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TREATMENT OF RESISTANT A/J MICE WITH METHYLPREDNISOLONE (MP) RESULTS IN LOSS OF RESISTANCE TO MURINE HEPATITIS STRAIN 3 (MHV-3) AND INDUCTION OF MACROPHAGE PROCOAGULANT ACTIVITY (PCA)

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ABSTRACT

BALB/cJ mice die of fulminant hepatitis within 7 days of exposure to murine hepatitis virus strain 3 (MHV-3) whereas A/J mice are fully resistant to the lethal effects of MHV-3 infection. Previous studies have implicated macrophage activation with production of a unique macrophage prothrombinase (PCA) and lymphocyte cytokine secretion in the pathogenesis of MHV-3 susceptibility and have demonstrated that immunosuppression induces susceptibility in resistant mice. This study was undertaken to determine whether macrophages, derived from resistant A/J mice and treated *in vitro* with methylprednisolone sodium succinate (MP), elaborated PCA following MHV-3 exposure and whether therapy with MP altered resistance of A/J mice to MHV-3 infection *in vivo*.

Macrophages, incubated with MP *in vitro*, expressed dose dependent increases in PCA following infection with MHV-3. No induction of PCA occurred in macrophages treated with MHV-3 or MP alone. Analysis of mRNA transcripts for mouse fibrinogen like protein (musfiblp), the MHV-3 specific prothrombinase, in macrophages which were incubated with MP prior to exposure to MHV-3 demonstrated significantly increased mRNA levels as compared to macrophages not incubated with MP prior to MHV-3 exposure. *In vivo*, A/J mice treated for 3 days with 500 mg/kg/day of MP prior to infection with MHV-3 demonstrated extensive hepatocyte necrosis and fibrin deposition in hepatic sinusoids on histologi-

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cal examination of liver tissue, elevated serum transaminases and 100% mortality within 10 days of infection. These results therefore provide further support for the role of increased PCA in the pathogenesis of MHV-3 related liver necrosis.

INTRODUCTION

Infection of inbred mice by the coronavirus murine hepatitis virus strain 3 (MHV-3) causes a strain and age dependent spectrum of disease[1,2]. BALB/cJ mice are fully susceptible whereas mature A/J mice are fully resistant and develop no clinical disease[2]. We have shown that susceptibility to MHV-3 correlates with the induction of a unique monocyte/macrophage prothrombinase (PCA). High levels of PCA are produced *in vivo* and *in vitro* by macrophages from susceptible BALB/cJ mice following MHV-3 exposure whereas macrophages from resistant A/J mice fail to produce increased PCA under similar circumstances[2,3]. We have recently isolated and cloned a gene (musfiblp) that encodes a polypeptide with prothrombinase-like activity and is induced during MHV-3 infection[4]. MHV-3 infection of susceptible macrophages resulted in a marked increase in musfiblp mRNA which was detected as early as 6 hours p.i. In contrast, induction of musfiblp mRNA was seen in macrophages from A/J mice but was markedly less than that seen in macrophages from BALB/cJ mice and was not only seen until 12 hours p.i.

Treatment of resistant mice with corticosteroids results in the loss of resistance to MHV and the development of lethal acute hepatitis[5]. The mechanism for this loss of resistance is not known but may reflect impairment of cellular immunity which is a known consequence of administration of corticosteroids[6].

These present studies were initiated to determine whether corticosteroids affect viral replication, transcription of musfiblp and expression of its functional gene product (PCA) in macrophages from resistant A/J mice.

METHODS AND RESULTS

I. In Vivo Studies

Mature female A/J mice, infected with 1 x 10^6 plaque forming units (PFU) of MHV-3 administered intraperitoneally (IP), demonstrated no histologic or biochemical evidence of hepatitis and all mice survived. Animals pretreated for 3 days with Methylprednisolone Sodium Succinate (MP) (Solu-medrol^R, Upjohn Co, Don Mills, Ontario) 500/mg/kg daily with continuation of therapy following MHV-3 exposure demonstrated marked elevations of serum alanine transaminase, a marker of liver necrosis (1500±450 vs 50±10 IU/l in control mice), extensive hepatocyte necrosis with fibrin deposition in hepatic sinusoids on histological examination of the liver and 100% mortality within 10 days of infection. Peak hepatic viral titers of $1.12x10^4$±$4.7x10^3$ PFU/gm liver and $8.9x10^3$±$3.8x10^3$ PFU/gm liver were seen in MP treated and untreated, MHV-3 infected, animals respectively at 6 days post infection (p.i.). The differences in peak viral titers between these two groups were not statistically significant (p=0.244). At 9 days p.i., viral replication was undetectable in animals not receiving MP whereas high titers of MHV-3 persisted in MP treated mice.

