ROLE OF MACROPHAGE PROCOAGULANT ACTIVITY IN MOUSE HEPATITIS VIRUS (MHV) INFECTION: STUDIES USING T CELL MHV-3 CLONES AND MONOCLONAL ANTIBODY 3D4.3

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I. INTRODUCTION

Murine hepatitis virus strain 3 (MHV) infection produces a strain dependent spectrum of disease[1,2]. Mice of the A and SJL strain are fully resistant to the effects of viral infection, whereas mice of semisusceptible strains (C3H/HeJ) develop acute hepatitis which progesses to varying degrees of chronic hepatitis. Mice of fully susceptible strains (Balb/cJ, C57BL/6J) die of fulminant hepatic failure. The resistance of the A strain mice cannot be explained by a lack of a cellular receptor for MHV as viral binding occurs on cells from these resistant mice[3,4]. Furthermore, restriction of viral replication does not explain resistance, as resistance occurs despite the presence of active viral replication in the resistant A strain. Bang and Warwick previously reported that differences in viral replication in cultures of macrophages reflects the relative susceptibility/resistance (S/R) to viral infection[5]. However, several laboratories have shown that MHV replicates in cultures of macrophages, endothelial cells and hepatocytes from both susceptible and resistant animals[4,6-8], although viral replication occurs to a lesser degree in cells derived from resistant animals[6,9]. Thus, absolute differences in viral replication do not account for the strain dependent S/R pattern seen in MHV infection.

Liver disease in this model correlates directly with the induction of macrophage procoagulant activity (PCA) by MHV both *in vivo* and *in vitro*[1,6,10]. Macrophages harvested from the fully susceptible strains of infected animals demonstrate significantly elevated levels of PCA. Furthermore, monocytes and peritoneal macrophages from uninfected mice can be induced to express marked increases in PCA following MHV stimulation *in vitro*. Macrophages from the semisusceptible strains of animals express intermediate levels of PCA, whereas in the fully resistant mice (A/J) which have no evidence of liver disease, the macrophages harvested from these mice express only baseline levels of PCA. *In vitro* stimulation of macrophages from the resistant animals with MHV does not elicit an increase in PCA above baseline. Thus, the induction of PCA by macrophages distinguishes between mice that are susceptible and those that are resistant to MHV infection.

MHV induction of PCA *in vitro* is rapid, with increases being observed within 1-1.5 hours, and maximal activity at 12-18 hours[1]. Viral growth in the susceptible 17 CL1 cell line is not detectable until 6 hours after infection, and not until 18 hours in

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monocytes/macrophages from susceptible Balb/cJ mice[1]. Therefore, the induction of PCA precedes the replication of infectious virus. Pretreatment of macrophages with actinomycin D and cycloheximide results in inhibition of PCA without affecting viral replication[11]. Thus, the induction of PCA is dependent upon host macrophage RNA and protein synthesis, and is not a viral protein product, although live virus is a prerequisite for its expression.

The induction of PCA in the susceptible animals corresponds to disturbances seen within the microcirculation of the liver[12,13]. Normal flow in the terminal vessels is swift and streamlined. In the sinusoids, erythrocytes and leukocytes traverse these spaces as single cells and travel so quickly that they cannot be seen clearly. Within 6-12 hours post-infection, velocity of flow is diminished, and by 24 hours aggregation of erythrocytes appear. Microthrombi within the microcirculation become prominent which progress to obliterate the sinusoids. By 48 hours, areas of focal necrosis can be seen corresponding to the areas of thrombosis, and by 72 hours, blood flow is marked ly reduced with obvious confluent necrosis. The initial abnormalities seen in the vascular bed precede in vivo viral replication by 24 hours. The deposition of fibrin is a prominent feature within the affected areas. These deposits are present in the hepatic sinusoids as well as in the areas of necrosis. Using a fluorescein-labeled anti-prothrombinase monoclonal antibody[14], increased expression of the enzyme can be seen within 24 hours in areas of inflammation and necrosis, and in the hepatic sinusoids. The prothrombinase is localized primarily on endothelial cells and Kupffer cells, but not on hepatocytes. In contrast, no discernible vascular or histological abnormalities can be seen in the livers of resistant A/J mice[15].

