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Loss of Resistance to Murine Hepatitis Virus Strain 3 Infection after Treatment with Corticosteroids Is Associated with Induction of Macrophage Procoagulant Activity

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Activation of the immune coagulation system has been implicated in the pathogenesis of liver injury following infection of inbred mice with murine hepatitis virus strain 3 (MHV-3). Following MHV-3 infection, macrophages isolated from MHV-3-susceptible and -semisusceptible inbred strains of mice express increased procoagulant activity (PCA), whereas macrophages from resistant strains express no increase in PCA over basal levels. The PCA induced by MHV-3 is a prothrombinase, encoded by the gene *Fgl-2*, which encodes a fibrinogen-like protein (musfiblp). In this study, MHV-3-resistant A/J mice treated with methylprednisolone prior to infection with MHV-3 developed elevated levels of alanine aminotransferase in serum and died within 10 days of infection, with histological findings of fulminant hepatitis. In vitro, macrophages isolated from A/J mice and pretreated with methylprednisolone produced a marked increase in functional PCA following infection with MHV-3. The PCA was shown to be a prothrombinase by its ability to cleave ^{125}I-prothrombin. Northern blot analysis of RNA transcripts from these macrophages demonstrated increased transcription of the *Fgl-2* gene relative to that in macrophages which had not been pretreated with methylprednisolone prior to MHV-3 infection. Methylprednisolone pretreatment of MHV-3-infected macrophages stabilized the *Fgl-2* mRNA. Thus, loss of resistance to MHV-3 secondary to methylprednisolone therapy is associated with increased transcription and stability of *Fgl-2* mRNA resulting in expression of the *Fgl-2* gene product, musfiblp. These results provide further insight into mechanisms of PCA regulation in response to MHV-3 infection in inbred strains of mice.

Fulminant viral hepatitis is a rapidly progressive disease which is associated with high mortality (18). Although the pathogenesis is unknown, there is evidence supporting a role for activation of the immune coagulation system and secondary microcirculatory disturbances with resultant intravascular thrombosis in the liver (4, 17, 26–28). Agents which interfere with activation of the classical or immune coagulation system have been shown to be beneficial in the setting of fulminant hepatitis (1, 32, 37).

Infection of inbred mice with murine hepatitis virus strain 3 (MHV-3), a member of the coronavirus family, produces a strain-dependent spectrum of liver disease which has been utilized as a model of fulminant hepatitis (19, 24, 43). Following infection with MHV-3, BALB/cJ and C57BL/6J mice develop a fatal hepatitis characterized histologically by sinusoidal thrombosis and hepatocellular necrosis, similar to that seen in humans with fulminant viral hepatitis, whereas A/J mice develop neither histological nor biochemical evidence of liver injury (19, 21, 25). Three lines of evidence suggest that activation of the immune coagulation system by MHV-3, as evidenced by increased macrophage/monocyte procoagulant activity (PCA), participates in the disease process in MHV-3-related hepatitis. First, induction of monocyte/macrophage PCA during MHV-3 infection correlates with the severity of liver injury (7, 20). Second, there is a genetic linkage between induction of PCA in response to MHV-3 and susceptibility to liver disease (8). Finally, treatment of susceptible mice with a monoclonal antibody to MHV-3-induced prothrombinase prevents the development of MHV-3-related fulminant hepatitis (22).

We recently reported the molecular cloning and sequencing of a cDNA isolated from a library prepared from macrophages which had been infected with MHV-3 (29). The sequence of this cDNA is essentially identical to a previously described sequence corresponding to a gene encoding a mouse fibrinogen-like protein (musfiblp) (16). Recently, this gene has been renamed *Fgl-2*. In macrophages, *Fgl-2*-specific mRNA is not normally expressed. However, following infection, expression can be detected in macrophages derived from resistant as well as susceptible strains of mice, with expression of this gene occurring earlier and in significantly greater amounts in macrophages derived from susceptible strains (29). Transfection of the RAW 264.7 cell line, a BALB/cJ macrophage continuous cell line which does not express PCA following exposure to MHV-3, with a cDNA containing the entire coding region of *Fgl-2* resulted in expression of functional PCA with prothrombinase activity following infection, confirming that *Fgl-2* is the gene encoding MHV-3-induced PCA (29).

It has been demonstrated that a loss of resistance and 100% mortality occur in mice from resistant strains which are treated with corticosteroids prior to infection with MHV (5, 11). However, the mechanism for this loss of resistance is not known. The current studies were initiated to determine whether the loss of resistance following corticosteroid therapy is associated with induction of *Fgl-2* gene transcription and PCA expression in resistant A/J mice.

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MATERIALS AND METHODS

Mice. Female A/J mice aged 6 to 8 weeks were purchased from Jackson Laboratories (Bar Harbor, Maine), housed in the animal colony at the University of Toronto, and fed a standard chow diet and water ad libitum prior to and during studies. Animals were maintained in microisolation cages, and serologic tests for evidence of prior MHV infection were consistently negative.

Cells and virus. The origin and growth of 17CL1, DBT, and L2 cells have been described previously (14, 33, 39). MHV-3 was obtained from the American Type Culture Collection, Rockville, Md., and plaque purified on monolayers of DBT cells. Stock virus was grown to a titer of 4.8×10^7 PFU/ml in 17CL1 cells, harvested by one cycle of freeze-thawing, and clarified by centrifugation at $4,500 \times g$ for 1 h at 4°C. Viral titers were determined on monolayers of L2 cells in a standard plaque assay (23, 39).

Chemicals. Methylprednisolone sodium succinate (Solu-medrol; Upjohn Co., Don Mills, Ontario, Canada) used in the in vitro studies was dissolved in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) at concentrations of 3.75 to 150 μg/ml, while methylprednisolone used in the in vivo studies was dissolved in sterile distilled water at a standard concentration of 10 mg/ml. All samples were filter sterilized with Millex-GS 0.22-μm-pore-size filter units (Millipore Products, Bedford, Mass.) and stored at 4°C until use.