II. In Vitro Studies

Peritoneal macrophages (2 X 10^6/ml) derived from A/J mice were preincubated with 0-30 μg of MP/ml 30 minutes prior to infection with 1000 PFU of MHV-3. Dose dependent

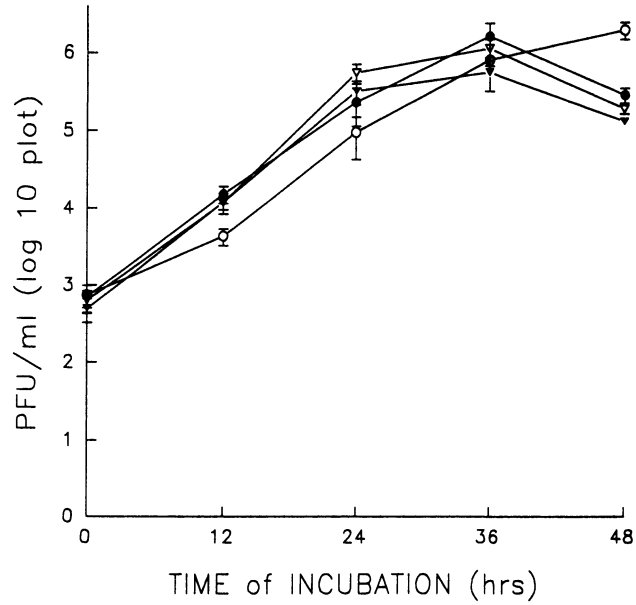

Figure 1. The effect of methylprednisolone (MP) on MHV-3 replication in A/J macrophages *in vitro* Macrophages from A/J mice were pretreated with 0 (○), 10 (●), 20 (▽) or 30 (▼) μg of MP/ml and infected with MHV-3 Viral titres were determined in standard plaque assay Results are mean ± 1 standard deviation of 3 experiments done in duplicate

enhanced viral replication was seen in the MP treated macrophages compared to MHV-3 infected and non MP treated macrophages in the first 24 hours of incubation. However, by 36 hours, there were no significant differences in viral titres in macrophages incubated with or without MP and, at 48 hours of incubation, viral titers were still increasing in MP untreated macrophages at a time when they were decreasing in the MP treated macrophages (Figure 1).

To assess the effect of MP on PCA expression, peritoneal macrophages (1 X 10^6/ml), harvested from A/J mice were preincubated for 30 minutes with 0 or 100 μg/ml of MP, infected with MHV-3 at a multiplicity of infection (MOI) of 1.0 and assayed for PCA. A/J macrophages infected with MHV-3 without prior *in vitro* MP exposure failed to express increased PCA above basal levels, as has been described previously[2,3]. In contrast, macrophages from A/J mice pretreated with MP *in vitro* expressed markedly increased levels of PCA following MHV-3 stimulation. An increase in PCA was seen as early as one hour p.i. reaching maximum levels at 6 hours p.i. and declining at 8 hours p.i. (Figure 2). Subsequent studies demonstrated that the PCA response of A/J derived macrophages to MP was dose dependent (Figure 3). Similar increases in PCA expression occurred in macrophages derived from A/J mice treated in vivo with 500 mg/kg/day of MP for 3 days prior to sacrifice (data not shown).

To determine the effect of MP on transcription of the recently identified PCA gene musfiblp, macrophages were preincubated with MP as described above and infected with MHV-3 at an MOI of 1.0. Macrophages were incubated for up to 8 hours following which total cellular RNA was isolated, resolved on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose membranes. The membranes were subsequently hybridized with a musfiblp specific probe. Constitutive expression of musfiblp mRNA was not observed in MP treated or untreated macrophages. In macrophages which had been pretreated with MP and infected with MHV-3, significantly increased levels of musfiblp mRNA were detected as early as 2 hours p.i. which continued to increase at 8 hours p.i.. In contrast, in non MP treated but MHV-3 infected macrophages, significantly increased mRNA was only seen at 8 hours p.i. (Figure 4).