II. Lymphocyte Response to MHV Infection and Regulation of Macrophage PCA *In vitro*

The proliferative response of splenic mononuclear cells (SMNC) from resistant and susceptible mice differs significantly in response to MHV infection[16]. SMNC from naive Balb/cJ mice are completely unresponsive to MHV stimulation, whereas, SMNC from the resistant A/J mice demonstrate an effective proliferative response that peaks at 7-9 days. Priming of resistant mice with MHV resulted in an augmented and earlier response (2-6 days) compared with the naive mice. These differences in the SMNC proliferative response between the resistant and susceptible strains is further emphasized by the absence of IL-2 production by lymphocytes from the Balb/cJ mice when infected with MHV. In contrast, mononuclear cells from resistant A/J mice effectively produce IL-2 after stimulation with MHV.

Recently, T helper cell subpopulations have been identified on the basis of cytokine secretion profile[17,18]. TH1 cells have been shown to produce IL-2 and interferon gamma (IFN-γ), while TH2 cells produce IL-4 and IL-5. Resistant A/J mice have now been shown to produce a predominantly TH1 cell population following MHV infection[19]. TH1 T cell clones generated from mice following immunization with the neurotropic coronavirus JHMV are capable of conveying significant protection to animals infected with the virus[20]. It is possible that the generation of a TH1 population accounts for the elimination of the virus in this strain.

These findings are consistent with other models of resistance and susceptibilty to invading pathogens[21-25]. In leishmaniasis infection, the resistance or susceptibility of mice depends upon the the relative preponderance of the T helper cell populations, with resistance being dependent upon the animal's ability to generate TH1 cells[26]. Treatment of resistant mice with anti-interferon results in a switch of TH1 to TH2 cells, decreased levels of IFN-γ , increased levels of IL-4 and IL-5, and conversion to a susceptible phenotype[27]. Therefore, it is very likely that IFN-γ plays a pivotal role in development of resistance to this infection.

These differences in lymphocyte response to MHV infection are significant in the regulation of PCA[11,16]. When macrophages from susceptible mice are stimulated with MHV in the presence of CD4+ syngeneic lymphocytes, the levels of PCA are 5-6 fold higher than the levels seen if the macrophages were stimulated with MHV alone. However, macrophages from resistant mice continue to express baseline levels of PCA when stimulated with MHV alone or in the presence of lymphocytes from H-2

compatible susceptible mice (Table I). In contrast to the augmentation in PCA seen in macrophages cultured with lymphocytes from susceptible mice, induction of PCA can be abrogated by lymphocytes from H-2 compatible resistant mice (Table II). This inhibition occurs in an antigen specific fashion as only lymphocytes from immunized resistant RI mice downregulated the induction of PCA in macrophages from susceptible mice. Lymphocytes harvested from naive animals did not have any regulatory effect on PCA induction[11].

Table 1. Role of Lymphocytes in Induction of PCA by MHV-3

Lymphocytes	(Macrophage)		
	AXB5 (R)	AXBl(SS)	AXB3)S)
AXB5(R)	690 ± 300	4900 ± 2100	10400 ± 2100
AXBl(SS)	745 ± 210	14800 ± 2900	21800 ± 1600
AXB3(S)	910 ± 200	51500 ± 9100	69400 ± 8400

Data are expressed as mU/10^6 macrophages and is total content PCA. 5 x 10^5 macrophages were stimulated with 10^6 PFU of MHV-3 in the presence of 3 x 10^6 Thy 1.2 + lymphocytes. R, resistant; S, susceptible; SS, semisusceptible