Peritoneal macrophages. Peritoneal macrophages, harvested from A/J mice 4 days after intraperitoneal administration of 1.5 ml of 3% aged Brewer's thioglycolate pH 6.9 (Difco Laboratories, Detroit, Mich.), were suspended in RPMI 1640 supplemented with 2 mM glutamine (Sigma Chemical Co., St. Louis, Mo.) (RPMI 0) as previously described (38). Cell suspensions contained greater than 95% macrophages as determined by morphology and nonspecific esterase staining, with viability exceeding 98% by trypan blue exclusion. Viability was not affected by incubation with up to 120 μg of methylprednisolone per ml (data not shown).

Biochemistry. Serum was analyzed quantitatively for alanine aminotransferase activity with a Worthington Statzyme GPT kit obtained from Cooper Biomedical, Inc., Malvern, Pa. (13).

Histology. Liver tissue which had been fixed by immersion in 10% formalin in 0.1 M phosphate buffer, pH 7.4, was dehydrated in graded alcohols and xylene and embedded in paraffin. Sections were cut and stained with Harris' hematoxylin and counterstained with eosin Y. The sections were subsequently washed with distilled water, dehydrated in graded alcohols and xylene, and mounted with Permount. Tissue sections were assessed for the presence of necrosis and inflammatory cell infiltration as previously described (22).

Immunofluorescence. Samples of liver tissue were snap frozen in liquid nitrogen and cut into sections approximately 4 μm thick. Sections were fixed for 5 min in acetone and air dried for 2 h, following which unoccupied sites were blocked with 5% horse serum in phosphate-buffered saline (PBS), pH 7.4. Following 2 h of incubation, samples were stained for 1 h with 3D4.3, an antiprothrombinase monoclonal antibody recently isolated in our laboratory (13), and conjugated with fluorescein isothiocyanate (Sigma Chemical Co.) as previously described (22, 40). The liver tissue samples were then washed three times, mounted in 90% glycerol in PBS, and examined on a phase-epifluorescence microscope equipped with a 40× Fluotar objective (E. Leitz, Inc., Rockleigh, N.J.).

PCA studies. Macrophages, suspended at a concentration of 10^6 cells per ml in RPMI 1640, were treated with either 0 or 100 μg of methylprednisolone per ml and 30 min later were infected with MHV-3 at a multiplicity of infection of 1.0. Similarly, macrophages were pretreated with either 0 or 100 μg of methylprednisolone per ml without being infected with MHV-3. Following incubation periods of up to 8 h, cells underwent a single freeze-thaw cycle and total content PCA was assayed by determining the ability of the suspensions to accelerate the spontaneous clotting time of normal recalcified human platelet-poor plasma as previously described (1). Results were quantitated by comparison with serial dilutions of standard rabbit brain thromboplastin (Sigma Chemical Co.). Medium and reagents were without procoagulant activity.

Prothrombin cleavage assays. Peritoneal macrophages, suspended at 4.0×10^6 cells per ml in RPMI 1640, were pretreated with 0 or 100 μg of methylprednisolone per ml and, 30 min later, infected with MHV-3 at a multiplicity of infection of 1.0. Following incubations of 6 h, 0.05-ml aliquots of these macrophages were incubated for 1 h at 37°C with 0.01-ml aliquots of 25 mM $CaCl_2$ and 0.01 ml of 100 nM ^{125}I-prothrombin which had been prepared as previously described (10, 35). Human factor Xa in the presence of Russell's viper venom was used as a positive control for ^{125}I-prothrombin cleavage. Reactions were terminated by addition of EDTA and sodium dodecyl sulfate (SDS) to achieve a 1% concentration, and the products were analyzed by electrophoresis on 10% polyacrylamide–0.1% SDS gels followed by fixing and drying of the gel and, finally, autoradiography (10, 35).

Northern (RNA) blot analysis. Macrophages were harvested from A/J mice and suspended at a concentration of 2.0×10^6 cells per ml in RPMI 1640, preincubated for 30 min in the presence or absence of 100 μg of methylprednisolone per ml, and infected with MHV-3 at a multiplicity of infection of 1.0. Following incubations of up to 6 h, macrophages were washed two times with cold 0.1 M PBS, pH 7.2, and pelleted in polypropylene tubes (17 by 100 mm; Falcon, Becton Dickinson Labware, Lincoln Park, N.J.). Total cellular RNA was isolated by using 8 M guanidine hydrochloride (9). RNA was resolved in a 1% agarose gel containing formaldehyde and transferred onto a nitrocellulose membrane (Bio-Rad, Oakville, Ontario, Canada).

A 1.3-kb *Fgl-2* cDNA was excised, separated on an agarose gel, and purified (29). The probe was labelled by using a random-primer DNA-labelling system (^{T7}QuickPrime Kit; Pharmacia Inc., Montreal, Quebec, Canada) with [α-^{32}P] dCTP (specific activity, >3,000 Ci/mmol; Amersham, Mississauga, Ontario, Canada). Membranes were prehybridized for 6 h at 42°C in a solution containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, 100 μg of denatured salmon sperm DNA per ml, and 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) buffer. Hybridization was subsequently carried out overnight at 42°C in the same solution with the addition of radiolabelled probe. The membranes were then washed twice for 15 min each time at room temperature with 5× SSPE–0.5% SDS; this was followed by two additional washes each for 15 min at 37°C with 1× SSPE–0.5% SDS and finally three washes for 15 min each at 65°C with 0.1× SSPE–1.0% SDS. Membranes were dried and exposed to Kodak XAR-5 film with intensifying screens for 24 h at −70°C. To confirm that equivalent amounts of RNA were loaded in each lane, membranes were probed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (41).

RNA stability studies. Peritoneal macrophages, suspended at a concentration of 2.0×10^6 cells per ml in RPMI 0, were infected with MHV-3 at a multiplicity of infection of 1.0 following a 30-min preincubation in the presence or absence of 100 μg of methylprednisolone per ml. Following a 6-h incubation, actinomycin D (10 μg/ml), an inhibitor of gene transcription, was added, and after further incubation for up to 6 h, samples were harvested, washed twice in ice-cold 0.1 M PBS (pH 7.2), and pelleted in polypropylene tubes (17 by 100 mm). Northern blot analysis was carried out as described above. Quantitation was performed with a Betagen scanner (29).

Statistical analysis. Data are expressed as means ± standard deviations. Statistical analysis utilized Student's *t* test or analysis of variance when appropriate. A *P* value of 0.05 or less was considered statistically significant.