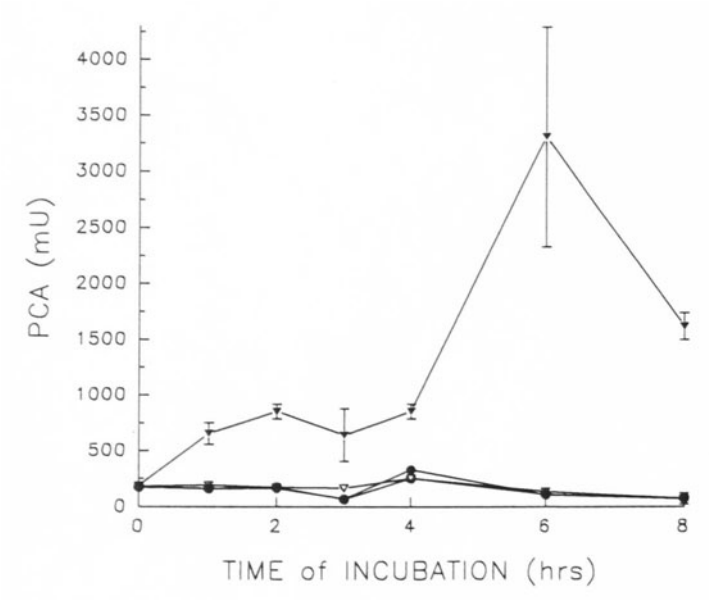

Figure 2. The effect of methylprednisolone (MP) on induction of procoagulant activity (PCA) by MHV-3 in A/J derived macrophages *in vitro*. Control macrophages (O), macrophages treated with 100 μg MP (●), MHV-3 infected macrophages (▽) and MHV-3 infected macrophages preincubated with 100 μgm MP (▼) were assessed for PCA in a one stage clotting assay. Results are mean ± 1 standard deviation of 3 experiments done in duplicate.

DISCUSSION

The mechanisms underlying the strain dependent spectrum of liver injury in mice following MHV-3 infection are unclear. Bang and Warwick suggested that susceptibility to MHV infection correlated with replication of MHV in isolated cultures of macrophages[7]. However, in more recent studies, MHV-3 replication has been demonstrated in macrophages[2] and hepatocytes[8] from both resistant and susceptible strains of mice. Thus, restriction of viral replication does not appear to explain resistance. Lamontagne et al have demonstrated that viral pathogenicity correlates with replication of MHV-3 in T and B lymphocytes and the subsequent consequences of loss of immunocompetence[9].

Susceptibility to MHV-3 in inbred strains of mice correlates with the ability of macrophages derived from these mice to produce PCA following MHV-3 exposure *in vitro*[2,3]. Induction of PCA in susceptible strains correlates with the MHV-3 related liver injury which

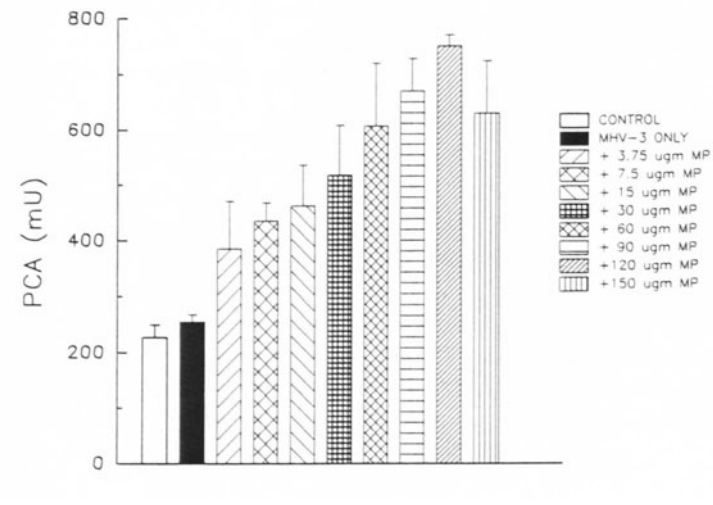

Figure 3. Enhancing effect of increasing concentrations of methylprednisolone (MP) on induction of procoagulant activity (PCA) by MHV-3 in A/J derived macrophages *in vitro*. Macrophages were preincubated with MP at 0 to 150 μg/ml and 30 minutes later infected with MHV-3. Following a 2 hour incubation, PCA was determined in a one stage clotting assay. Results are mean ± 1 standard deviation of 3 experiments done in duplicate.