Table 2. Suppression of Induction of PCA in Susceptible AXB3 RI Mice by Lymphocytes from MHV-3 Primed Resistant AXB5 RI Mice

Ratio of Lymphocytes (AXB5/AXB3)	PCA (mU/10^6macrophages)	
	Source of Lymphocytes	
	Naive	Immunized
0	54150+4200	55450+6100
1	56550+2180	51800+3200
2	53500+2500	40400+2200
3	52000 +3000	17550+ 1800
4	54500+4500	640+550

5 x 10^5 macrophages from susceptible AXB3 mice were stimulated with 10^6 PFU of MHV-3 in the presence of 3 x 10^6 host lymphocytes and increasing numbers of splenic lymphocytes from resistant AXB5 mice which had been primed (immunized) or not primed (naive) with 10^7 PFU of MHV-3 14 days prior to harvesting of lymphocytes. Following a 12 hour incubation cells were frozen and assayed for total content PCA.

Five T cell lines were derived from draining popliteal lymph nodes from resistant A/J mice, which had been immunized with MHV-3. These cell lines were maintained in culture by periodic stimulation with antigen presenting cells, MHV-3 and T cell growth factors. MHV structural proteins were expressed in MC57 cells utilizing recombinant vaccinia virus vectors. Three cell lines stimulated with recombinant full length MHV-3 structural proteins in the presence of syngeneic antigen presenting cells proliferated to the nucleocapsid protein whereas 2 cell lines proliferated only to live MHV-3. By FACS analysis for cell lines were CD4+ and one was CD8+. All CD4+ lines produced IL-2 and two produced IFN-γ (TH1).

One TH1 T cell clone generated from the MHV-immunized animals was shown to downregulate PCA induction[19] (Table III). Furthermore, using a series of transwell cell culture experiments, the inhibitory mechanism of these T cell clones was shown to involve a soluble mediator. However, a cell-to-cell inhibitory action could not be excluded as the induction of PCA was similarly inhibited when the clones and the macrophages were in direct contact. It is possible that IFN-γ plays a role by way of its direct anti-viral effect, or by facilitating macrophage elimination of the virus. For example, the treatment of resistant A/J mice with anti-IFN-γ and other immunosuppressive agents results in susceptiblity of the animal to MHV infection and death of the animal[28].

Table 3. Relationship of Induction of PCA to Development of Hepatitis in Inbred Strains of Mice Following MHV Infection

Strain of Animal	Disease	Induction of PCA
A/J	−	−
C3H	+	+
Balb/cJ	+++	+++

Currently, attempts to produce T cell clones from susceptible Balb/cJ mice have been unsuccessful, due partly to the sensitivity of this strain to MHV which is required for priming, and partly due to the poor proliferative response of the lymphocytes to in vitro stimulation, both to live and UV-irradiated virus. However, it is possible that these animals produce primarily a TH2 cell response to MHV infection, which is consistent with the lack of IL-2 production by the MHV-stimulated lymphocytes[16]. Expansion of TH2 cells results in the production of IL-4 and IL-10[23,29,30] which inhibit the mechanisms necessary for elimination of the virus. This could account for the inability of the Balb/cJ mice to survive MHV infection. Furthermore, Lamontagne has shown that MHV infection in susceptible mice results in thymic atrophy with depletion of all T lymphocyte populations, although there may be a greater depletion of CD4+ CD8- T cells[31]. If there was a greater depletion of TH1 cells, this would support the hypothesis that alterations in regulatory T cells accounts for differences in susceptibility and resistance.

CD4+ T cell lines generated from susceptible C57BL/6 mice are capable of inducing a strong delayed-type hypersensitivity (DTH) reaction[32,33]. As the induction of PCA has been linked to the DTH reaction[1,2], this finding is consistent with the a model of PCA, DTH, and T cell regulation.