RESULTS

In vitro studies. (i) Effect of methylprednisolone pretreatment on induction of PCA in response to MHV-3 infection in vitro. A/J macrophages infected with MHV-3 in vitro without prior treatment with methylprednisolone or treated with methylprednisolone and not infected with MHV-3 expressed only baseline levels of PCA, similar to levels expressed by macrophages which were neither treated with methylprednisolone nor infected with MHV-3. However, macrophages which were pretreated for 30 min with 100 μg of methylprednisolone per ml prior to MHV-3 infection expressed significantly increased levels of PCA, with peak PCA levels of 3,300 ± 980 mU/10^6 macrophages recorded following 6 h of incubation with MHV-3 ($P < 0.05$) (Fig. 1).

In subsequent experiments, macrophages, suspended at a concentration of 10^6 cells per ml, were treated with increasing concentrations of methylprednisolone prior to infection with MHV-3 and incubated for 6 h. Pretreatment of macrophages with methylprednisolone resulted in a dose-dependent augmentation of the PCA response to MHV-3. Pretreatment for 30 min with methylprednisolone at a concentration equal to or greater than 3.75 μg/ml followed by incubation for 6 h with MHV-3 resulted in an augmented macrophage PCA response. At a concentration of methylprednisolone of 3.75 μg/ml, the PCA response was 1,695 ± 378 mU/ml, compared with 135 ± 40 mU/ml in macrophages incubated with MHV-3 without pretreatment with methylprednisolone ($P < 0.05$). With increasing concentrations of methylprednisolone, the PCA response to MHV-3 continued to rise to a maximal PCA response of 3,350 ± 88 mU/ml following treatment with 120 μg of methylprednisolone per ml (Fig. 2).

(ii) In vitro MHV-3 replication. Viral titers were determined in peritoneal macrophages which were pretreated for 30 min with 0, 30, 60, or 100 μg of methylprednisolone per ml and incubated for up to 48 h following infection with MHV-3. Methylprednisolone pretreatment of macrophages had no effect on MHV-3 replication in these cells at all time points studied (data not shown).

(iii) Prothrombin cleavage assays. Macrophages which were

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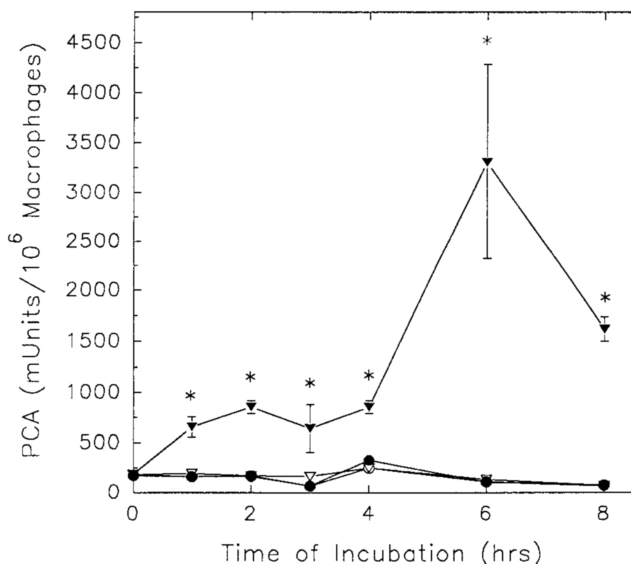

FIG. 1. Effect of methylprednisolone on induction of PCA in response to MHV-3 in A/J-derived peritoneal macrophages in vitro. Macrophages pretreated for 30 min with 100 μg of methylprednisolone per ml were incubated in either the absence (●) or presence (▼) of MHV-3. Two additional groups, not treated with methylprednisolone, either were not (○) or were (▽) infected with MHV-3. Results represent PCA expression following 0, 2, 4, 6, and 8 h of incubation and are the arithmetic means ± standard deviations from three studies repeated in triplicate. *, statistical significance ($P < 0.05$) versus macrophages neither treated with methylprednisolone nor infected with MHV-3.

neither treated with methylprednisolone nor infected with MHV-3 did not exhibit prothrombinase activity, as demonstrated by the inability to cleave ^{125}I-prothrombin. Similarly, macrophages infected with MHV-3 without being pretreated with methylprednisolone did not demonstrate induction of prothrombinase activity following incubation for up to 8 h. However, macrophages which were both treated with methylprednisolone and infected with MHV-3 elaborated prothrombinase activity as early as 1 h post-MHV-3 infection, which progressively increased, as evidenced by increased ^{125}I-prothrombin cleavage at 2, 4, 6, and 8 h postinfection with MHV-3. Thus, only A/J macrophages which were both treated with methylprednisolone and infected with MHV-3 demonstrated prothrombin cleavage or prothrombinase activity (Fig. 3).

(iv) *Fgl-2* mRNA expression. No constitutive expression of *Fgl-2* mRNA was seen in uninfected macrophages derived from A/J mice regardless of methylprednisolone treatment (results not shown). In contrast, low levels of *Fgl-2* RNA were detected in MHV-3-infected and methylprednisolone-untreated macrophages within 6 h of incubation with MHV-3. Treatment of A/J-derived macrophages with methylprednisolone for 30 min prior to infection with MHV-3 resulted in an earlier and markedly increased expression of RNA encoding for *Fgl-2*. Transcription of *Fgl-2* was detectable as early as after 1 h of incubation with MHV-3 (Fig. 4). Levels of *Fgl-2* mRNA in macrophages both pretreated with methylprednisolone and infected with MHV-3 reached a peak at 6 h postinfection with a slight decline at 8 h postinfection. Thus, treatment of A/J macrophages with methylprednisolone prior to infection with MHV-3 results in increased accumulation of *Fgl-2* mRNA following infection with MHV-3.

