Monoclonal Antiprothrombinase (3D4.3) Prevents Mortality from Murine Hepatitis Virus (MHV-3) Infection

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Summary

The induction of monocyte/macrophage procoagulant activity (PCA) has been implicated in the pathogenesis of murine hepatitis virus strain 3 (MHV-3) infection and disease. Previously, we have shown that induction of PCA by MHV-3 correlated with resistance/susceptibility to infection in different mouse strains. In this study, all BALB/cJ mice that were infected with 10^3 plaque-forming units of MHV-3 developed severe liver disease and died within 96–120 h. Examination of the livers of these animals showed marked hepatic necrosis, deposition of fibrin, and cellular expression of PCA by direct immunofluorescence staining in areas of necrosis as well as in hepatic sinusoids. Splenic mononuclear cells recovered from these mice expressed high concentrations of PCA with time after infection. Infusion into mice of a high-titered monoclonal antibody that neutralized PCA (3D4.3) attenuated the development of hepatic necrosis and enhanced survival in a dose-dependent manner. All of the animals receiving 100 μg, and 44% and 22% of the animals that received 50 and 25 μg per day, respectively, survived for 10 d and made a full recovery. Administration of the antibody resulted in a dose-dependent reduction in fibrin deposition, PCA expression as detected by direct immunofluorescence staining and by a functional assay. In animals treated with high concentrations of antibody, titers of antibody to PCA fell from 87 ± 15 μg/ml to 100 ± 7 ng/ml during the active phase of the disease, consistent with sequestration due to binding of the immunoglobulin to cells expressing PCA. Surviving animals, when rechallenged with MHV-3, had a 40% mortality, consistent with the known rates of metabolism of immunoglobulin. This further suggested that protection was by a passive mechanism. The results reported here demonstrate that a neutralizing antibody to PCA protects animals from fulminant hepatitis and death associated with MHV-3 infection, and supports the notion that PCA is a potent inflammatory mediator that plays a pivotal role in the pathogenesis of liver injury resulting from MHV-3 infection.

Murine hepatitis virus strain 3 (MHV-3),[1] a member of the coronavirus family, is a universally fatal infection in fully susceptible BALB/cJ mice even when mice are infected with as little as 0.1 PFU (1). Recent investigations have implicated the induction of monocyte/macrophage procoagulant activity (PCA), a prothrombinase, in the pathogenesis of MHV-3 infection and disease (2–4). Previously, we demonstrated that MHV-3 induction of PCA correlated with resistance/susceptibility to infection in different mouse strains (2–4). Furthermore, using recombinant inbred strains of mice, we showed that genetic linkage between resistance/susceptibility to MHV-3 infection and induction of PCA was controlled by two non-H2-linked recessive genes (5). After infection with MHV-3, disturbances of the hepatic microcirculation associated with sinusoidal thrombosis occurred coincident with the rise in PCA (6, 7). Intravenous infusion of macrophages, induced to express high amounts of PCA by MHV-3, resulted in rapid (2–5 min) death from disseminated intravascular coagulation in both susceptible and resistant mice (our unpublished observations). Together, these observations suggest that coagulative necrosis occurring as a result of induction of PCA may be a crucial feature of MHV-3-induced hepatic injury.

[1] *Abbreviations used in this paper:* MHV-3, murine hepatitis virus strain 3; PCA, procoagulant activity; p.i., postinfection; SMNC, splenic mononuclear cells.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/09/0689/09 $2.00
Volume 176 September 1992 689–697

We have recently produced a panel of mAbs to MHV-induced macrophage PCA (8). The antibodies did not react with purified viral proteins nor did they inhibit viral replication. One of these mAbs (3D4.3), an IgG2ak, strongly inhibited PCA expression in a one-stage clotting assay and inhibited conversion of prothrombin to thrombin (8). This antibody had no reactivity with murine, rabbit, or human tissue factor. The monoclonal bound to a 70-kD protein that is distinct from murine and human tissue factor (42 kD) (9, 10).

The purpose of this study was to determine whether treatment with this specific murine antiprothrombinase would modify the morbidity and/or mortality associated with murine hepatitis virus infection.

Materials and Methods

Virus. The origin and growth of MHV-3 have been previously described (2). MHV-3, obtained from the American Type Culture Collection (Rockville, MD) (ATCC-VR262), was plaque purified on monolayers of DBT cells. Stock virus was grown to a titer of 2.8×10^7 PFU/ml in 17 CL1 cells. The virus was harvested by one cycle of freeze-thawing and clarified by centrifugation at 4,500 *g* for 1 h at 4°C. Virus was then assayed on monolayers of L2 cells in a standard plaque assay as previously described (2). To induce MHV-3 infection, each mouse received 10^3 PFU of MHV-3 by intraperitoneal injection.

Mice. BALB/cJ mice 8–10 wk of age, were obtained from Charles River Laboratories, (St. Constant, Quebec). Animals were housed in the D class facility at the University of Toronto fed a diet of standard chow and water ad libitum. Mice were killed on days 1, 3, 5, 7, 10, and 14 after infection. Blood was obtained by axillary bleeding. Splenic mononuclear cells were harvested and assayed for PCA both by immunofluorescence staining and in a one-stage clotting assay. Livers were harvested for viral titers, histopathology, and PCA by immunofluorescence staining as described below. All mice used were screened by an ELISA for exposure to MHV, and were found to be negative (11).

