

# Granulomatous peritonitis and pleuritis in interferon-gene knockout mice naturally infected with mouse hepatitis virus

MP FRANCE<sup>a</sup>, AL SMITH<sup>b</sup>, R STEVENSON<sup>c</sup> and SW BARTHOLD<sup>d</sup>

**Objective** To investigate a disease outbreak in a colony of laboratory mice with targeted disruption of the gene for interferon-

**Format** A case report based on necropsy, histopathology, serology and immunohistochemistry.

**Results** Affected mice exhibited depression and variable ascites. Necropsy revealed a granulomatous peritonitis and pleuritis with extensive adhesions although parenchymal lesions were minimal. Serum samples had high concentrations of antibody to mouse hepatitis virus and immunohistochemical examination revealed the presence of mouse hepatitis virus antigen in granuloma macrophages. Sero-logical testing for other infectious agents and bacterial culture were negative and wild type mice kept in the same facility remained healthy. Despite the association between the disease and mouse hepatitis virus infection, the precise role played by mouse hepatitis virus was not determined. While the disease is superficially similar to feline infectious peritonitis (another coronavirus-induced serositis), differences exist between the histopathological findings in these two conditions.

**Conclusion** This unusual disease process illustrates how new diagnostic challenges can arise in novel mouse genotypes created through molecular genetics. Furthermore, the association between the disease and mouse hepatitis virus illustrates the importance of maintaining laboratory animals under specific-pathogen free conditions.

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Key words: Mouse, mouse hepatitis virus, interferon-, granuloma, gene knockout, peritonitis, pleuritis, macrophage.

MHV is a coronavirus distinguishable into many strains and has a high rate of mutation.<sup>1,2</sup> It is widely regarded as the most important pathogen of laboratory mice due to its high prevalence<sup>3-5</sup> and its ability to interfere with both in-vivo and in-vitro research.<sup>1,6-9</sup> MHV-related effects on research may be insidious since natural infection is usually subclinical in immunocompetent hosts.<sup>2,6,8</sup> In immunodeficient hosts, however, infection is often fatal and reports continue to appear in which valuable colonies have been devastated following incursion by MHV.<sup>10-12</sup> Indeed, a severe disease consistent with MHV infection greatly impeded early propagation of the athymic (nude) mouse, now probably the most widely used immunodeficient mouse genotype in biomedical research.<sup>13</sup>

In recent years, the range of mice available to biomedical researchers has expanded greatly through the application of molecular genetics. In particular, the development of mice in which a single, targeted gene has been rendered non-functional (gene knockout mice) has resulted in the creation of several hundred novel genotypes.<sup>14</sup> Many such mice have been developed for immunological studies and often the resulting phenotypes exhibit a degree of immunodeficiency. This situation raises the prospect of atypical infectious disease problems due to peculiar host responses or opportunistic infections. This report describes the investigation of an outbreak of an unusual granulomatous peritonitis and pleuritis associated with natural MHV infection in knockout mice lacking a functional gene for the important immunomodulatory cytokine IFN-. The findings draw attention to both the emerging challenges of disease diagnosis in animals with novel genotypes and the importance of maintaining SPF husbandry conditions when working with these valuable animals.

## Materials and methods

### Mice

Mice rendered deficient in IFN- through targeted disruption of the IFN- gene (IFN- knockout mice) and backcrossed to the C57BL/6 strain were derived from stock originally produced by Dalton et al.<sup>15</sup> The main breeding colony was kept under SPF conditions. A second breeding and experimental colony was then established in a separate facility where mice were kept in open top cages under 'conventional' conditions. While regular health monitoring was not undertaken in this facility, MHV, rotavirus, murine encephalomyelitis virus, *Spironucleus muris*, *Giardia muris* and pinworm had been detected there in the past. Despite this, all mice apart from the IFN- knockouts appeared clinically normal. Food (commercial rodent pellets), water, bedding (vermiculite) and caging were unsterilised. None of the mice examined in this investigation had been used for experimental work.

