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Pattern of Disease after Murine Hepatitis Virus Strain 3 Infection Correlates with Macrophage Activation and Not Viral Replication

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Murine hepatitis virus strain 3 (MHV-3) produces a strain-dependent pattern of disease which has been used as a model for fulminant viral hepatitis. This study was undertaken to examine whether there was a correlation between macrophage activation and susceptibility or resistance to MHV-3 infection. Peritoneal macrophages were isolated from resistant A/J and susceptible BALB/cJ mice and, following stimulation with MHV-3 or lipopolysaccharide (LPS), analyzed for transcription of mRNA and production of interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), transforming growth factor β (TGF-β), mouse fibrinogen-like protein (musfiblp), tissue factor (TF), leukotriene B_4, and prostaglandin E_2 (PGE_2). Macrophages from BALB/cJ mice produced greater amounts of IL-1, TNF-α, TGF-β, leukotriene B_4, and musfiblp following MHV-3 infection than macrophages from resistant A/J mice, whereas in response to LPS, equivalent amounts of IL-1, TNF-α, TGF-β, and TF were produced by macrophages from both strains of mice. Levels of mRNA of IL-1, TNF-α, and musfiblp were greater and more persistent in BALB/cJ than in A/J macrophages, whereas the levels and kinetics of IL-1, TNF-α, and TF mRNA following LPS stimulation were identical in macrophages from both strains of mice. Levels of production of PGE_2 by MHV-3-stimulated macrophages from resistant and susceptible mice were equivalent; however, the time course for induction of PGE_2 differed, but the total quantity of PGE_2 produced was insufficient to inhibit induction of musfiblp, a procoagulant known to correlate with development of fulminant hepatic necrosis in susceptible mice. These results demonstrate marked differences in production of inflammatory mediators to MHV-3 infection in macrophages from resistant A/J and susceptible BALB/cJ mice, which may explain the marked hepatic necrosis and fibrin deposition and account for the lethality of MHV-3 in susceptible mice.

An incomplete understanding of the pathogenesis of fulminant viral hepatitis has limited the development of successful medical approaches to its treatment (35, 46). Recent studies using a model of hepatitis induced by infection with murine hepatitis virus strain 3 (MHV-3) have provided significant insight into the mechanisms underlying this disease process (13, 19, 33). One of the unique features of MHV-3-induced fulminant hepatitis is its strain-dependent pattern of disease. While low doses of MHV-3 cause hepatocellular necrosis and death in susceptible BALB/cJ mice, A/J mice are totally resistant and semisusceptible C3H mice develop acute hepatitis which progresses to chronic hepatitis (36).

MHV-3-induced fulminant hepatitis is characterized pathologically by sinusoidal thrombosis and associated hepatocellular necrosis (17, 36, 42). Several lines of evidence suggest that stimulation of the immune coagulation system by MHV-3 participates in the disease process. First, induction of monocyte/macrophage procoagulant activity (PCA) correlates well with the severity of the disease during infection (18, 36). Second, administration of exogenous prostaglandin E_2 (PGE_2) completely inhibits induction of PCA and prevents the development of hepatic necrosis (1, 55). Finally, treatment of mice with a monoclonal antibody to the MHV-3-induced PCA prevents the lethality associated with MHV-3 infection (22, 37). Considered together, these studies suggest a causal relationship between macrophage activation, subsequent fibrin deposition through the production of PCA, and the pathogenesis of the disease. We have now identified the MHV-3-induced PCA as mouse fibrinogen-like protein (musfiblp), a protease with direct prothrombin cleaving activity (13, 30, 49). This molecule is distinct from the lipopolysaccharide (LPS)-induced macrophage procoagulant tissue factor (TF), the cellular receptor and essential cofactor for the serine protease factor VII (40).