(v) *Fgl-2* mRNA stability assays. To determine whether the increase in *Fgl-2* mRNA levels observed after administration of corticosteroids was at least in part due to a posttranscriptional mechanism, *Fgl-2* mRNA stability studies were carried out. Following 6 h of incubation with MHV-3, baseline *Fgl-2* mRNA levels were significantly greater in macrophages treated with methylprednisolone prior to MHV-3 infection than in macrophages not pretreated with methylprednisolone. Over the sub-

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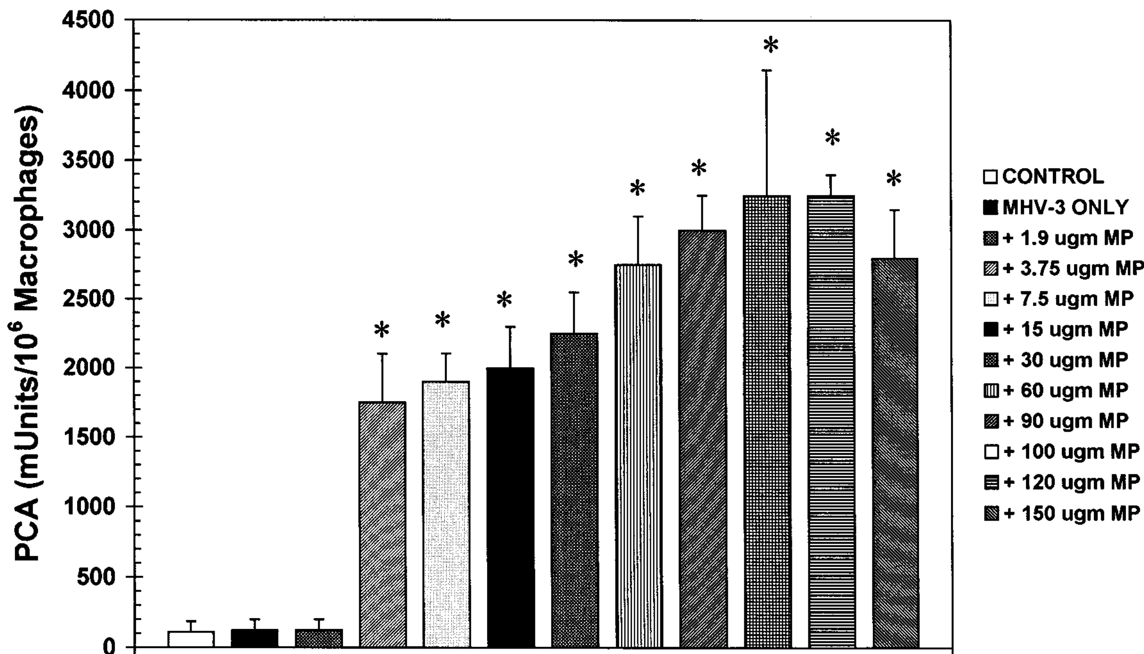

FIG. 2. Effects of increasing concentrations of methylprednisolone on induction of PCA by MHV-3 in A/J-derived macrophages. Macrophages were pretreated for 30 min with methylprednisolone (MP) and infected with MHV-3. PCA expression was determined following 6 h of incubation. Results represent the means ± standard deviations from three studies repeated in triplicate. *, statistical significance ($P < 0.05$) versus macrophages neither treated with methylprednisolone nor infected with MHV-3 (control).

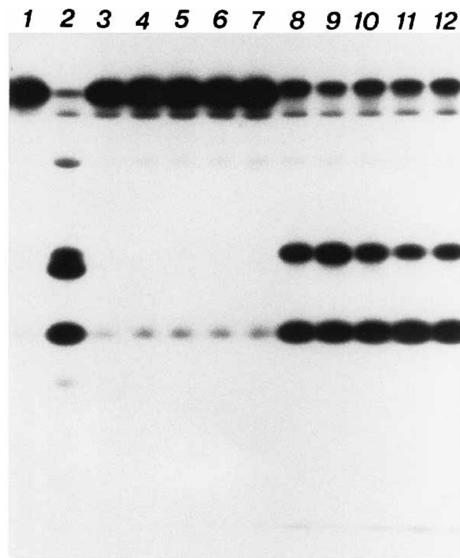

FIG. 3. Effect of methylprednisolone on induction of prothrombinase by MHV-3 in A/J-derived peritoneal macrophages. Macrophages, either pretreated or not pretreated for 30 min with methylprednisolone at 100 μg/ml, were infected with MHV-3 and incubated for up to 8 h. Macrophages were then incubated for 60 min with ^{125}I-prothrombin and 25 mM $CaCl_2$. SDS and EDTA were added, and the samples were electrophoresed on an SDS–10% polyacrylamide slab gel with results displayed by autoradiography. Lane 1, ^{125}I-prothrombin incubated with RPMI 1640 (negative control); lane 2, ^{125}I-prothrombin incubated with human factor Xa and Russell's viper venom (positive control); lanes 3 to 7, ^{125}I-prothrombin incubated with macrophages which were not pretreated with methylprednisolone but were incubated with MHV-3 for 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), 6 h (lane 6), or 8 h (lane 7); lanes 8 to 12, ^{125}I-prothrombin incubated with macrophages which were pretreated with methylprednisolone and incubated with MHV-3 for 1 h (lane 8), 2 h (lane 9), 4 h (lane 10), 6 h (lane 11), or 8 h (lane 12).

sequent 6 h, following treatment with actinomycin D, *Fgl-2* mRNA levels decayed more rapidly in the MHV-3-infected and methylprednisolone-untreated macrophages than in the MHV-3-infected and methylprednisolone-treated macrophages (Fig. 5). Quantitation by beta scanning indicated that methylprednisolone pretreatment stabilized *Fgl-2* mRNA, with greater than 60% activity seen at 6 h, when less than 5% of initial PCA activity was seen in RNA extracted from macrophages which had been infected with MHV-3 but not pretreated with methylprednisolone. Thus, the increased accumulation of *Fgl-2* mRNA in response to MHV-3 after corticosteroid treatment is at least in part attributable to increased stability of the mRNA.

In vivo studies. (i) Survival. A/J mice infected with 10^6 PFU of MHV-3 demonstrated no subjective or objective evidence of disease, and all mice survived for 14 days, at which time they were sacrificed. Histologic or gross examination revealed no signs of liver injury. In contrast, A/J mice which had been treated with methylprednisolone at a concentration of 500 mg/kg of body weight per day beginning 3 days prior to infection with 10^6 PFU of MHV-3 developed clinical signs of disease by day 2 postinfection, characterized by ruffled fur and lethargy. By day 3, oral intake was markedly reduced; by day 4, mice in this group began to die; and by day 10 postinfection, all mice had died (Fig. 6). Postmortem examinations demonstrated pale mottled livers in these mice, consistent with fulminant MHV-3 hepatitis. A/J control mice which received neither methylprednisolone nor MHV-3 or received methylprednisolone alone at concentrations as high as 500 mg/kg/day for 14 days all survived and demonstrated no histological or biochemical liver abnormalities.