III. Inhibition of PCA by Anti-Prothrombinase Monoclonal Antibody

Recently, a panel of monoclonal antibodies (mAb) specific for the MHV-induced procoagulant, prothrombinase, has been produced in our laboratory[25]. These antibodies do not react with rodent, rabbit or human tissue factor (TF), or MHV. Most of the mAb inhibit PCA expression in a one stage clotting assay and inhibited the conversion of prothrombin to thrombin, supporting the concept that MHV-induced PCA is a prothrombinase. However, all of the mAb tested react with proteins of 140, 74, and 70 kD on non-reduced gels, and 74 and 70 kD on reduced gels. (Fig.1). This would suggest that the antibodies recognize different epitopes on the protein, and that most of the epitopes are on the protein's functional structure. The molecular weights of the proteins recognized by these mAb are clearly distinct from TF which is a 47 kD protein[34]. Immunofluorescence studies demonstrate that the mAb bind only to MHV-stimulated macrophages that functionally express elevated levels of PCA.

Infusion of one of the high titred neutralizing mAbs into susceptible mice infected with 10^3 plaque forming units of MHV results in a dose dependent increase in survival, with a 100% survival in animals receiving 100 μg of the mAb. (Fig. 2.) All untreated, MHV-infected mice died within 96 hours. In the mice receiving the mAb, the hepatic necrosis, inflammatory cell infiltrate, and fibrin deposition was markedly attenuated; functional expression of macrophage PCA was markedly decreased, and by immunofluorescence staining, livers from the mAb-treated animals had diminished antigenic expression of prothrombinase in macrophages and endothelial cells in the hepatic sinusoids. In contrast to the livers of untreated mice in which there was exten-

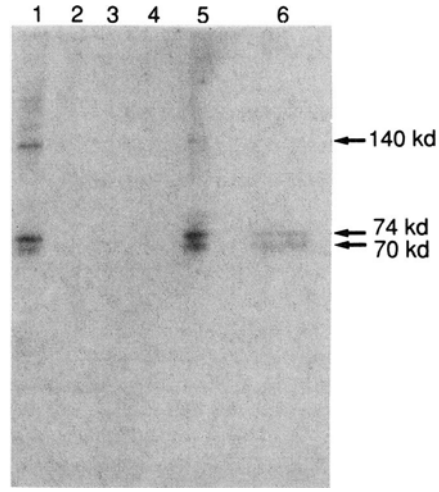

Figure 1. Immunoblot analysis of MHV-3-induced prothrombinase. 1 μg of nonreduced (lanes 1 and 5) or reduced (lane 6) MHV-3-induced macrophage prothrombinase, nonreduced unstimulated macrophages that were devoid of prothrombinase (lane 2), purified MHV-3 (lane 3), or mouse crude tissue factor (lane 4) was resolved by electrophoresis on a 4-20% polyacrylamide gel, transferred to nitrocellulose and reacted with a 1/10 dilution of hybridoma culture supernatant 3D4.3.

sive fibrin deposition in the sinusoids and in the areas of necrosis, animals treated with the mAb had a dose-dependent reduction in the deposition of fibrin. No fibrin was seen in the animals receiving the highest dose of mAb (100 μg). In the treated animals, there was a rapid fall in the titre of antibody to prothrombinase, consistent with sequestration due to binding of the immunoglobulin to cells expressing the prothrombinase[15].

In animals treated with lower doses of mAb (25 and 50 μg), there were no differences in viral titres from those observed in untreated mice early in the course of the infection. (Fig. 3). However, in the surviving mice, there was complete elimination of the virus by the 10th day post-infection. In the animals that received the highest dose

(100 μg) of mAb, viral titres were significantly reduced in the first 3 days, and approached those titres seen in resistant A strain mice, with elimination of the virus by the fifth day post-infection[15].