In several other animal models of liver injury, including those due to CCl_4, endotoxin, galactosamine, and acetaminophen, the hepatic injury is associated with fibrin deposition, sinusoidal thrombosis, and accumulation of inflammatory cells (3, 24, 34, 60). In the hepatocellular necrosis associated with these pathologic processes, resident macrophages within the liver (Kupffer cells) exhibit morphologic features of activation and release a number of inflammatory mediators, including tumor necrosis factor (TNF), interleukin-1 (IL-1), proteolytic enzymes, and eicosanoids, as well as superoxide anions and nitric oxide (34). In liver necrosis induced by *Corynebacterium parvum* and endotoxin, a correlation has been demonstrated between the amount of liver injury and serum levels of lipid peroxidation products (4), while pretreatment with superoxide dismutase reduces the extent of the liver damage and mortality (3). Furthermore, liver injury associated with alcohol and endotoxin correlates with macrophage production of eicosanoids and TNF-α (43).

Macrophages are known to generate a wide range of mediator molecules which may contribute either directly or indirectly to the development of fulminant hepatitis by inducing PCA (60). TNF and IL-1 production by macrophages can stimulate endothelial cell TF production and increase neutrophil-endothelial interactions, thereby potentially promoting micro-

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vascular thrombosis (7, 48, 57). Leukotriene B_4 (LTB_4), another macrophage product, has also been shown to contribute to hepatocellular necrosis (54).

Given the importance of the macrophage in the pathogenesis of hepatic necrosis following MHV-3 infection, this study was carried out to determine whether differences in the production of the procoagulants TF and musfiblp, the proinflammatory mediators TNF, LTB_4, and IL-1, and the anti-inflammatory mediators transforming growth factor β (TGF-β) and PGE_2 in macrophages from resistant and susceptible strains of mice could account for the different patterns of disease seen following MHV-3 infection in vivo.

MATERIALS AND METHODS

Mice. Female BALB/cJ and A/J mice, 6 to 8 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, Maine) and were stored in the animal colony at the University of Toronto. They were fed a standard chow diet and water ad libitum prior to and during the studies.

Virus. MHV-3 was plaque purified on monolayers of DBT cells. It was grown to a titer of 1.5×10^7 PFU/ml in 17 CL1 cells. Viral titers were determined on monolayers of L2 cells in a standard plaque assay as previously described (36).

Peritoneal macrophages. Peritoneal macrophages were harvested from A/J and BALB/cJ mice 4 days after intraperitoneal administration of 1.5 ml of 3% thioglycolate (Difco Laboratories, Detroit, Mich.) as previously described (37). After being washed, the macrophages were resuspended at 10^6/ml in RPMI 1640 (ICN Biomedicals Inc., Costa Mesa, Calif.) supplemented with 2 mM glutamine (Sigma Chemical Co., St. Louis, Mo.) and 2% heat-inactivated fetal calf serum (Flow Laboratories, Mississauga, Ontario, Canada). Cell suspensions contained greater than 95% macrophages as determined by morphology, nonspecific esterase staining, and staining with antibody MAC-1 (22). Viability exceeded 98% by trypan blue exclusion.

PCA. Macrophages were evaluated for functional PCA in a one-stage clotting assay as previously described (36). Following incubation, samples to be assayed for PCA were washed three times with unsupplemented RPMI 1640 and then resuspended in unsupplemented RPMI 1640 at a concentration of 10^6/ml. The cells were then subjected to three cycles of freeze-thawing to obtain maximal total cellular PCA (36). Samples were assayed for the ability to shorten the spontaneous clotting time of normal citrated human platelet-poor plasma. Additional studies were performed with human plasmas which were congenitally deficient in coagulation factor VII, X, or II or fibrinogen (Helena Laboratories, Beaumont, Tex.) to determine the nature (factor dependence) of the procoagulant (36). Milliunits of PCA were assigned by reference to a standard curve generated with serial log dilutions of a standard rabbit brain thromboplastin (Dade Division, American Hospital Supply Co., Miami, Fla.). Media and reagents were without activity.

TNF. TNF activity was detected by using a semiautomated L929 fibroblast modified lytic assay as described by Kunkel et al. (32). The amount of cell lysis was determined by using a micro enzyme-linked immunosorbent assay autoreader. Units of TNF activity were determined by comparison with the amount of lysis generated by serial dilutions of recombinant TNF (Zanzyme, Boston, Mass.).