(ii) Viral replication. The patterns of viral replication in the first 6 days of infection were similar in liver tissue recovered from mice infected with MHV-3 and either treated or not treated with methylprednisolone. Viral replication could first be detected by day 3 postinfection, with peak viral titers reached on day 6. By day 9, when viral replication was no longer detected in liver tissue obtained from MHV-3-infected and methylprednisolone-untreated mice, viral titers in the surviving methylprednisolone-treated and MHV-3-infected mice remained at high levels, which persisted until the death of the animals (Fig. 7).

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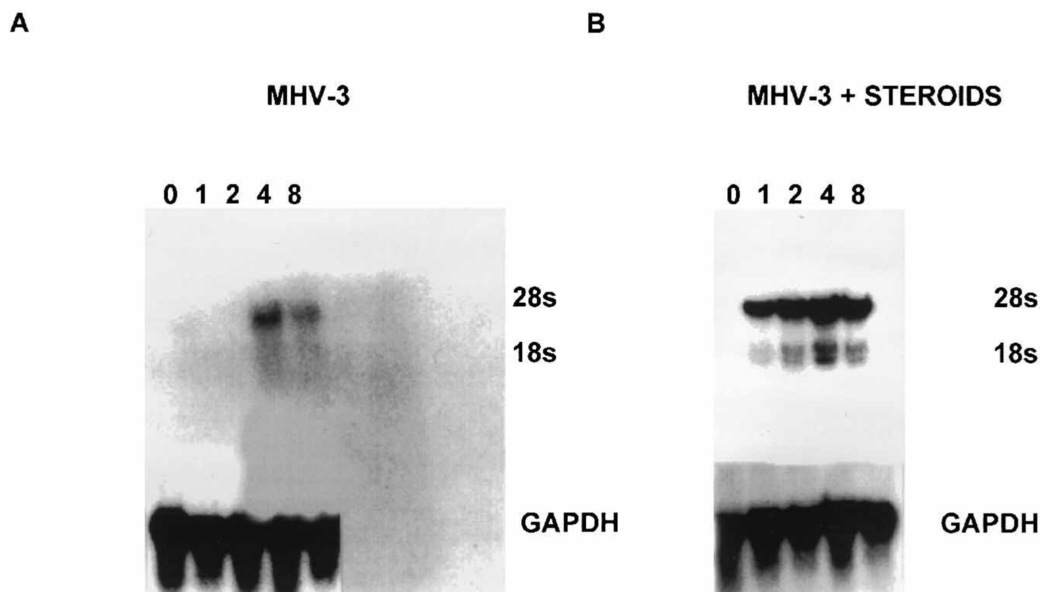

FIG. 4. Effect of methylprednisolone on transcription of *Fgl-2* in A/J-derived macrophages stimulated with MHV-3. Ten micrograms of total RNA per lane, isolated from A/J-derived peritoneal macrophages which were not (A) or were (B) pretreated for 30 min with methylprednisolone and infected with MHV-3 for 0, 1, 2, 4, or 8 h, was hybridized with a 1.3-kb cDNA probe for *Fgl-2*. A GAPDH cDNA probe was used to ensure equal loading of each lane.

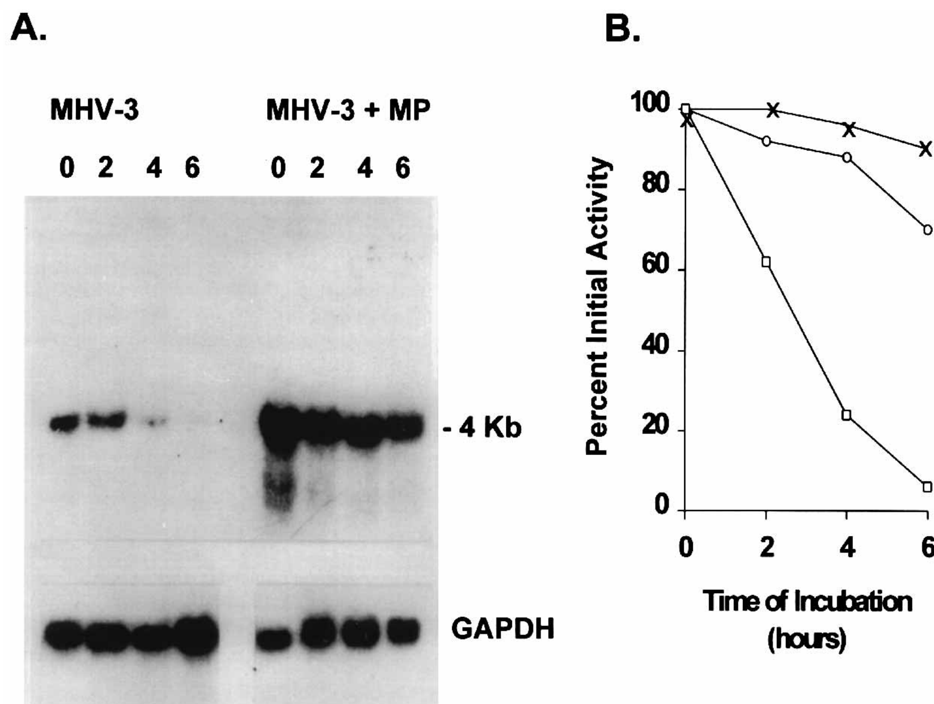

FIG. 5. Methylpredisolone increases stability of *Fgl-2* mRNA. (A) Ten micrograms of total RNA per lane was isolated from A/J-derived peritoneal macrophages which either were not or were pretreated for 30 min with 100 μg of methylprednisolone (MP) per ml, infected with MHV-3 for 6 h, and then incubated with actinomycin D (10 μg/ml), an inhibitor of gene transcription, for 0, 2, 4, or 6 h. RNA was hybridized with a 1.3-kb cDNA probe for *Fgl-2*. A GAPDH cDNA probe was used to ensure equal loading of each lane. (B) Quantification of *Fgl-2* mRNA by a Betagen scanner. ×, GAPDH; ○, *Fgl-2* plus MP; □, *Fgl-2*.