Anti-PCA mAb. The preparation of mAb 3D4.3 has been previously described (8). After injection of 10^6 hybridoma cells into Pristane-primed CAF_1 mice, ascites was harvested. Animals were treated with ascitic fluid containing 25, 50, or 100 μg of mAb daily for 7 d preinfection and 7 d postinfection (p.i.) by intraperitoneal injection. As a standard control, 100 μl of ascites from animals that were infected with SP2 myeloma cells was injected into six separate mice. All ascitic fluid whether from SP2- or 3D4.3-injected mice had no reactivity with MHV-3 either in the ELISA or plaque reduction assays (8). To ensure that the protective effect of ascites containing mAb to PCA was specific, mAb to PCA (3D4.3) was isolated from hybridoma supernatants by affinity chromatography using goat anti–mouse IgG immobilized on Sepharose 4B (Pharmacia, Montreal, Quebec). Immunoblot analysis of the column eluate confirmed that IgG was the only Ig present. Subclass analysis by ELISA, as previously described (8), confirmed that IgG2ak was the only subclass present. 100 μg of the purified mAb was injected into 10 mice for 7 d preinfection and for 7 d p.i. as described above.

Histology. Histology was assessed by a blinded observer as previously described (8). Briefly, livers were cut into 1.0 × 0.2-cm blocks and fixed by immersion into 10% formalin in 0.1 molar phosphate buffer, pH 7.4. After fixation, the tissue was dehydrated in graded alcohols and xylene, then embedded in paraffin. 4-μm sections were cut, stained with Harris' hematoxylin for 4 min, and counterstained with eosin Y for 30 s. The sections were then washed with distilled water, dehydrated in graded alcohols and xylene, and mounted with Permount. For each group five animals were used. To quantitate the effect of the mAb on liver histology, a digitalized image analysis system (HP-88; Hewlett Packard Co., Ltd., Mississauga, Ontario) with customized software was used. This constitutes a modification of a technique described previously (12). The areas of necrosis were encircled as well as the entire section yielding a percentage figure representing the proportion of diseased liver present in that particular section. For each animal, three random sections were assayed in this fashion, and the mean ± SD was calculated. Fibrin deposition was assessed by the Morris-Lendrum Picro-Mallory stain as previously described (13).

Viral Titers. Livers that had been snap frozen at −70°C were homogenized in DMEM supplemented with 2% FCS and 4 mM glutamine as a 10% homogenate at 4°C as previously described (14). Viral titers of liver homogenates were then determined on monolayers of L2 cells in a standard plaque assay (2, 13).

Isolation of Splenic Mononuclear Cells (SMNC). Spleens were harvested aseptically and cells teased from splenic tissue and suspended in 8 ml of DMEM as previously described (15). SMNC were isolated over Ficoll-Hypaque gradients (density, 1.0749) (Pharmcia) by centrifugation at 1,800 *g* for 12 min at 22°C. Cells at the interface were collected. Viability was >98% as assessed by trypan blue exclusion. Cells were washed three times and resuspended in DMEM at a concentration of 4×10^6 SMNC/ml.

Procoagulant Activity. Samples of frozen thawed SMNC were assayed for the capacity to shorten the spontaneous clotting time of human citrated platelet-poor plasma in a one-stage clotting assay as previously described (12). Equal volumes (80 μl) of the cellular homogenate were admixed with citrated normal human platelet-poor plasma, and then 80 μl of 25 mM $CaCl_2$ was added at 37°C to start the reaction. The time in seconds for the appearance of a fibrin gel was then recorded. To establish arbitrary units, a rabbit brain thromboplastin standard at 36 mg dry mass/ml (Dade Division, American Hospital Supply, Miami, FL) was assigned a value of 100,000 mU. The assay was used over the range of 1–100,000 mU, and the results were linear with normal human plasma substrate. Data were converted to PCA per 10^6 splenic macrophages and expressed as the mean and standard deviation from six mice done in triplicate. Media and buffers were all without activity in this assay.

Inhibition of Viral Replication. mAb purified from hybridoma cell cultures containing 3D4.3 and ascites were assayed for their ability to inhibit replication of MHV-3 in a standard plaque reduction assay as previously described (8). Briefly, 100 PFU of virus was admixed with dilutions of purified antibody or ascites, media as a negative control, or a high titered anti-MHV-3-neutralizing antibody as a positive control, for 30 min at 4°C. The mixture was then added to a monolayer of L2 cells in culture medium, overlayed with 2% agarose, and incubated at 37°C in a 5% CO_2 environment for an additional 48 h. The effect of antibody on viral replication was assessed by reduction of viral plaques (8).

Immunofluorescence. Blocks of liver tissue were snap frozen in liquid nitrogen. Cryostat sections (~4 μm thick) were fixed for 5 min in acetone and air dried for 2 h as previously described (14). Unoccupied sites were then blocked with 5% horse serum in PBS, pH 7.4, for 2 h. mAb 3D4.3 was conjugated with FITC (Sigma Chemical Co., St. Louis, MO) according to the method of Thi and Feltkamp (16). The fluoresceinated antibody did not react with normal liver or uninduced normal peritoneal macrophages. Tissues

were then stained with FITC-conjugated mAb 3D4.3 for 1 h at room temperature, washed three times, mounted in 90% glycerol in PBS, and viewed on a phase-epifluorecence microscope equipped with a ×40 Fluotar objective (E. Leitz, Inc., Rockleigh, NJ).