IL-1. Induction of IL-1 by MHV-3 in macrophages recovered from A/J or BALB/cJ mice was assessed by using the mouse thymocyte proliferation assay (45). Briefly, 10^6 C3H/HeJ mouse thymocytes were cultured in a final volume of 200 μl in RPMI 1640 supplemented with 10% fetal calf serum, 4 mM glutamine, 5×10^{-5} M mercaptoethanol (Sigma), and 100 U of penicillin and 100 μg of streptomycin (Flow Laboratories) per ml in 96-well flat-bottom microtest plates (Falcon Laboratories, Grand Island, N.Y.) with dilutions of UV-inactivated macrophage supernatants in the absence or presence of concanavalin A (2.5 mg/ml). Cultures were incubated for 66 h in a humidified CO_2 atmosphere at 37°C, pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, Mass.), and harvested at 72 h onto glass fiber filters. Total cell-associated radioactivity was measured in a Beckman scintillation counter, and bioassay data are expressed as units per milliliter, derived from a standard curve with mouse recombinant IL-1β (Genzyme, Boston, Mass.). Rabbit anti-mouse IL-1β antibodies (Genzyme) neutralized greater than 92% of experimental IL-1 activity, whereas rabbit anti-mouse IL-6 antibodies (Genzyme) had no effect on IL-1 activity.

LTB_4. LTB_4 was determined in a competitive inhibition radioimmunoassay (RIA) (New England Nuclear) (51). Rabbit anti-LTB_4 serum was diluted 1:1,000 in RIA buffer (50 mM Tris [pH 8.6] containing 0.1% gelatin), and aliquots (0.1 ml) were mixed with standard or sample (0.1 or 0.2 ml) in glass tubes (10 by 75 mm). ^{3}H-LTB_4 (specific activity, 30 Ci/mmol) in RIA buffer (0.1 ml containing approximately 7,500 dpm) was added to give a total incubation volume of 0.4 ml, and the mixture was incubated at 4°C for 18 to 24 h. Free LTB_4 was adsorbed onto dextran-coated charcoal as described for RIA of 6-keto-prostaglandin $F_{1\alpha}$ (15); after centrifugation, the supernatant containing the antibody-antigen complex was collected, and the radioactivity was determined in a liquid scintillation counter. Although maximum binding of ^{3}H-LTB_4 to the antiserum occurred after 1 h of incubation at 0 to 4°C, the incubation was continued overnight to ensure a consistent equilibrium between free acid and lactone in standards and sample. Buffer at pH 8.6 rather than 7.4 was used to minimize lactonization of LTB_4. Specificity of the RIA was determined by measuring the inhibition of ^{3}H-LTB_4 binding to the antiserum by a series of related hydroxy acids and prostaglandins; relative cross-reactions were calculated from the mass of the compound which caused 50% inhibition of binding.

The reproducibility of the assay was determined by repeated measurement of standard solutions of LTB_4 and samples containing LTB_4.

TGF-β. Levels of TGF-β were determined by measuring the inhibition of growth of the TGF-β-sensitive mink lung epithelial cell line Mv1Lu (CCL-64; American Type Culture Collection, Rockville, Md.) (14). Briefly, mink lung epithelial cells were maintained in Eagle's minimum essential medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 4 mM glutamine, 1.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 10% fetal calf serum. Then 5×10^4 cells were plated in 200 μl of medium in 96-well plates (Corning Glass Works, Corning, N.Y.) and incubated for 20 h at 37°C in a 5% CO_2 atmosphere. After 20 h, the cells were washed and resuspended in 100 μl of medium and 100 μl of UV-inactivated supernatants from peritoneal macrophages from A/J or BALB/cJ mice which had been incubated in the presence or absence of MHV-3 for up to 24 h. Prior to addition to the culture wells, the supernatants were heated at 75°C for 5 min to inactivate the latent TGF-β present in the supernatant (9). The cells were then pulse labeled with [^{3}H]thymidine (1.0 μCi per well; Amersham, Mississauga, Ontario, Canada) and incubated for an additional 8 h. Cells were then harvested onto glass fiber filters, and [^{3}H]thymidine incorporation was measured in an LKB-Wallac liquid scintillation counter. Growth inhibition was measured by the incorporation of [^{3}H]thymidine and converted to nanograms of TGF-β per milliliter by comparison with a standard curve constructed by using recombinant human TGF-β (R and D Systems, Minneapolis, Minn.). Supernatants were devoid of replicating virus as determined by plaque assay (36). UV-inactivated MHV-3 (10^8 viral particles per ml) had no effect on the growth of the Mv1Lu cell line.