### Serology

Serum samples taken from affected IFN- knockout mice at necropsy were frozen at -70°C then shipped overnight in ice to the Murine Virus Monitoring Service, Adelaide, South Australia for ELISA testing. ELISA antigen was obtained from the University of Missouri, Columbia, USA, or was manufactured in-house as follows. Permissive cell lines were infected with virus (strain A59 in the case of MHV) and after 72 h (approximately

<sup>a</sup>Department of Veterinary Anatomy and Pathology, University of Sydney, New South Wales 2006

<sup>b</sup>Department of Pathology, Loyola University, Chicago, IL 60153 USA

<sup>c</sup>Murine Virus Monitoring Service, 101 Blacks Plains Road, Adelaide, South Australia 5086

<sup>d</sup>Center for Comparative Medicine, University of California, Davis, CA 95616 USA

ELISA	Enzyme-linked immunosorbent assay
IFN-	Interferon-
MHV	Mouse hepatitis virus
FIP	Feline infectious peritonitis
SPF	Specific-pathogen free

90% cytopathic effect) the cells were lysed by freeze-thawing; cell debris was pelleted at 3000 *g* for 20 min and virus-containing supernatant was concentrated by ultracentrifugation then purified by ultracentrifugation on a 20% sucrose cushion. Negative control antigen was prepared likewise from uninfected cells. Antigen concentration was determined by titration against known positive and negative sera. Antigens were diluted in bicarbonate buffer (pH 9.8) and coated onto 96-well microtitre plates at 4°C overnight and then either stored at -80°C or washed four times with phosphate-buffered saline containing 0.05% Tween-20 for immediate use. Test and control sera were diluted 1:100 in phosphate-buffered saline containing 0.5% low-fat skim milk powder and 100 µL added to coated microtitre plate wells. After incubation and washing, 100 µL of previously titrated affinity isolated goat anti-mouse IgG and IgM-horseradish peroxidase conjugate (Biosource International, CA, USA) was added to each well. After re-incubation and washing, 100 µL of K-Blue substrate (Graphic Scientific, Queensland) was added to each well, the reaction stopped after 10 min with 2M H<sub>2</sub>SO<sub>4</sub> and the plates read at 450 nm on an automated plate reader. Corrected optical density values were compared to values from positive and negative reference sera (BioReliance, USA) and the results expressed as a percentage of reference sera values. Equivocal (15 to 30%) and positive (> 30%) sera were then re-tested by the immunofluorescence antibody method using slides provided by the University of Missouri, Columbia, USA, or prepared with air-dried, acetone-fixed, virus-infected cells. Test and control sera were diluted 1:10 in phosphate-buffered saline and 15 µL added to appropriate wells on the slide. After incubation, washing and air drying, 15 µL of previously titrated goat anti-mouse IgG and IgM-fluorescein conjugate (Biosource International, CA, USA) was added to each well. After re-incubation and washing, the slides were glycerol mounted and examined by fluorescence microscopy.

#### *Necropsy, histopathology and immunohistochemistry*

Mice were euthanased with CO<sub>2</sub> and necropsied immediately. Ascitic fluid was collected from five mice and cytocentrifuge preparations made then stained with Diff-Quick (Lab-Aids, New South Wales). Swabs of peritoneal lesions and exudate from four mice were cultured aerobically on blood agar. Tissues were fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin. Selected sections were also stained with Warthin-Starry silver, Brown and Brenn, Grocott's methenamine silver, periodic acid-Schiff, Ziehl Neelsen and Giemsa methods. Immunohistochemical staining for MHV was performed on 5 µm trypsinised sections of formalin fixed, paraffin-embedded tissue mounted on silane-coated slides. Sections were incubated with polyvalent mouse ascitic fluid containing anti-MHV antibody then labelled using biotinylated goat anti-mouse antibody followed by a streptavidin-horseradish peroxidase conjugate and diaminobenzidine substrate.<sup>16,17</sup>