ELISA for Anti-PCA Antibody. Titres of antibody to PCA were determined in a standard ELISA as previously described (8). 96-well enzyme immunoabsorbant assay (EIA) plates (Dynatech, McLean, VA) were coated with 50 μl/well of MHV-3-stimulated PCA-positive macrophage membranes or unstimulated PCA-negative membranes (4×10^6 macrophage/ml) at 4°C overnight. The plates were then washed three times with PBS, pH 7.5, containing 0.05% Tween 20 (washing buffer), and the unoccupied sites were blocked with 200 μl of 5% Ig-free horse serum (Flow Laboratories, Mississauga, Ontario) that had been dissolved in washing buffer for 2 h at room temperature. 50-μl/well dilutions of sera from treated animals were then added to the plates and incubated for 1 h at 37°C. After washing three times, 50 μl/well of alkaline phosphatase–conjugated goat anti–mouse Ig in PBS containing 0.1% BSA and 0.05% Tween 20 was added for 1 h at 37°C. After three washings, *P*-nitrophenyl phosphate in 0.1 M 2-amino-2-methyl-1, 3-proponediol buffer, pH 10.3 (Zymed Laboratories, San Francisco, CA), substrate was then added. The plates were then incubated at 22°C for 3 h and read at 405 nM with a plate reader (Titertek MCC/340; ICN/Flow, Mississauga, Ontario). Antibody levels were expressed (μg/ml) by comparison to a standard curve.

Statistical Analysis. Statistical analysis was carried out using analysis of variance and the Wilcoxon ranked sum test. A p value of 0.05% or less was considered statistically significant.

Results

Survival. Mice infected with 1,000 PFU of MHV-3 (n = 16) all succumbed to the infection within 5 d (Fig. 1). This was consistent with previous data reported by our group as well as others (1, 2). In contrast, there was survival in some animals that were treated with ascites containing antibody to PCA (Fig. 1). Animals treated with ascites containing 25 μg/d had a 22% survival; 44% of those treated with ascites containing 50 μg/d and 100% of animals treated with 100 μg/d survived. All animals survived when treated with 100 μg of monoclonal anti-PCA (IgG2ak), which had been purified from hybridoma supernatant, confirming that the beneficial effect of ascites was due to the monoclonal anti-PCA. Mice immunized with 100 μl of ascites from SP2-injected animals and infected with MHV-3 all died within 5 d (data not shown).

Liver Histology. MHV-3-infected mice that did not receive antibody developed histologic evidence of severe liver disease. By 24 h p.i., small, discrete foci of necrosis with a sparse PMN infiltrate could be seen. At 48 h, these lesions became both more pronounced and more numerous (Fig. 2 *A*), and by 72–96 h, confluent liver necrosis was apparent (Fig. 2 *B*). In contrast, mice infected with MHV-3 but treated with antibody to PCA showed a marked reduction in liver disease in all groups (25, 50, and 100 μg) (Fig. 2 *C*) compared with nontreated and MHV-3-infected animals. Moderate necrosis was seen in animals treated with 25 μg of the mAb, whereas only a few foci of necrosis were observed in the animals treated with 50 μg of antibody. In the animals treated with 100 μg of anti-PCA, the livers appeared near normal, although there were a few small foci of inflammatory cells with no necrosis (Fig. 2 *D*). Morphometric image analysis showed that the proportion of the liver that was necrotic was significantly different between the MHV-3-infected, anti-PCA-treated, and untreated groups at 48, 72 and 96 h (Fig. 3). The histology of livers from all survivors at 10 and 14 d postinfection appeared normal. Fibrin deposits were seen in hepatic sinusoids as well as in areas of necrosis of untreated and MHV-3-infected mice (Fig. 4). In animals treated with 25 and 50 μg of anti-PCA, a marked reduction in fibrin was noted. No fibrin was seen in the livers of infected mice treated with 100 μg of mAb. Treatment with anti-PCA alone resulted in no detectable histological evidence of liver disease in noninfected animals.

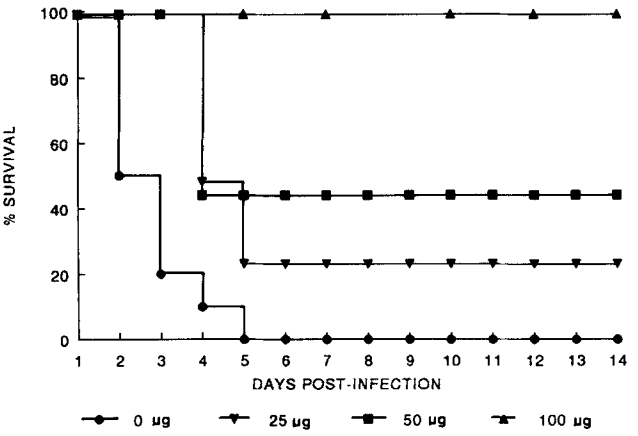

Figure 1. The effect of antibody to procoagulant activity (3D4.3) on survival of mice infected with murine hepatitis virus strain 3 (MHV-3). Mice were either not pretreated (●) or pretreated with 25 (▼), 50 (■), or 100 μg (▲) of mAb 3D4.3 for 7 d before infection with 10^3 PFU of MHV-3. Antibody was continued in treated animals for 7 d p.i. Survival was then studied (n = 9/group).

Viral Titers. By 24 h p.i., large amounts of infectious virus were recovered from liver homogenates of MHV-3-infected and untreated animals, and these persisted until the death of the animals (Fig. 5). In animals treated with 25 and 50 μg of antibody to PCA, there was no significant difference in viral titers from those observed in untreated mice at days 1 and 3. Viral titers remained high at day 5, however, viral titers decreased by day 7 and no virus could be detected after day 10. In animals treated with the highest dose of antibody to PCA (100 μg/d), viral titers were markedly reduced ($p < 0.05$) and approached those seen in resistant A/J mice (3, 5).

Using the method of Reed and Meunch, the MHV-3 recovered from infected and antibody-treated animals was as pathogenic as stock MHV-3 or virus recovered from infected and untreated mice (data not shown) (17).