PGE_2. Supernatants from control and MHV-3-infected macrophages in culture were collected and assayed for PGE_2 by RIA (New England Nuclear) (32). Tubes were counted in a gamma counter with an efficiency of 44% for 1 min. Average counts were determined for a set of duplicates, and the normalized percent bound (% B/B_0) for each standard and sample was determined as [(net cpm of sample/standard)/net cpm of standard] × 100.

Northern (RNA) blot analysis. The levels of mRNA transcripts of TF, musfiblp (prothrombinase), TNF, and IL-1 were assayed by Northern blot analysis. Macrophages were pelleted in 15-ml polypropylene tubes (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.), and total cellular RNA was isolated by 8 M acid-guanidium hydrochloride extraction in a modified procedure described by Arnstein and Cox (2) or by cesium chloride ultracentrifugation (25). RNA was resolved on a 1% agarose gel containing formaldehyde and transferred onto a nitrocellulose membrane (Bio-Rad, Oakville, Ontario, Canada). A 1-kb tissue factor cDNA (50), a 1.3-kb musfiblp cDNA (49), a 1.4-kb TNF cDNA (10), and a 2-kb IL-1 cDNA (38) were excised from plasmids and purified by agarose gel electrophoresis. The probes were labeled by using a random-priming DNA labeling system (Pharmacia Inc., Montreal, Quebec, Canada) with [α-^{32}P]dCTP (specific activity, >3,000 Ci/mmol; Amersham).

Membranes were prehybridized for 5 h at 42°C in a mixture containing formamide (50%), 5× Denhardt's solution, 0.2% sodium dodecyl sulfate, 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 carried out overnight at 42°C in the same mixture. The membranes were then washed under low- to medium-stringency conditions, and the membranes were exposed to Kodak XAR-5 film with intensifying screens for 24 h at −70°C. To confirm that equivalent amounts of RNA had been added in all lanes, the membrane was probed with an α-tubulin cDNA.

Endotoxin contamination. Media were tested for endotoxin contamination by the standard *Limulus* amoebocyte lysate assay (Association of Cape Cod, Woods Hole, Mass.) and were found to contain less than 0.003 ng of endotoxin per ml, which constituted the lower limit of the test.

Statistical analysis. Statistical analysis was carried out by using analysis of variance and the Wilcoxon rank sum test, and a P value of 0.05 or less was considered to be statistically significant. Results are reported as the mean and standard deviation for at least four separate experiments, each performed in triplicate.

RESULTS

Viral titers. The patterns of viral growth as determined by measurement of sequential viral titers were similar in macrophages from resistant A/J and susceptible BALB/cJ mice as described previously (42).