## Results

Clinical signs were observed in IFN- knockout mice of both sexes (including previously bred females) ranging in age from 10 to 24 weeks. C57BL/6 mice housed in adjacent cages remained normal. Affected animals exhibited depression and variable ascites, and some also developed mild diarrhoea. Detailed morbidity figures were not available although a total of 10 mice,

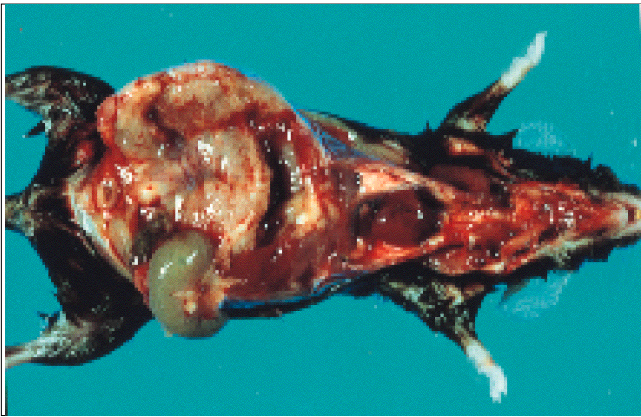
all similarly affected, were submitted for necropsy over a period of 3 weeks, after which all remaining IFN- knockout mice were euthanased. Clinical signs were not observed in IFN- knockout mice kept under SPF conditions in the main breeding colony.

Necropsy revealed a variable amount of cloudy peritoneal fluid and thick, white plaques on the serosae of abdominal viscera. Many of these plaques had formed rubbery adhesions between adjacent structures and in advanced cases had virtually formed a cocoon around groups of organs (Figure 1). The spleen was often the organ most affected and was always markedly enlarged. Adhesions and effusion were also found in the thoracic cavity in some animals. Culture of abdominal fluid from four mice resulted in no growth after 5 days. However, cytological examination of fluid from one mouse which was not cultured revealed small coccoid and occasional rod-shaped bacteria within neutrophil cytoplasm. In this specimen, approximately 90% of the cells were neutrophils. This was in contrast to the findings in smears from other mice in which the majority of cells were macrophages and lymphocytes and no bacteria were seen.

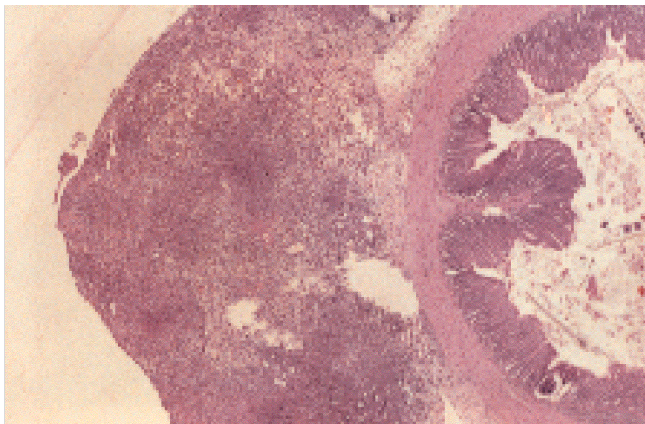
Histological examination revealed that the serosal plaques and adhesions consisted of sheets of macrophages forming granulomas, which in some mice had a necrotic centre. Some adhesions consisted only of a thin layer of fibrous tissue. Many granulomas were heavily infiltrated with neutrophils, especially at the periphery, and variable numbers of lymphocytes and plasma cells were scattered through the granulomas and surrounding connective tissue. Multinucleate giant cells were very rare. A striking feature of the inflammatory process was that it was almost entirely confined to the serosa and adjacent folds of peritoneum or pleura (Figure 2). Solitary parenchymal lesions were seen in the liver and kidney of one mouse although these were subcapsular and appeared to be extensions of serosal lesions. The only organ exhibiting substantial parenchymal damage was the pancreas although it was only affected in advanced cases and appeared to be the result of extension of inflammation from adjacent mesentery into the pancreatic interstitium. Occasional granulomas were present in visceral lymph nodes and splenic enlargement was found to be due to intense extramedullary myelopoiesis. Dilated lymphatic vessels were prominent adjacent to some foci of inflammation. Occasionally, near areas of intense inflammation, small muscular arteries showed a localised vasculitis or hyaline degeneration. In general, however, blood vessels appeared normal. There were no significant findings in sections of limb joints, brain or other structures of the head. No bacteria or fungi were seen in sections stained for micro-organisms.