One possible explanation for the decrease in the viral replication seen in the treated mice is that the antibodies are reacting with the MHV receptor. This is not likely in that the anti-prothrombinase mAb do not demonstrate any in vitro neutralizing of viral infectivity in plaque reduction assays[14], whereas antibodies to the MHV receptor inhibit infectivity[35]. Furthermore, the MHV receptor has a molecular weight of 110 kD which is different from the prothrombinase molecule (140 kD)[35]. A second possibility for the in vivo inhibition of viral replication in the infected mice by the mAb may be related to the normal cleavage of the MHV S protein. Cleavage of the S

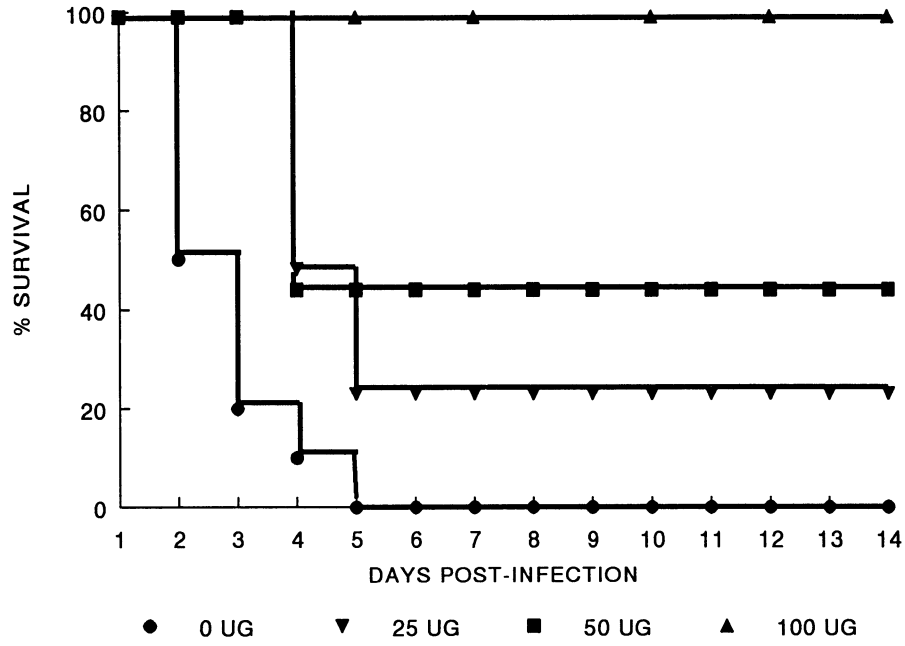

Figure 2. The effect of antibody to procoagulant activity (3D4.3) on survival of mice infected with murine hepatitis virus strain 3 (MHV-3). Mice were either not pretreated (●) or pretreated with 25 (▼), 50 (■), or 100 μg (▲) of mAb 3D4.3 for 7 days before infection with 10^3 PFU of MHV-3. Antibody was continued in treated animals for 7 days p.i. Survival was then studied (n=9/group).

protein by proteases is necessary to activate the membrane fusing properties of the S protein, and thus the virus itself[36,37]. This fusion property facilitates the spread of virus to uninfected cells by cell-cell fusion. It is possible that the MHV-induced prothrombinase, a serine protease, mediates at least in part the proteolytic cleavage of the S protein. This is consistent with the observations that infection of macrophages from A strain mice, which does not result in induction of PCA, does not result in syncitia formation, and that inhibition of PCA of macrophages from Balb/cJ mice with PGE also reduces syncitia formation[9,38].

The mechanism of the protective effect of the anti-prothrombinase mAb is not entirely known. However, utilization of the mAb results in the inhibition of PCA which correlates with the prevention of activation of the immune coagulation system

and deposition of fibrin. In concordance with these observations are the findings of Taylor et al. who attenuated the effects of septic shock using mAb against TF.[39] Thus, it seems reasonable to conclude that interference and inhibition of the action of PCA plays an important role in abrogation of the disease.