PCA. The time course for induction of functional PCA by MHV-3 in peritoneal macrophages isolated from BALB/cJ and A/J mice is shown in Fig. 1A. Macrophages from susceptible

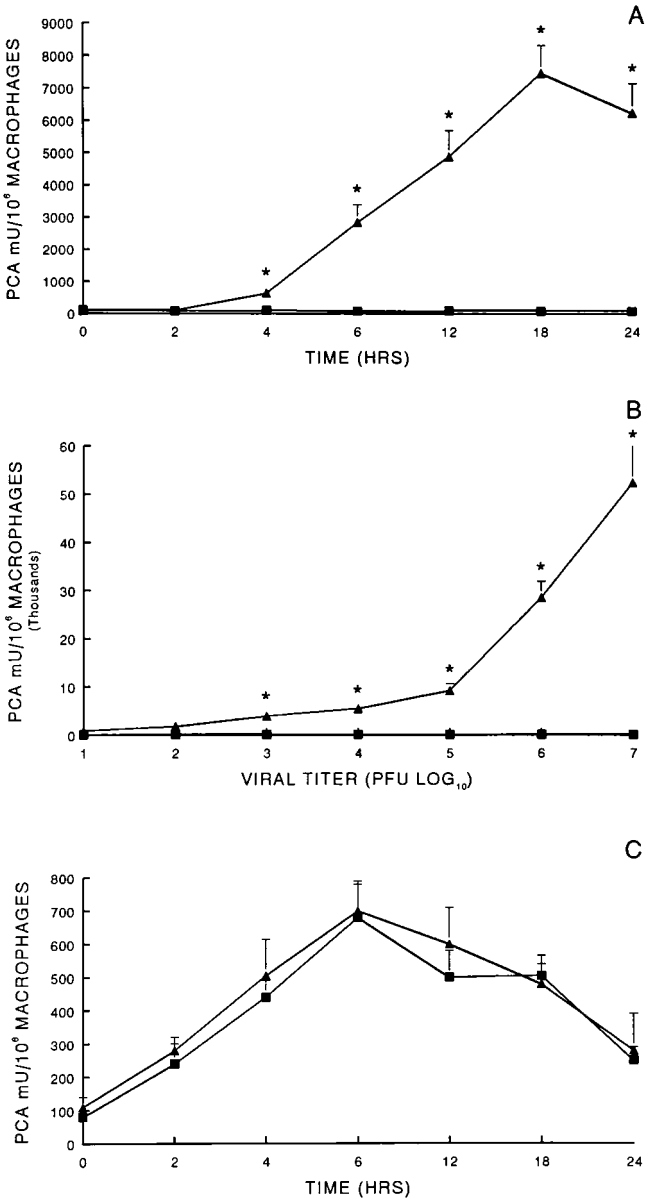

FIG. 1. Induction of PCA in macrophages from A/J and BALB/cJ mice by MHV-3 and LPS. (A) One million macrophages from A/J (■) or BALB/cJ (▲) mice were stimulated with MHV-3 at an MOI of 0.1. Cells were harvested at times shown, and following freeze-thawing, maximal cellular PCA was measured. (B) One million macrophages were stimulated with various doses of MHV-3 and harvested after 18 h for measurement of PCA activity. (C) Macrophages were stimulated with 10 μg of LPS per ml and assayed for total content of PCA. Results represent the mean ± standard deviation of three separate experiments done in duplicate. (* indicates $P < 0.05$ compared with control unstimulated cells.)

mice infected with MHV-3 demonstrated a significant rise in PCA as early as 4 h postinfection (p.i.) (645 ± 140 mU/10^6 macrophages), reaching a maximum level of 7,480 ± 840 mU/10^6 macrophages at 18 h p.i., in comparison with basal values of 120 ± 30 mU/10^6 macrophages in unstimulated controls. Induction of PCA by MHV-3 in macrophages from BALB/cJ mice was dose dependent, with induction of 920 ± 140 mU/10^6 macrophages seen following stimulation with 10 PFU and reaching a maximum of 52,400 ± 16,500 mU/10^6 macrophages with 10^7 PFU of MHV-3 (Fig. 1B). No induction of PCA above

TABLE 1. Effect of coagulation factor-deficient plasmas on PCA expression[a]

Plasma	PCA (mU/10^6 cells)	
	LPS	MHV-3
Normal	740 ± 145	7,480 ± 840
Deficient in:		
Factor VII	<10	7,530 ± 620
Factor X	<1	7,160 ± 510
Factor II	<1	<10
Fibrinogen	<1	<1