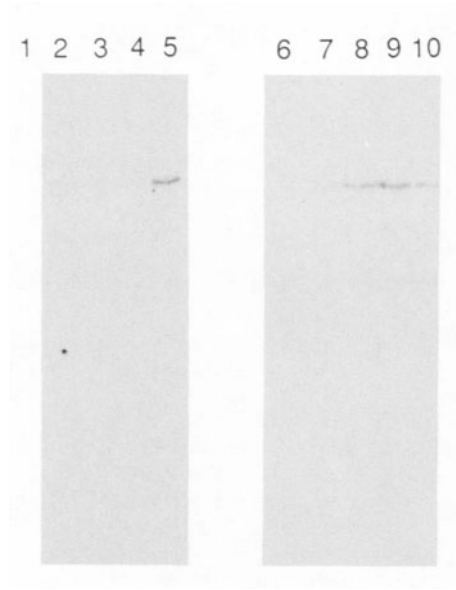

Figure 4. Effect of methylprednisolone (MP) on transcription of musfiblp mRNA in A/J derived macrophages stimulated with MHV-3. 10 μg total RNA, isolated from A/J macrophages which were not pretreated with MP (Lanes 1-5) or were pretreated with MP (Lanes 6-10) and infected with MHV-3 for 0 hrs (Lanes 1 and 6), 2 hours (Lanes 1 and 7), 4 hours (Lanes 3 and 8), 6 hours (Lanes 4 and 9) or 8 hours (Lanes 5 and 10), were hybridized with a 1.3 kb random-primed musfiblp cDNA probe.

is characterized by sinusoidal thrombosis and abnormalities of microcirculatory flow[10]. Susceptible mice which were treated with high titered neutralizing antibody to PCA were protected from MHV-3 related liver injury *in vivo*[11]. Together, these studies support the concept that induction of procoagulant synthesis, as manifested by increased PCA, plays an important role in the pathogenesis of MHV-3 related liver disease.

Corticosteroids are known to inhibit immune function. They inhibit macrophage cytotoxic function and processing and presentation of antigen to T cells; decrease the activity of natural killer cells and induce apoptosis in immature B and T cell precursors and mature T cells[12]. Their effects on T lymphocytes include inhibition of production of IL-2 associated with a shift of the cytokine response from a TH1 to a TH2 profile[13]. We have recently reported that TH1 lymphocytes inhibit induction of PCA by macrophages in response to MHV-3 *in vitro* and also can prevent the lethality of MHV-3 infection *in vivo*[14]. Körner et al. have also reported the importance of TH1 cells in the resistance to MHV JHM infection[15].

We have confirmed that corticosteroid therapy *in vivo* results in loss of resistance to MHV-3 in A/J mice. We have now demonstrated that treatment of A/J macrophages with corticosteroids prior to infection with MHV-3 results in induction of PCA whereas non corticosteroid treated and MHV-3 infected A/J macrophages fail to express PCA. This was shown both by demonstration of increased transcription of the musfiblp gene thought to encode PCA and by expression of functional PCA in the one stage clotting assay in corticosteroid treated and MHV-3 infected macrophages. These results suggest that differences in expression of PCA following MHV-3 exposure in resistant and susceptible mice cannot be simply explained by differences in the coding sequence of musfiblp and suggests that differences in musfiblp gene transcription or stability of message may account for the disparate expression of functional PCA between resistant and susceptible mice.

These findings in combination with the fact that corticosteroid treated resistant A/J mice develop microvascular thrombosis and hepatic necrosis and die following MHV-3 infection similar to fully suscepible BALB/cJ, mice further supports the concept that induction of macrophage PCA is pivotal to the pathogenesis of MHV-3 disease.

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