PCA. In untreated, but MHV-3-infected animals, a sharp increase in splenic macrophage PCA was noted at 24 h p.i. (Fig. 6). Maximal PCA was seen at 48 h and PCA remained elevated until the animals' death on day 4. Animals treated with low concentrations of antibody to PCA (25 and 50 μg/d) expressed high amounts of PCA at early time points, but by day 7, PCA levels fell and only basal levels of PCA were detected in SMNC by day 10. In contrast, no expression of

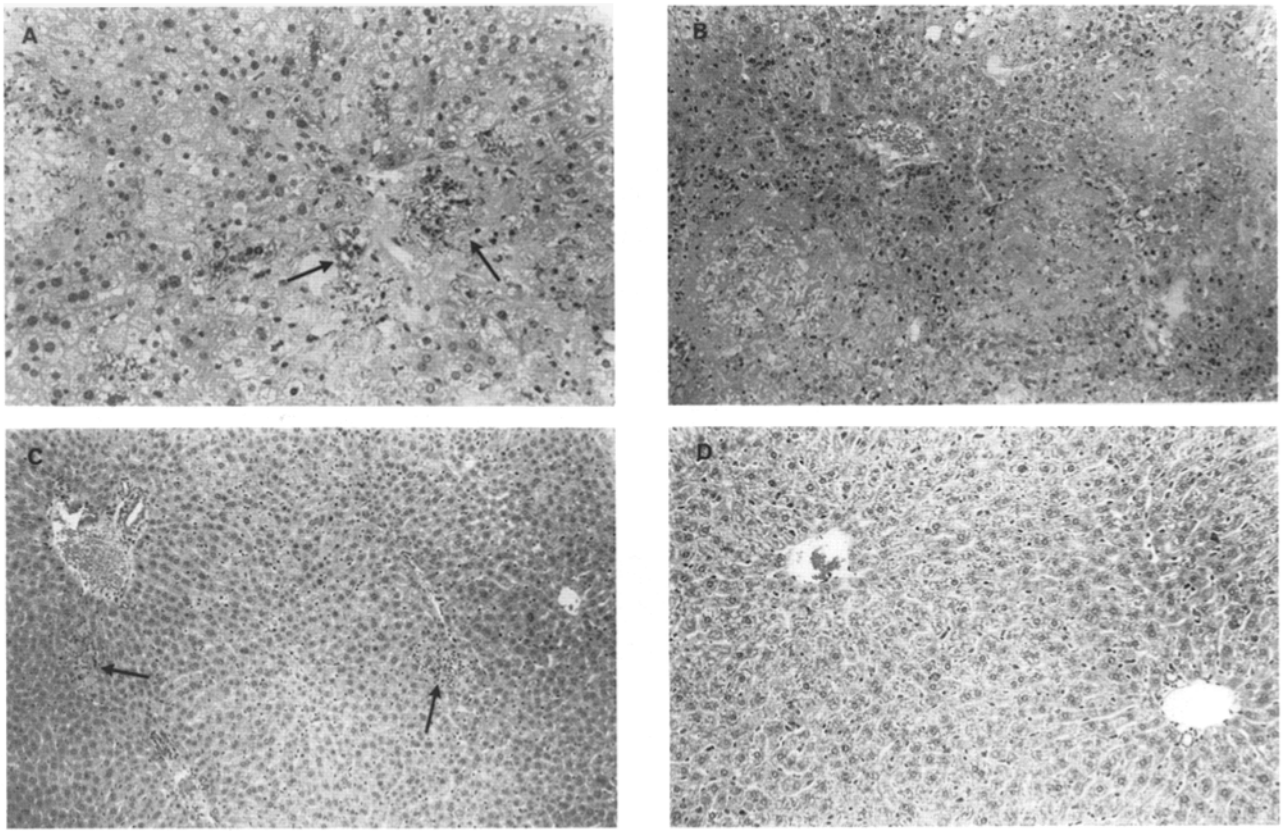

Figure 2. The effect of mAb 3D4.3 on liver histology after MHV-3 infection. Liver sections after infection with MHV-3 demonstrated scattered, small foci of inflammatory cells at 48 h (*A*), and widespread necrosis at 72 h (*B*). In contrast, pretreatment and continual treatment with the mAb 3D4.3 (100 μg/d) resulted in near normal histology at 48 h, with only an occasional focus of inflammation at 72 h (*C*), and normal histology at 7 d (*D*) (hematoxylin and eosin stain: ×225).

PCA could be detected in animals treated with 100 μg of antibody to PCA during the course of infection (Fig. 6).

Immunofluorescence. By direct immunofluorescence staining, PCA could be detected at 24 h in livers from MHV-3-infected and untreated mice. PCA was seen in areas of inflammation and necrosis, and also in hepatic sinusoids, localized primarily in endothelial cells and Kupffer cells, but not expressed by hepatocytes (Fig. 7). Livers from animals treated with antibody to PCA had significantly less PCA expression, although small amounts of PCA could be seen in macrophages and endothelial cells in hepatic sinusoids in animals treated with high-dose antibody to PCA (100 μg/d), even as late as day 14 (Fig. 7).