[a] One million BALB/c macrophages were stimulated with LPS (10 μg/ml) or MHV-3 (MOI of 0.1) for 18 h, harvested, and assayed for the ability to shorten the spontaneous clotting time of recalcified normal platelet-poor citrated human plasma or human plasmas deficient in factor II, VII, or X or fibrinogen in a one-stage clotting assay. Results represent the mean ± 1 standard deviation for three separate experiments.

the basal activity of 120 ± 25 mU/10^6 macrophages was seen in macrophages from resistant A/J mice at all titers of virus used (10 to 10^7 PFU) and at all time points studied (0 to 48 h) (Fig. 1A and B).

Data shown in Table 1 demonstrate that MHV-3-induced PCA was independent of factors VII and X but dependent on factor II, consistent with previous studies characterizing the MHV-3-induced PCA as a prothrombinase (13, 22, 36). In contrast to cells stimulated with MHV-3, LPS induced a rapid and equivalent rise in PCA in peritoneal macrophages from both A/J and BALB/cJ, an activity which was evident at 1 to 2 h and reached a maximum at 8 to 12 h (Fig. 1C). Studies using factor-deficient plasma indicated that the nature of the LPS-induced PCA was consistent with that of TF (Table 1). LPS-stimulated cells were incapable of initiating coagulation of factor VII-deficient plasma, indicating that no prothrombinase activity was induced by LPS.

Northern blot analyses were performed with the cDNA probes for TF and musfiblp. RNA hybridizing to the musfiblp-specific probe was not detected in RNA isolated from uninfected macrophages from either A/J or BALB/cJ mice. In MHV-3-infected macrophages from BALB/cJ mice, musfiblp-specific mRNA was first detected at 2 h, peaked at 8 h, and was still detectable at 12 h p.i. (data not shown). The kinetics of induction of musfiblp mRNA was similar to that previously reported (49). Transcription of musfiblp RNA was also observed in A/J macrophages which had been infected with MHV-3; however, the level of maximum induction occurred later (12 h p.i.) and was 35-fold less than that observed in BALB/cJ macrophages (data not shown), similar to previously reported results (49). RNA isolated from A/J and BALB/cJ macrophages which had been stimulated with LPS did not hybridize with musfiblp but hybridized with the TF cDNA probe (data not shown).

TNF. The production of TNF by peritoneal macrophages from BALB/cJ and A/J mice stimulated with 10 μg of LPS per ml is depicted in Fig. 2A. Low basal levels of TNF were detected in macrophages from both BALB/cJ (20 ± 10 U/ml) and A/J (25 ± 15 U/ml) mice (Fig. 2). The pattern of TNF production in response to LPS stimulation did not differ between resistant and susceptible strains. TNF production in macrophages from BALB/cJ and A/J mice was characterized by early increases from 20 ± 10 and 25 ± 15 U/ml, respectively, to 590 ± 90 and 485 ± 120 U/ml, respectively, at 6 h of incubation. TNF continued to rise to reach maximum levels of 1,150 ± 105 and 1,100 ± 200 U/ml, respectively, at 24 h (Fig. 2A).