Sera from two affected mice gave strong positive reactions when tested for antibodies to MHV but were negative to 13 other agents (murine cytomegalovirus, mouse parvoviruses, *Mycoplasma pulmonis*, lymphocytic choriomeningitis, pneumonia virus of mice, reovirus 3, Sendai virus, Theiler's murine encephalomyelitis virus, mouse rotavirus, ectromelia virus, mouse adenovirus types 1 and 2, polyoma virus and *Clostridium piliforme*, the agent of Tyzzer's disease). Sera from a further eight affected mice were then tested and again found to be strongly positive to MHV and negative to a reduced panel of agents (murine cytomegalovirus, mouse parvoviruses, *Mycoplasma pulmonis* and lymphocytic choriomeningitis). Immunohistochemical staining demonstrated MHV antigen in the centre of granulomas, most of which appeared to be within macrophages (Figure 3).

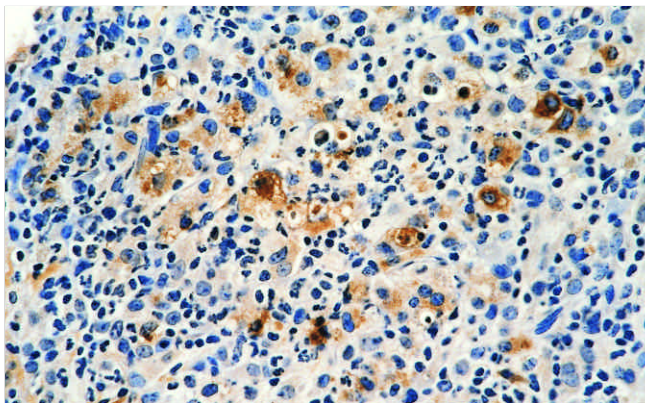




**Figure 1.** IFN- $\gamma$  knockout mouse with advanced granulomatous peritonitis and mild pleural adhesions. Many of the abdominal viscera are obscured by the inflammatory process.



**Figure 2.** Granulomatous serositis involving ascending colon, IFN- $\gamma$  knockout mouse. The mucosa and muscle layers of the colonic wall (right) are essentially normal. Haematoxylin and eosin, x 100.



**Figure 3.** Immunohistochemical staining for MHV antigen in a serosal granuloma. Antigen (brown) is present in the cytoplasm of many cells, most of which are probably macrophages. Immunoperoxidase, x 1000.

## Discussion

In recent years, the use of transgenic and gene knockout mice has revolutionised the study of gene function *in vivo*. Applications have ranged from the study of basic cellular processes right through to the creation of homologues of important human and animal diseases. Many of these genotypes, however, have yet to be well characterised and often the resulting phenotypes have produced surprising results which challenge prior understanding.<sup>14</sup> It is to be expected, therefore, that some of these animals will respond to commonplace infections in novel ways.

The present case describes a striking granulomatous disease of the peritoneum and pleura in IFN- $\gamma$  gene knockout mice and several findings indicate that MHV was important in its pathogenesis. These include appearance of the disease following introduction of the mice to an MHV-endemic facility in open top cages, seropositivity to MHV, and the demonstration of viral antigen in granuloma macrophages by immunohistochemistry.