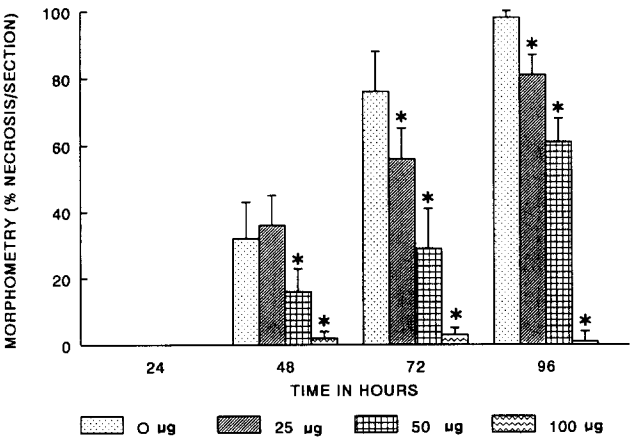

Figure 3. Morphometric analysis of liver histology. A marked difference in the proportion of disease to normal liver is seen at 48 h ($p < 0.05$), 72 h, and 96 h between untreated animals infected with MHV-3 and animals treated with 25, 50, or 100 μg of mAb 3D4.3 *Statistical significance as compared with untreated animals.

Antibody to PCA. Sera were collected from animals at all time points and analyzed in an ELISA for the presence of antibody to PCA as previously described (8). No antibody to PCA could be detected in sera from normal control animals or in animals infected with MHV-3 who had not received antibody to PCA (data not shown). Sera from animals treated with ascites containing 25–100 μg of antibody to PCA contained large amounts of anti-PCA before MHV-3 infection. After MHV-3 infection, the concentration of circulating antibody fell, but remained at >100 ng/ml in animals treated with 100 μg/d. In animals that were treated with 25 and 50 μg of antibody, by day 7, antibody was undetectable (<1 ng/ml) (Fig. 8).

Antibody to PCA either in ascites or purified from supernatants did not neutralize MHV-3 in a standard plaque reduction assay as previously described (8) (Table 1).

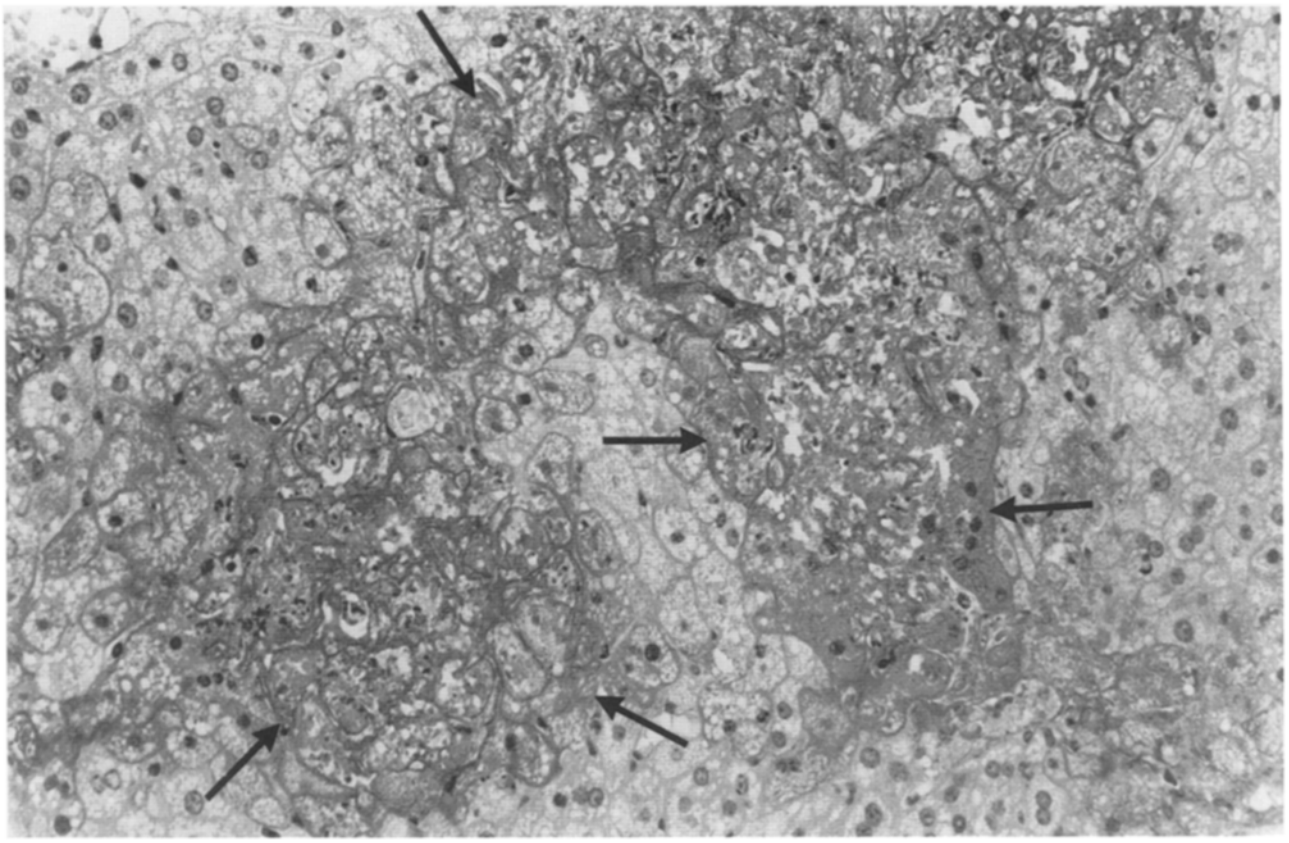

Figure 4. Deposition of fibrin in liver after MHV-3 infection. Large deposits of fibrin were observed in sinusoids and areas of necrosis (*arrows*) at 48 h after MHV-3 infection in untreated mice (Picro-Mallory Stain: ×425).

