Host genetic control of mouse hepatitis virus type 4 (JHM strain) replication. — I. Restriction of virus amplification and spread in macrophages from resistant mice. *J. Gen. Virol.*, 65, 1543-1549.

Knobler, R.L., Brainard, G.C., Perreault, M., D'Imperio, C., Phenix, P. & Lublin, F.D. (1988), Immune effects of intracerebral infection with mouse hepatitis virus. *Ann. NY Acad. Sci.*, 540, 642-644.

Lai, M.M.C., Brayton, P.R., Armen, R.C., Patton, C.D., Pugh, C. & Stohlman, S.A. (1981), Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic MHV3. *J. Virol.*, 39, 823-829.

Lamontagne, L., Dupuy, C., Leray, D., Chausseau, J.P. & Dupuy, J.M. (1985), Coronavirus-induced immunosuppression: role of mouse hepatitis virus 3-lymphocyte interaction. *Prog. Leuk. Biol.*, 1, 29-42.

Lamontagne, L. & Dupuy, J.M. (1984), Persistent *in vitro* infection with mouse hepatitis virus type 3 in mouse lymphoid cell lines. *Infect. & Immun.*, 44, 716-723.

Lamontagne, L., Descoteaux, J.P. & Jolicœur, P. (1989a), Mouse hepatitis virus 3 replication in T and B lymphocytes correlates with viral pathogenicity. *J. Immunol.*, 142, 4458-4466.

Lamontagne, L., Décarie, D. & Dupuy, J.M. (1989b), Host cell resistance to mouse hepatitis virus type 3 is expressed *in vitro* in macrophages and lymphocytes. *Viral Immunol.*, 2, 37-45.

Lamontagne, L. & Jolicœur, P. (1991), Mouse hepatitis virus 3-thymic cell interactions correlating with viral pathogenicity. *J. Immunol.*, 146, 3152-3160.

Le Prevost, C., Levy-Leblond, E., Virelizier, J.L. & Dupuy, J.M. (1975a), Immunopathology of mouse hepatitis virus type 3 infection. — I. Role of humoral and cell-mediated immunity in resistance mechanism. *J. Immunol.*, 114, 221-225.

Le Prevost, C., Virelizier, J.L. & Dupuy, J.M. (1975b), Immunopathology of mouse hepatitis virus type 3 infection. — III. Clinical and virologic observations of a persistent infection. *J. Immunol.*, 115, 640-645.

Leray, D., Dupuy, C. & Dupuy, J.M. (1982), Immunopathology of mouse hepatitis virus type 3 infection. — IV. MHV3-induced immunosuppression. *Clin. Immunol. Immunopathol.*, 23, 223-228.

Levy-Leblond, E., Oth, D. & Dupuy, J.M. (1979), Genetic study of mouse sensitivity to MHV3 infection: influence of the H-2 complex. *J. Immunol.*, 112, 1359-1362.

Levy, G.A., Leibowitz, J.L. & Eddington, T.S. (1982), Lymphocyte-instructed monocyte induction of the coagulation pathway parallels the induction of hepatitis by the murine hepatitis virus. *Prog. Liver Dis.*, 7, 393-409.

Levy, G. & Abecassis, M. (1989), Activation of the immune coagulation system by murine hepatitis virus strain 3. *Rev. Infec. Dis. II supp.*, 4: S712-721.

Martin, J.P., Chen, W., Obert, G. & Koehren, F. (1990), Characterization of attenuated mutants of MHV3: importance of the E2 protein in organ tropism and infection of isolated liver cells. *Adv. Exp. Med. Biol.*, 276, 403-410.

Makino, S., Fleming, J.O., Keck, J.G., Stohlman, S.A. & Lai, M.M.C. (1987), RNA recombination of coronavirus: localization of neutralizing epitopes and neuropathogenic determinants on the carboxyl terminus of peplomes. *Proc. Natl. Acad. Sci.* USA, 84, 6567-6571.

McNaughton, M.R. & Patterson, S. (1980), Mouse hepatitis virus strain 3 infection in C57, A/Sn and A/J mice and their macrophages. *Arch. Virol.*, 66, 71-76.

Osmond, D.G. & Nossal, G.J.V. (1974), Differentiation of lymphocytes in mouse bone marrow. — II. Kinetics of maturation and renewal of anti-globulin-binding cells studied by double labeling. *Cell. Immunol.*, 13, 137-143.

Park, Y.H. & Osmond, D.G. (1987), Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. *J. Exp. Med.*, 165, 444-450.

Piazza, M., Piccinino, F. & Matano, F. (1965). Hematologic changes in viral (MHV3) murine hepatitis. *Nature*, 205, 1034-1035.

Raff, M.C. & Owen, J.T.T. (1971), Thymus-derived lymphocytes: their distribution and role in the development of peripheral lymphoid tissues of the mouse. *Eur. J. Immunol*, 1, 27-30.

Routledge, E., Stauber, R., Pfleiderer, M. & Siddell, M.G. (1991), Analysis of murine coronavirus surface glycoprotein functions by using monoclonal antibodies. *J. Virol.*, 65, 254-270.

Sissons, J.P.G. & Oldstone, M.B.A. (1980), Antibody-mediated destruction of virus-infected cells. *Adv. Immunol.*, 29, 209-259.

Sissons, J.P.G., Schreiber, R.D., Cooper, N.R. & Oldstone, M.B.A. (1982), The role of antibody and complement in lysing virus-infected cells. *Med. Microbiol. Immunol.*, 170, 221-228.

Sissons, J.P.G. & Borysiewicz, L.K. (1985), Viral immunopathology. *Br. Med. Bull.*, 41, 34-40.

Starkey, P.M., Sargent, I.L. & Redman, C.W.G. (1988), Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. *Immunology*, 65, 129-134.

Talbot, P.J., Salmi, A.A., Knobler, R.L. & Buchmeier, M.J. (1984), Topographical mapping of epitopes on the glycoproteins of murine hepatitis virus 4 (strain JHM): correlation with biological activities. *Virology*, 132, 250-256.

Williamson, J.S.P. & Stohlman, S.A. (1990), Effective clearance of mouse hepatitis virus from the central nervous system requires both $CD4^+$ and $CD8^+$ T cells. *J. Virol.*, 64, 4589-4595.