(iii) Biochemistry. In MHV-3-infected but methylprednisolone-untreated A/J mice, no increase in alanine aminotransferase activity, a marker of hepatocellular necrosis, was seen, similar to results in methylprednisolone-untreated and MHV-3-uninfected mice. In contrast, marked increases in serum alanine aminotransferase were noted in methylprednisolone-treated and MHV-3-infected A/J mice within 3 days of infection. Maximal alanine aminotransferase levels of 740 ± 145 IU/liter were seen on day 6 postinfection and did not significantly change until time of death of these animals by day 10.

(iv) Histology. Livers obtained from control mice or mice treated with methylprednisolone at 500 mg/kg/day for 14 days showed normal architecture. Similarly, no histological evidence of hepatocellular injury was seen in A/J mice infected with MHV-3 and which had not received methylprednisolone. In contrast, in mice treated with methylprednisolone and infected with MHV-3, histologic evidence of hepatocellular necrosis associated with portal and periportal infiltrations of mononuclear and polymorphonuclear cells was observed by day 3 following infection with MHV-3 and progressed to a marked coagulative necrosis by day 6 (Fig. 8).

(v) Immunofluorescence. By direct immunofluorescence staining, expression of PCA was detected in liver tissue from the methylprednisolone-treated and MHV-3-infected mice in areas of inflammation as well as in hepatic sinusoids adjacent to areas of hepatic necrosis within 3 days of viral infection. In the sinusoids, PCA expression localized primarily to endothelial and Kuppfer cells. PCA expression was more pronounced in livers harvested from methylprednisolone-treated and MHV-3-infected mice at 6 and 9 days postinfection (Fig. 9). Expression of PCA was not detected by immunofluorescence staining in the livers of methylprednisolone-untreated and MHV-3-infected A/J mice or control uninfected A/J mice which either were or were not treated with methylprednisolone.

DISCUSSION

MHV-3 infection in inbred strains of mice serves as a model to study the pathogenesis of virally induced liver injury. Despite similar patterns of growth of virus, BALB/cJ and A/J mice

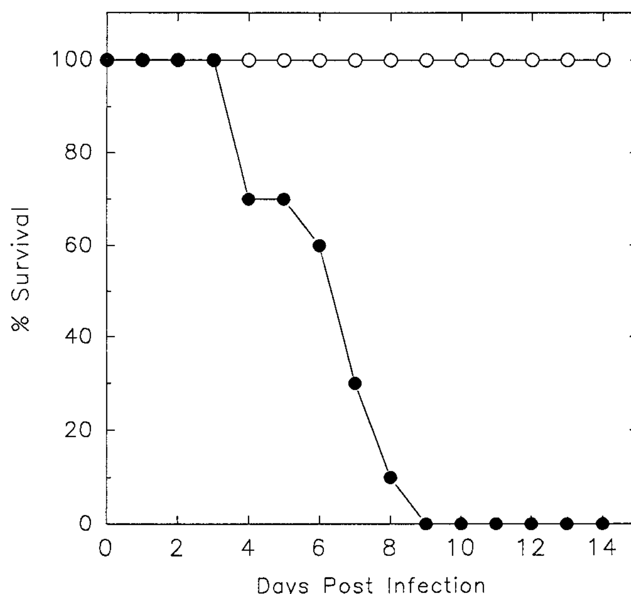

FIG. 6. The effects of methylprednisolone on survival in A/J mice infected with MHV-3. A/J mice (10 in each group), pretreated for 3 days with either 500 mg of methylprednisolone per day (●) or normal saline (○) injected intraperitoneally, were infected with 10^6 PFU of MHV-3. Treatment with either methylprednisolone or normal saline was continued following infection with MHV-3, and survival was studied for 14 days postinfection.

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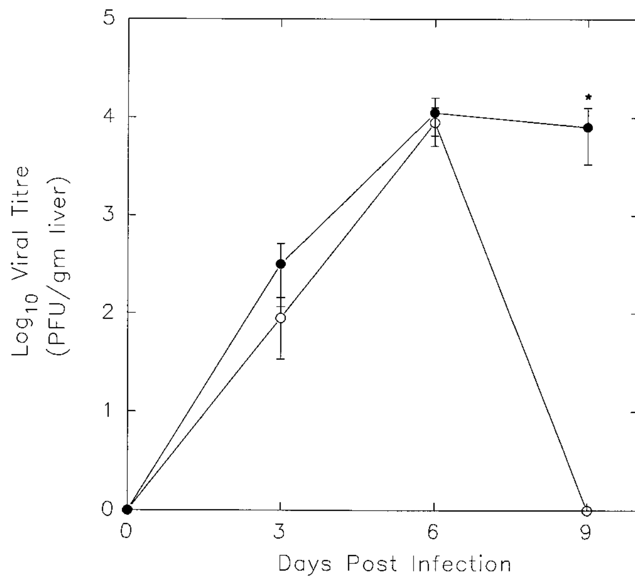

FIG. 7. Effect of methylprednisolone on intrahepatic viral replication in A/J mice infected with MHV-3. A/J mice, pretreated with either methylprednisolone (500 mg/kg/day) (●) or normal saline (○) for 3 days, were infected with 10^6 PFU of MHV-3. Treatment with normal saline or methylprednisolone was continued following MHV-3 infection. Results represent the arithmetic means ± standard deviations of viral titers in livers harvested from three mice at each time point. *, statistical significance ($P < 0.05$) versus methylprednisolone-untreated and MHV-3-infected mice.

demonstrate markedly different responses to MHV-3 (19, 24, 43). Following infection, A/J mice develop neither clinical, histological, nor biochemical evidence of liver injury; clear the virus by 10 to 14 days; and survive. In contrast, BALB/cJ mice develop severe hepatitis characterized by neutrophil and mononuclear cell infiltrates of the hepatic parenchyma, sinusoidal fibrin deposition, and coagulative hepatocellular necrosis (19, 21, 25). Thus, factors other than viral replication must be important in the pathogenesis of MHV-3-induced liver disease. Resistance to MHV-3 may be dependent upon immune defense mechanisms either alone or in combination with innate macrophage factors alone or in combination with the absence of functional cellular receptors (2).