Published reports of MHV-related lesions in the peritoneum and pleura are rare although early accounts noted the accumulation of giant cells in these tissues,<sup>18</sup> and ascites and peritonitis due to MHV has been described in nude mice.<sup>19,20</sup> Furthermore, the experience of one of the present authors (SWB) suggests that such lesions are more common than is reported. In all these cases, however, the peritoneal and pleural changes are accompanied by substantial hepatic parenchymal lesions, unlike in the present case. More recently, a condition almost identical to the present case has been reported in two separate situations where IFN- $\gamma$  deficient gene knockout mice were exposed to MHV. In the first, a granulomatous pleuro-peritonitis developed following natural exposure in IFN- $\gamma$ /interleukin 10 double knockout mice, although interestingly IFN- $\gamma$  single knockout mice were not affected.<sup>21</sup> In the second report, IFN- $\gamma$  knockout mice developed ascites, peritonitis and pleuritis with granulomatous inflammation following experimental inoculation of MHV by the intraperitoneal route.<sup>22</sup> While the findings of these two reports suggest that pleuroperitonitis can be the major lesion induced by MHV in animals deficient in IFN- $\gamma$ , other studies show that this is not always the case. In particular, several studies with gene knockout mice deficient in various components of the IFN- $\gamma$  pathway (including the IFN- $\gamma$  gene) reported no significant serosal lesions despite an increased susceptibility to parenchymal damage by MHV<sup>11,23-25</sup> and similar findings were made in earlier studies using mice treated with anti-IFN- $\gamma$  antibodies.<sup>26,27</sup> It is possible that in the present case and in the other two with prominent pleuroperitonitis that factors such as age of host and genetic background and the dose and strain of virus contributed to the pathogenesis since all these can affect the outcome of MHV infection.<sup>1,2,6</sup> Even so, it is curious that some of the hallmark lesions of MHV-related disease such as epithelial and endothelial syncytia were lacking. Route of inoculation can also influence disease manifestation, although this does not seem to be important in establishing peritonitis since natural exposure and intraperitoneal routes both led to a similar distribution of lesions. Overall it seems likely that factors in addition to IFN- $\gamma$  deficiency and simple exposure to MHV are required to produce the sort of disease seen here.

The search for additional infectious agents in this investigation was limited. In particular, bacteriological culture did not include suitable techniques for the detection of mycobacteria which are important causes of granulomatous inflammation

and can be difficult to demonstrate histologically. Anaerobic and other fastidious organisms would also have gone undetected with the culture methods used. While bacteria were seen in smears of abdominal fluid from one mouse, that particular fluid sample was not cultured. Moreover, the very high proportion of neutrophils in that sample compared to samples from other mice suggests that a secondary septic peritonitis might have been present. This is further supported by the fact that despite large numbers of organisms in smears of fluid, no bacteria were seen in sections of solid granulomas. Virus isolation was not attempted so it is unknown whether the viral antigen seen immunohistochemically represented infectious virus particles or components of degenerate virions. Serological testing was largely aimed at detecting antibodies to those murine viruses which have been found to affect macrophage function although testing was not available for lactate dehydrogenase elevating virus, an agent which can replicate in peritoneal macrophages and has subtle but complex effects on the host.<sup>6</sup> The possibility that other, undetected infectious agents contributed to this disease therefore cannot be excluded.

The extent to which the granulomatous inflammation was restricted to the peritoneal and pleural serosae is one of the most striking features of this disease. This finding may relate in part to the IFN- $\gamma$  deficient state of the animals. The peritoneal cavity contains resident and blood monocyte-derived macrophages which form an important line of defense.<sup>28,29</sup> Since IFN- $\gamma$  plays an important role in the activation of macrophages in the peritoneum and elsewhere,<sup>30</sup> impaired activation resulting from IFN- $\gamma$  deficiency could predispose to granuloma formation. The presence of MHV might then exacerbate this situation since MHV itself can affect peritoneal macrophage function<sup>8,31</sup> and it has been found that peritoneal macrophage numbers increase during natural MHV infection.<sup>31</sup> Loss of autocrine synthesis of IFN- $\gamma$  by peritoneal macrophages resulting in an inability to induce an antiviral state<sup>32</sup> might further contribute to this proposed sequence of events.