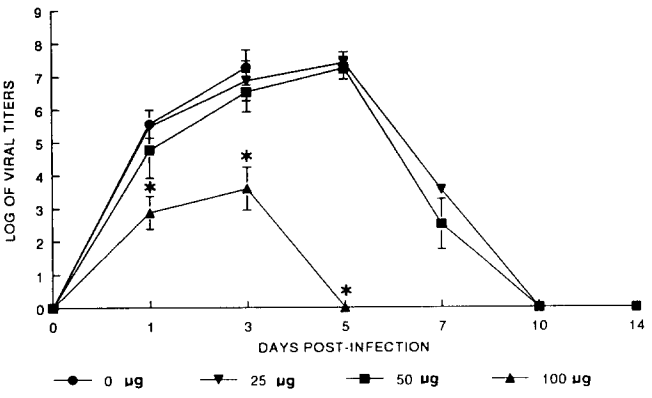

Figure 5. The effect of treatment with mAb to PCA on viral replication. High titers of virus were recovered from untreated and MHV-3-infected animals at all time points (●). In contrast, in a dose-dependent fashion, mAb 3D4.3 at concentrations of 25 (▼), 50 (■), and 100 µg (▲) attenuated the titer of virus recovered from the liver. In the animals treated with 25 and 50 µg of 3D4.3, no difference in viral titer was seen as compared to untreated mice on days 1, 3, and 5. However, titers of virus fell by day 7 and were not detected on day 10. Animals treated with 100 µg of 3D4.3 had statistically significantly lower levels of virus in their livers at all time points studied as compared with MHV-3-infected and untreated animals. *Statistical significance, $p < 0.05$.

To determine whether treated animals that had survived the acute infection developed long-term resistance to MHV-3 infection, mice previously infected with MHV-3 and treated with 100 µg of antibody (n = 15) were infected with 1,000 PFU of MHV-3, 21 d after their last exposure to virus. PCA antibody titers in this group were ~50% of that present during antibody therapy, consistent with known rates of disappearance of IgG. The MHV-rechallenged mice experienced a 50% mortality rate, suggesting that protection was by passive immunization of antibody, not an acquired, active immune process (Fig. 9). In addition, no antibody to MHV-3 was detected in these mice either before or after rechallenge with virus.

Discussion

MHV-3 infection produces fulminant hepatic failure and death in BALB/cJ mice (1, 3). The availability of a mAb to PCA that neutralized acceleration of coagulation in vitro (8) provided us an opportunity directly to examine the role of PCA in vivo in murine hepatitis virus strain 3 infection. All BALB/cJ mice which were infected with 10^3 PFU of MHV-3

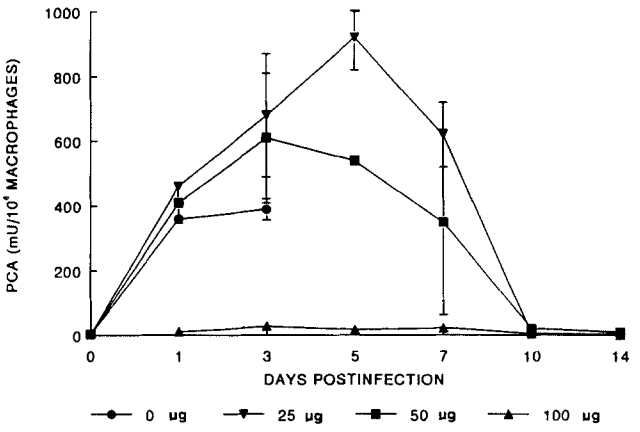

Figure 6. The effect of treatment with mAb 3D4.3 on MHV-3 induction of macrophage PCA in vivo. High amounts of PCA were seen in MHV-3-infected and untreated mice (●). Similarly, high titers of PCA were seen on days 1, 3, and 5 in animals treated with 25 (▼) and 50 μg (■) of antibody. By day 7, the PCA was falling and was no longer detected on days 10 and 14. Animals treated with 100 μg (▲) of mAb 3D4.3 had no detectable levels of PCA in splenic mononuclear cells. $p < 0.05$.

developed severe liver disease and died within 96–120 h. Administration of the mAb to PCA attenuated the hepatic necrosis, fibrin deposition and enhanced survival in a dose-dependent manner. All of the animals receiving 100 μg, and 44% and 22% of the animals that received 50 and 25 μg/d, respectively, survived for 10 d and made a full recovery. Furthermore, the same protective effect was seen in mice treated with IgG2ak purified from hybridoma culture supernatants (3D4.3). The increased survival of the treated animals was specific for anti-PCA, since ascites from mice injected with SP2 cells alone, which contained no antibody to PCA, failed to protect mice from MHV-3 infection. Although antibody-treated, MHV-3-infected animals demonstrated clinical evidence of viral hepatitis early in the course of the infection, by 7–10 d their behavior appeared normal and liver sections showed little or no apparent disease.

Examination of the livers of antibody-treated animals showed marked reduction in hepatic necrosis and inflammatory cells (neutrophils and mononuclear cells), each of which are prominent features of MHV-3 infection (3, 6, 7). PCA expression is a feature of MHV-3 infection in this mouse strain (2–4), and in untreated MHV-3-infected mice, PCA was detected by immunofluorescence staining in areas of necrosis as well as in hepatic sinusoids. Both macrophages and endothelial cells expressed detectable PCA, but hepatocytes did not. It is likely that the hepatic necrosis is secondary to ischemic changes resulting from induction of PCA, leading to the deposition of fibrin. Administration of mAb resulted in a dose-dependent reduction in the deposition of fibrin and in expression of PCA as detected by immunofluorescence staining in the liver and by a direct functional assay in SMNC.