We have previously demonstrated that MHV-3 causes induction of a unique procoagulant (PCA) in murine cells of the monocyte/macrophage line in a strain-dependent pattern (8, 20). Synthesis of PCA correlates with disease susceptibility and is responsible for the coagulative liver necrosis seen in susceptible mice following MHV-3 infection (10, 20, 25). Supportive evidence for the role of PCA in the pathogenesis of MHV-3-related liver disease has been derived from recent studies in which susceptible mice which were treated with a high-titer neutralizing antibody to the MHV-3-induced PCA were protected from MHV-3-related liver injury in vivo (22). Together, these studies support the concept that activation of the immune coagulation system as evidenced by induction of PCA synthesis plays an important role in the pathogenesis of MHV-3-related liver disease.

We have recently identified the gene, *Fgl-2*, responsible for production of PCA in murine macrophages infected with MHV-3 and have shown that this gene is located on mouse chromosome 5 (29, 31). In macrophages derived from susceptible BALB/cJ mice, expression of *Fgl-2* mRNA was detected within 3 h of MHV-3 infection, peaked at 6 h, and returned to baseline by 24 h. By comparison, expression of *Fgl-2* RNA transcripts in macrophages derived from resistant A/J mice was markedly delayed and 120-fold lower than that seen in BALB/cJ mice (29). Despite transcription of small amounts of *Fgl-2* mRNA, no functional expression of the gene product of *Fgl-2* (PCA) was seen in macrophages derived from A/J mice in response to MHV-3 (29). In contrast, infection of macrophages derived from susceptible BALB/cJ mice resulted in rapid expression of functional PCA within 2 h of infection which persisted for 24 h (29). The data reported here demon-

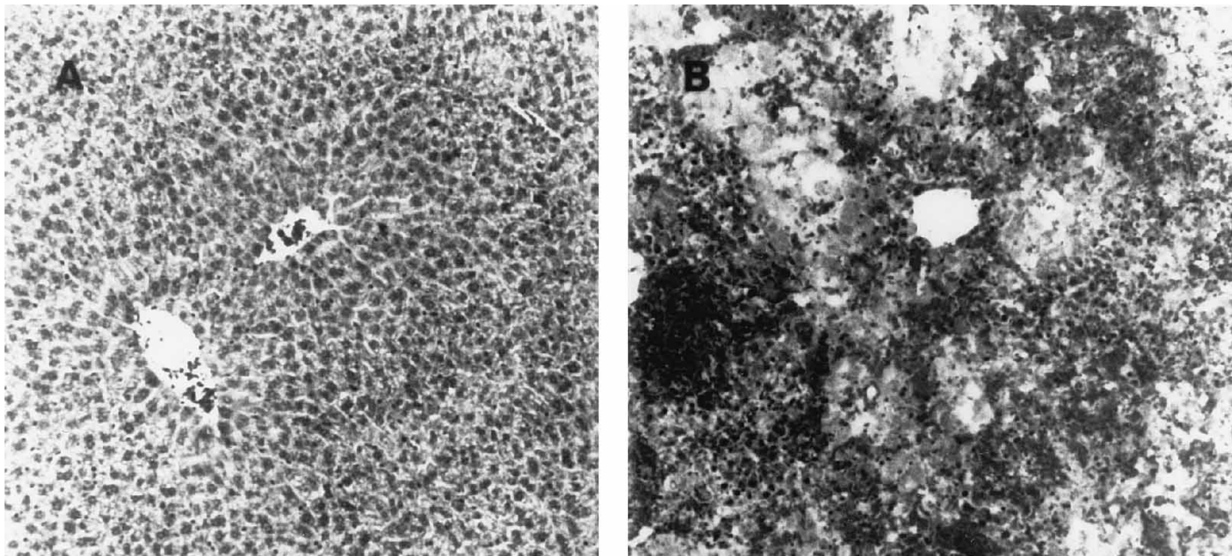

FIG. 8. Effects of methylprednisolone on liver histology in A/J mice infected with MHV-3. Liver tissue was obtained from A/J mice infected with 10^6 PFU of MHV-3 following 3 days of pretreatment with either methylprednisolone (500 mg/kg/day) or normal saline with continuation of therapy following MHV-3 infection. (A) Liver tissue obtained at sacrifice on day 14 from an A/J mouse infected with MHV-3 and treated with normal saline prior to and following MHV-3 infection. No histological abnormalities are present. (B) Liver tissue obtained on day 7 post-MHV-3 infection from an A/J mouse that was treated with methylprednisolone prior to and following infection with MHV-3. Extensive hepatocellular necrosis associated with inflammatory cell infiltrate is present and is consistent with fulminant acute hepatitis.

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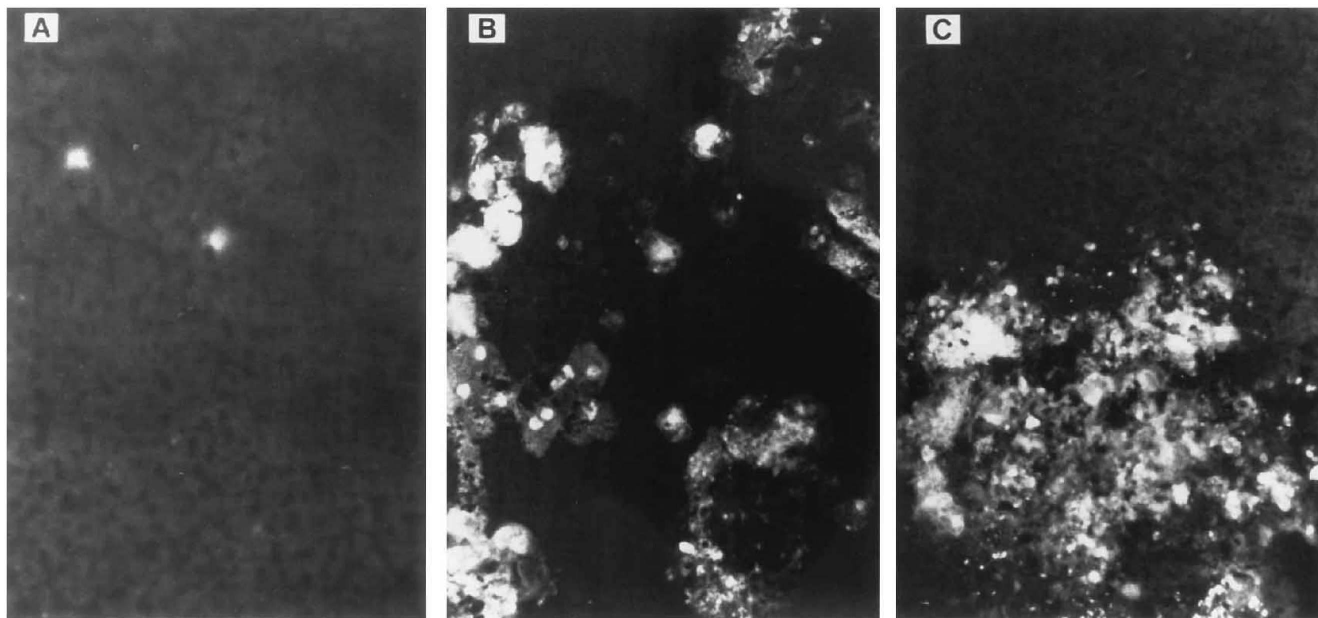