In their discussion of MHV-induced pleuroperitonitis in IFN- $\gamma$  knockout mice, Kyuwa et al suggest the possible involvement of B-1 cells.<sup>22</sup> B-1 cells are a class of B lymphocyte which is enriched in the peritoneal and pleural cavities and evidence that IFN- $\gamma$  plays a role in the regulation of B-1 cells<sup>33</sup> adds to the appeal of their hypothesis. Further studies into this disease may therefore be of value in the investigation of general immune mechanisms in the peritoneal and pleural cavities.

The disease in these mice bears a superficial similarity to another coronaviral disease, feline infectious peritonitis. Specifically, serosal inflammation and peritoneal and sometimes pleural effusions can be a major feature of FIP. In addition, different strains of both murine and feline coronaviruses tend to produce either enteric or systemic disease, and replication in macrophages is important to the pathogenesis of infection in both host species.<sup>1,2,34</sup> There are, however, some morphological differences between the two. In typical FIP, effusions are associated with vascular and perivascular lesions which were not a major feature in the present case. Furthermore, the solid lesions which predominate in the so-called 'dry' form of FIP (often loosely termed granulomas) are distinct from the 'classic' granulomas seen here and are not exclusively subserosal in distribution. Thus while the findings in the present case suggest that the role of IFN- $\gamma$  in FIP may warrant investigation, it seems likely that some differences exist in the pathogenesis of these two diseases.

Regardless of the precise pathogenesis of this condition, the clear involvement of MHV serves to illustrate the sort of problem which can arise when laboratory animals are not kept under appropriate standards of SPF husbandry. As in this case, effective management of such problems may require undertaking the costly process of depopulation and restocking. The appropriate use of microisolator caging combined with regular health monitoring can greatly reduce the likelihood of incurring such costs.<sup>3</sup>

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## CORRECTION

Brown AT, Gregory AR, Ellis TM and Hearnden MN. Comparative immunogenicity of two bivalent botulinum vaccines. *Aust Vet J* 1999;77:388-391.

Tables 1 and 2 should read as follows:

Table 1. ELISA reactivity (OD<sub>450</sub>) of vaccinated groups of weaners, mean (SE).

Vaccine group		24/04/1997			27/06/1997			7/11/1997		
		n	Type C	Type D	n	Type C	Type D	n	Type C	Type D
CSL	Overall	75	0.37 (0.04)	0.43 (0.04)	74	0.9 (0.06)	1.16 (0.07)	71	0.5 (0.04)	0.6 (0.06)
	Unbranded	39	0.50 (0.08)	0.55 (0.08)	38	0.93 (0.07)	1.25 (0.10)	36	0.54 (0.07)	0.68 (0.10)
	Branded	36	0.25 (0.03)	0.30 (0.03)	36	0.90 (0.10)	1.08 (0.10)	35	0.45 (0.04)	0.53 (0.07)
Websters	Overall	75	0.39 (0.04)	0.46 (0.04)	75	1.1 (0.05)	1.85 (0.06)	75	1.01 (0.06)	1.49 (0.08)
	Unbranded	32	0.43 (0.07)	0.52 (0.06)	32	1.02 (0.07)	1.76 (0.09)	32	0.87(0.08)	1.34 (0.12)
	Branded	43	0.36 (0.04)	0.42 (0.05)	43	1.16 (0.06)	1.91 (0.08)	43	1.12 (0.08)	1.60 (0.10)

Table 2. Analysis of deviance results for the covariate (maternal antibody), factors (vaccine type, branding group) and the factor interaction for each final antibody response.

Antibody	Factor/covariate	Change in deviance	d.f.	p
Final type C	Maternal antibody (covariate)	0.23	1	ns
	Vaccine	9.88	1	< 0.005
	Branding	0.52	1	ns
	Vaccine X branding	1.04	1	ns
Final type D	Maternal antibody (covariate)	0.11	1	ns
	Vaccine	28.82	1	< 0.0001
	Branding	0.61	1	ns
	Vaccine X branding	1.67	1	ns

ns = not significant at the 0.05 level