Previously, we have shown that MHV-3 infection of peritoneal macrophages in vitro results in the production of PCA, TNF, leukotriene B_4 (18), and IL-1 (19). Thus, expression

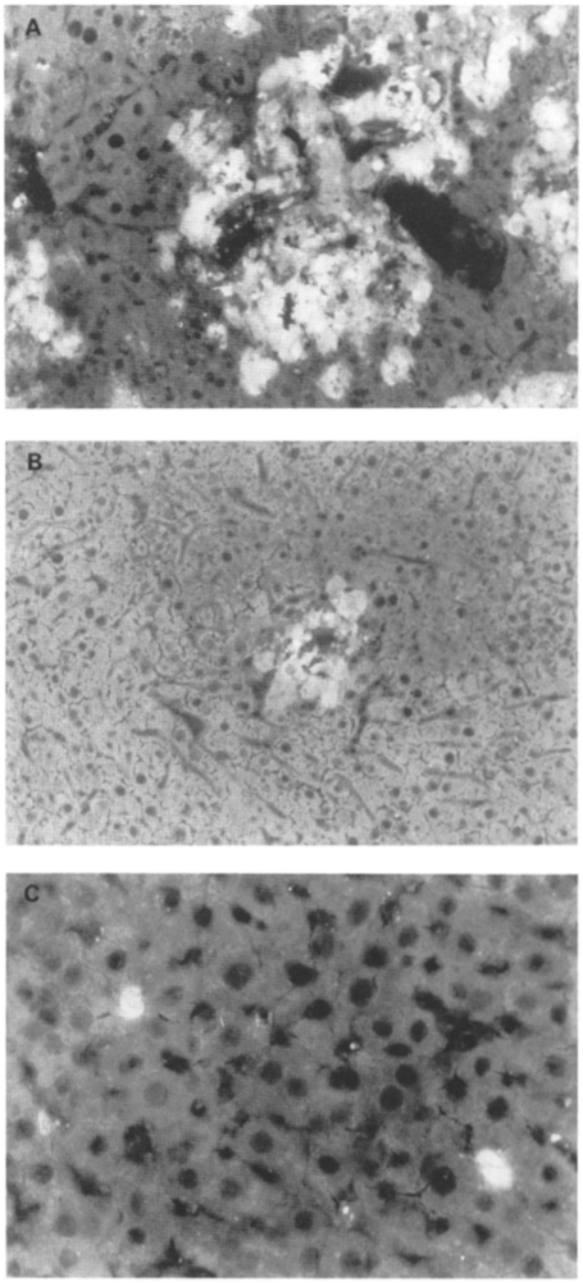

Figure 7. Direct immunofluorescence studies of livers from MHV-3-infected mice. (*A*) At 48 h, high amounts of PCA could be seen in areas of necrosis as well as in macrophages and endothelial cells in MHV-3-infected and untreated animals. (*B*) However, marked reduction in the amount of PCA was seen in 48 h in animals treated with 100 μg of mAb 3D4.3, and (*C*) at 10 d, only small amounts of PCA were seen in Kupffer cells and in endothelial cells in sinusoids with no PCA in hepatocytes (×500).

of PCA by endothelial cells might either be due to direct induction by MHV-3 or due to induction by IL-1 and/or TNF, which have previously been shown to induce PCA in endothelial cells in vitro (20, 21).

Treatment of mice with 100 μg of mAb to PCA not only increased survival and reduced hepatic necrosis, but also resulted

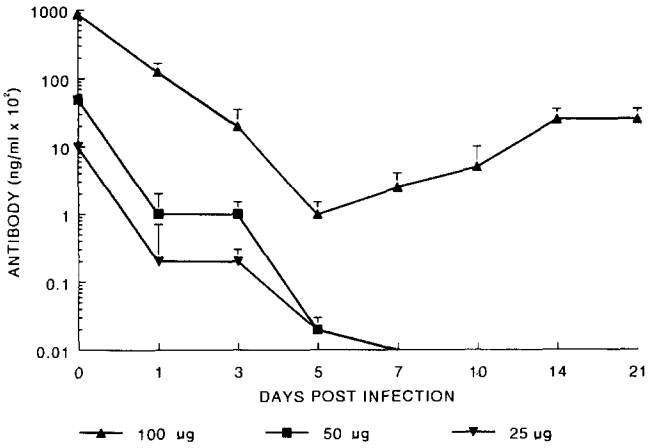

Figure 8. Antibody to PCA in serum from MHV-3-infected mice. Antibody to PCA was determined in a standard ELISA as described in Materials and Methods. Large amounts of antibody to PCA were detected before infection in mice treated with 100 (▲), 50 (■), and 25 μg (▼) of mAb 3D4.3. By day 7 p.i., antibody was only detected in mice treated with 100 μg/d. In contrast, no antibody to PCA was seen in animals infected with MHV-3, which were not treated with antibody (data not shown). The data represent the mean ± SD of three determinations from four different animals at each time point.

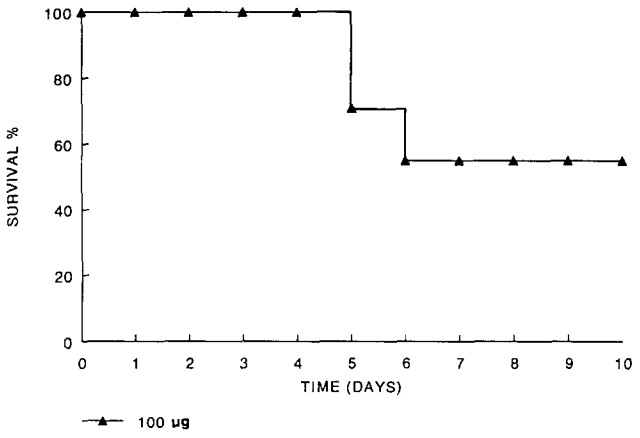

Figure 9. Effect of antibody 3D4.3 on survival to MHV-3 infection. Animals that survived MHV-3 infection and that had been previously treated with 100 μg of antibody (n = 15) were infected with 10^3 PFU of MHV-3 21 d after their first exposure to virus. Animals were then monitored for survival.

in a marked reduction of viral replication. In the untreated mice, high titers of virus were recovered early in the course of infection and persisted until death. The viral titers recovered from the livers of mice treated with 100 μg of antibody were much lower and similar to the amounts of virus recovered from the livers of MHV-3-infected resistant A/J strain mice (3).