IV. CONCLUSION

The interactions between the virus and the host result in a complex series of events that result in either resolution of the infection, or death of the host. Differences in viral processing, generation of second messengers, and production of inflammatory mediators and cytokines contribute to the disease outcome.

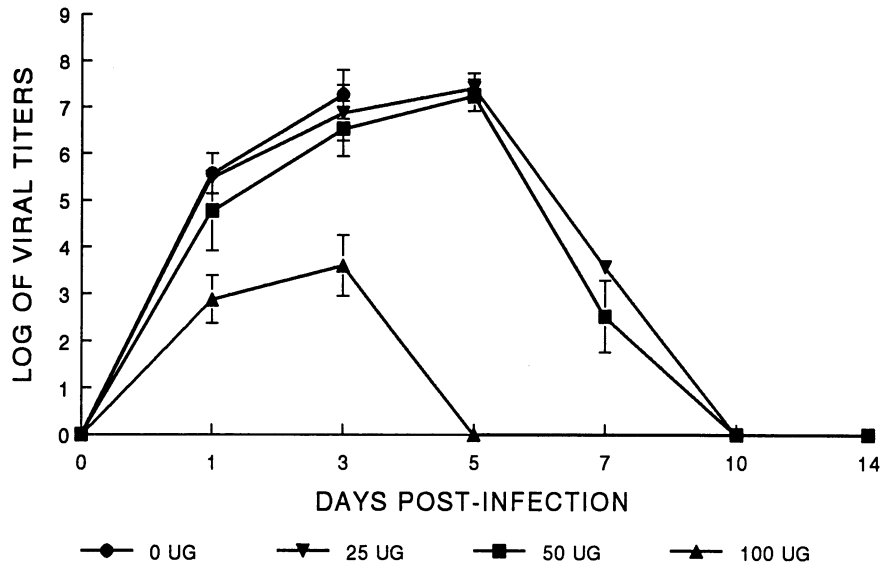

Figure 3. The effect of treatment with mAb to PCA on viral replication. High titers of virus were recovered from untreated and MHV-3-infected animals at all time points (●). In contrast, in a dose-dependent fashion, mAb 3D4.3 at concentrations of 25 (▼), 50 (■), and 100 μg (▲) attenuated the titer of virus recovered from the liver. In the animals treated with 25 and 50 ug 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 ug of 3D4.3 had statistically significantly lower levels of virus in thier livers at all time points studied as compared with MHV-3-infected and untreated animals. *Statistical significance, $p<0.05$.

The activation of the immune coagulation system with the induction of monocyte/macrophage PCA by MHV has been shown to be important in the pathogenesis of MHV-induced hepatitis. Modulation of PCA can result in both abrogation of the liver pathology, and survival of the susceptible animal. Prevention of mortality in MHV-infected susceptible mice using a specific anti-prothrombinase mAb that neutralizes PCA underscores the importance of PCA in the pathogenesis of MHV-induced fulminant hepatitis. Although PGE also inhibits PCA and prevents liver necrosis in MHV-infected mice, it has many other non-specific immunosuppressive properties which may contribute the paradoxical observation of mortality despite hepatoprotection.

Regulation of MHV-induced PCA resides at the level of both macrophage and lymphocyte. Absolute restriction for the induction of PCA is at the level of the macrophage but T lymphocyte cooperation is required for final expression of PCA.

The relative balance of TH1/TH2 T cell subpopulations in response to MHV infection may be an important determinant in resistance or susceptibility and development of liver disease. Methods of altering the relative T cell subpopulations could influence the induction of macrophage PCA and affect the outcome of MHV infection.

Information gained in the study of this model will further the understanding of the mechanisms of induction and regulation of PCA during MHV infection. As the expression of PCA by macrophages and endothelial cells has been implicated in the pathogenesis of several human diseases, knowledge gained will ultimately lead to better treatment of diseases in which PCA has been shown to be involved.

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