FIG. 9. Effects of methylprednisolone on intrahepatic expression of prothrombinase in mice infected with MHV-3. Immunofluorescence studies were carried out with liver tissue harvested from MHV-3-infected A/J mice which either were or were not pretreated with 500 mg of methylprednisolone per kg per day beginning 3 days prior to infection with 10^6 PFU of MHV-3. Sections were stained with 3D4.3, an antiprothrombinase monoclonal antibody, and conjugated with fluorescein isothiocyanate. (A) Liver tissue harvested at 3 days postinfection from a methylprednisolone-untreated and MHV-3-infected mouse demonstrates no detectable immunofluorescence, confirming an absence of intrahepatic prothrombinase expression in livers harvested from mice infected with MHV-3 without also being treated with methylprednisolone. (B) Liver tissue harvested at 3 days postinfection from a methylprednisolone-treated and MHV-3-infected A/J mouse demonstrates expression of prothrombinase in areas of inflammation as well as in hepatic sinusoids. In sinusoids, fluorescence localized primarily to endothelial and Kuppfer cells. (C) Liver tissue harvested at 5 days postinfection from a methylprednisolone-treated and MHV-3-infected A/J mouse demonstrates extensive expression of prothrombinase in areas of inflammation as well as in hepatic sinusoids adjacent to areas of hepatic necrosis.

strate that the coding sequence of the prothrombinase (*Fgl-2*) must be intact in A/J mice. This suggests that differences in expression of the gene following MHV-3 infection in BALB/cJ and A/J mice must reside in part in differences in the signal transduction pathway, differences in the promoter region, and/or differences in mRNA degradation.

In the current studies, we have confirmed that treatment of A/J mice with methylprednisolone results in a loss of resistance to MHV-3. All A/J mice treated with methylprednisolone and infected with MHV-3 died within 10 days of infection. Postmortem findings were consistent with acute hepatitis characterized by coagulative hepatic necrosis. Immunofluorescence studies, using a monoclonal antibody to the MHV-3-induced prothrombinase (3D4.3), demonstrated PCA in liver tissue harvested from mice which had been pretreated with methylprednisolone and infected with MHV-3 in areas of hepatic necrosis as well as in hepatic sinusoids adjacent to areas of hepatic necrosis within 48 h of infection with MHV-3. In vitro, macrophages derived from A/J mice which were pretreated with methylprednisolone and infected with MHV-3 expressed increased levels of functional PCA, as determined by a single-stage clotting assay. Prothrombin cleavage studies confirmed that this increased PCA was a direct prothrombinase. The prothrombin cleavage activity was associated with increased and earlier transcription of the *Fgl-2* gene. In addition, RNA stability studies demonstrated that methylprednisolone resulted in increased stability of the *Fgl-2* RNA, suggesting that a second mechanism underlying corticosteroid-related up-regulation of *Fgl-2* expression was posttranscriptional stabilization of an otherwise labile mRNA.

Regulation of mRNA turnover plays an important role in modulating gene expression and has been demonstrated as being important in the adjustment of expression of cytokines, transcription factors, and proto-oncogenes (15, 34, 36). Many mRNAs which undergo rapid turnover contain a common sequence, AUUUA, which signals for rapid degradation (36). We have found one such sequence in the 3′ untranslated region of the *Fgl-2* gene cDNA sequence. At present, the mechanisms responsible for the rapid degradation of the *Fgl-2* mRNA are not known. The AUUUA sequence might direct the nascent *Fgl-2* mRNA to a cytoplasmic compartment where rapid degradation would occur. Alternatively, the sequence may be recognized by an RNA-binding protein involved in the specific degradation of transiently expressed RNA sequences (36). Potentially, the synthesis of this RNA-binding protein is inhibited in the presence of corticosteroids, leading to Fgl-2 stabilization and, ultimately, PCA expression. Whether these mechanisms are operative for the *Fgl-2* gene remains to be proven.

Recent studies have shown that corticosteroids alter the T-helper lymphocyte response to antigens from a Th1 to a Th2 response (6, 12, 42). We have shown previously that resistance to MHV-3 is dependent on a Th1 response and that Th1 lymphocytes derived from resistant mice exposed to MHV-3 inhibit procoagulant expression in MHV-3-infected macrophages in vitro and protect susceptible mice from the lethal effects of MHV-3 in vivo (3). Furthermore, we have now shown that MHV-3-specific Th2 lymphocytes isolated from susceptible mice increase the transcription of *Fgl-2* by macrophages, and adoptive transfer of these lymphocytes failed to protect naive BALB/cJ mice from MHV-3 infection (30). Thus, an additional mechanism for loss of resistance to MHV-3 by corticosteroids may be changing of the T-helper response to MHV-3 in resistant mice from a Th1 to a Th2 response, resulting in augmented *Fgl-2* transcription and ultimately procoagulant expression.

In summary, corticosteroid therapy of MHV-3-resistant A/J

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mice is associated with a loss of resistance to MHV-3 and increased accumulation of the *Fgl-2* mRNA associated with posttranscriptional stabilization of the *Fgl-2* mRNA, leading to expression of the *Fgl-2* gene product prothrombinase. These studies provide further insight into mechanisms of PCA regulation in response to MHV-3 infection in inbred strains of mice and further define the importance of the *Fgl-2* product PCA in the pathogenesis of MHV-3-induced fulminant liver failure.

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