One possible explanation for the decrease in viral replication observed in mice treated with antibody 3D4.3 is that this antibody reacts with the MHV receptor recently described by Holmes and coworkers (22, 23). We regard this explanation as unlikely, since 3D4.3 does not show any neutralization of viral infectivity in a plaque reduction assay, whereas antibody to the MHV receptor does inhibit infectivity. Furthermore, the MHV receptor has a molecular mass of 110 kD, considerably different than that of the PCA molecule (74 kD). A second possible explanation for the effect of anti-PCA antibody on MHV-3 growth in mice is related to the normal cleavage of the MHV S protein. It has been shown that cleavage of S by proteases is necessary to activate the membrane-fusing properties of the S protein (24). This fusion property facilitates the spread of virus to uninfected cells by cell-cell fusion and also increases the specific infectivity of MHV when compared with virus in which S has not been cleaved (24). It is possible that in infected macrophages, PCA, a serine protease, mediates at least in part the proteolytic cleavage of S into S1 and S2, and thereby activates the fusion properties of this molecule. Consistent with this idea is the observation that infection of A/J macrophages, which do not produce PCA in response to MHV infection, does not result in the appearance of syncytial giant cells (our unpublished observations). Thus, antibody to PCA could inhibit the spread of virus to uninfected cells by decreasing the activation of the fusion properties of S.

Although the mechanism by which anti-PCA treatment protects the susceptible animals is not clear, the use of antibody to PCA neutralizes PCA, thereby preventing activation of the coagulation system and inhibiting fibrin formation. The antibody could also result in complement-mediated destruction of MHV-3-infected cells that express membrane-bound PCA or promote macrophage activation with restriction of viral growth. In animals treated with 100 μg of antibody for 7 d before infection and 10 d p.i., concentrations of antibody to PCA in sera fell from 87 μg/ml before MHV-3 infection to 100 ng/ml during the active phase of disease (day 5), consistent with sequestration perhaps due to binding of the Ig to cells expressing PCA. Furthermore, in these mice, levels of PCA in SMNC remained at basal levels. In animals

Table 1. *Effect of 3D4.3 on Viral Replication*

Amount of antibody added	No. of plaques	Percent inhibition
μg		
0	105 ± 7	0
25	111 ± 12	0
50	99 ± 16	0
100	122 ± 14	0

100 plaques were added to L2 cells in presence or absence of antibody 3D4.3, and after 72 h, plaques were counted by crystal violet staining. Percent inhibition = 100 × [(A − B)/A]; where A = number of plaques on plates where no antibody was added, and B = number of plaques on plates where antibody 3D4.3 added. Polyclonal antibody to MHV-3 resulted in 100% plaque reduction to a titer of 1/10,000.

treated with lower amounts of antibody (50 or 25 μg/d) to PCA, hepatic necrosis and survival were only partially attenuated, and antibody was not detected (<1 ng/ml) after day 7 p.i. Together, the data strongly support the notion that anti-PCA antibody neutralizes PCA in vivo during the infection and that this may be the basis for its protective effect.

In a previous report we have demonstrated that dimethyl prostaglandin E_2 inhibited procoagulant activity and prevented fulminant viral hepatitis, yet all animals still succumbed to the infection (13). In PGE-treated mice, viral replication proceeded at a rate similar to that in untreated animals. Recently, we have demonstrated that although PGE_2 inhibited functional PCA, antigenic expression of PCA was not altered as determined by Western immunoblotting (25). We have proposed that PCA may exert its effect through activation of the coagulation system with microvascular and macrovascular thrombosis (6, 7), but the present results, in concert with the inhibition studies of PCA by PGE_2, suggest that PCA has other sites of action as well. The protective effects of the mAb to PCA occurred even though the BALB/cJ mice failed to mount an antiviral humoral response. Our observations are consistent with a recent report by Korner et al. (26) and support the notion that the murine antiviral antibody response may not be required for protection from acute viral infection.

Cytokines can play a potent role in the course of inflammatory injuries in vivo, and interference with their action can alter the course of certain inflammatory diseases (27, 28). Treatment of rats with recombinant antibody to TNF has been shown to protect animals from the hypotension, hypothermia, and mortality of Gram-negative sepsis (29), and treatment of rabbits with an IL-1R antagonist reduced the mortality associated with endotoxin shock (30, 31). Our studies support the notion that induction of PCA during MHV-3 infection in mice is an integral and potentially central step in the disease. A previous report by Taylor et al. (32) demonstrated that lethal *Escherichia coli* septic shock can be prevented by blocking tissue factor (a distinct procoagulant) with mAb, demonstrating the importance of cellular coagulants in the pathophysiology of other infectious diseases. We conclude that PCA is a potent inflammatory mediator that plays a pivotal role in hepatic injury resulting from MHV-3 infection.

This work was supported by a grant from the Medical Research Council of Canada (MA6787), grants from the Arthritis Society of Canada and Kidney Foundation of Canada, a grant-in-aid from the American Heart Association, and National Institutes of Health grant AI-31069.

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Received for publication 18 December 1991 and in revised form 15 May 1992.

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