Induction of TNF in macrophages from resistant and sus-

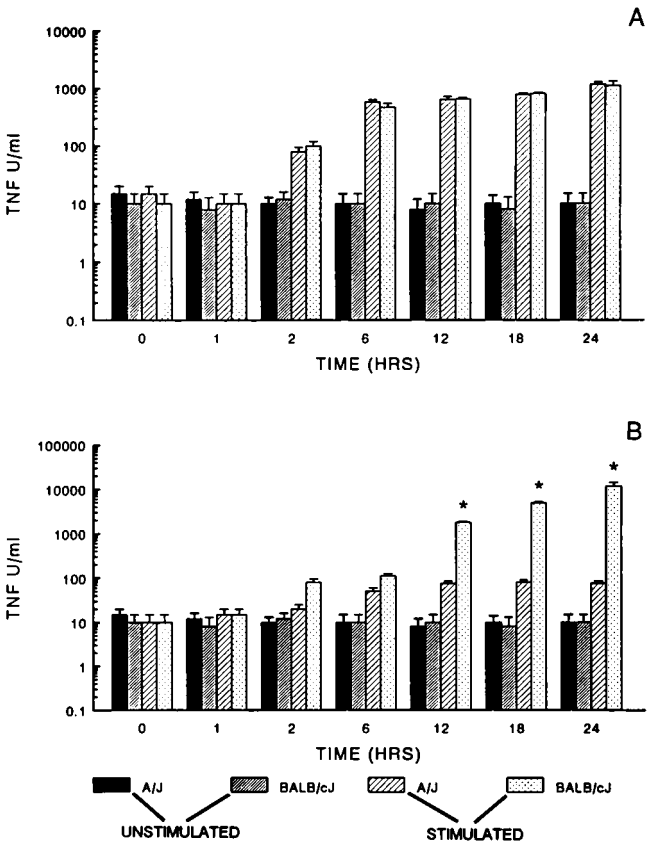

FIG. 2. Induction of TNF by LPS (A) and MHV-3 (B). One million macrophages from A/J or BALB/cJ macrophages were stimulated with MHV-3 at an MOI of 0.1 or LPS at 10 μg/ml. TNF levels in the supernatants were measured by an L929 fibroblast modified lytic assay after incubation for the times shown. Results represent the mean ± standard deviation for three separate experiments done in triplicate. (* indicates $P < 0.05$ compared with stimulated A/J macrophages.)

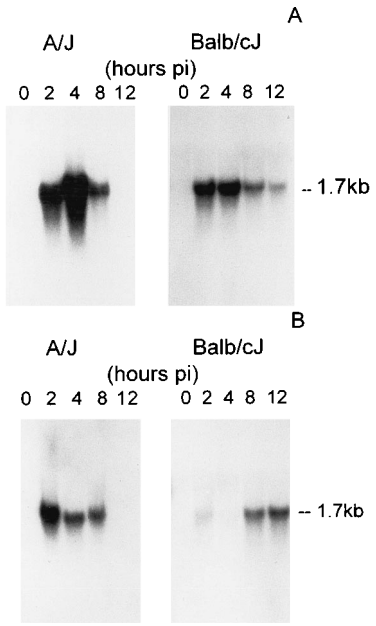

FIG. 3. TNF-α mRNA levels in BALB/c and A/J macrophages stimulated with MHV-3 or LPS. Ten micrograms of total RNA isolated from peritoneal macrophages from BALB/cJ and A/J macrophages which had been stimulated with 10 μg of LPS per ml (A) or 10^5 PFU of MHV-3 (B) for periods of time up to 12 h was added to each lane, and the RNA was hybridized with a TNF-α cDNA as described in Materials and Methods. An α-tubulin cDNA was used to ensure equal levels of mRNA in all lanes (data not shown).

ceptible mice in response to stimulation with 10^5 PFU of MHV-3 is plotted against time in Fig. 2B. A marked increase in TNF was observed in BALB/cJ macrophages, with a maximum response of 9,800 ± 2,200 U/ml observed at 24 h p.i. The level of TNF induction achieved in A/J macrophages was less than that seen in BALB/cJ macrophages and remained constant from 12 to 24 h p.i. rather than continuing to increase as was seen in macrophages from BALB/cJ mice.

By Northern blot analysis, LPS induced an equivalent increase in mRNA from both resistant A/J and susceptible BALB/cJ macrophages by 2 h which could still be detected at 8 h (Fig. 3A). Following MHV-3 infection, maximal TNF mRNA levels in macrophages from A/J mice were detected at 2 h, falling thereafter but being still detectable at 8 h. In contrast, in macrophages from BALB/cJ mice, TNF mRNA was detected in small amounts at 2 h, but continued to increase up to 12 h p.i. (Fig. 3B).

IL-1. Supernatants from macrophages were analyzed for production of IL-1 in response to both LPS (10 μg/ml) (Fig. 4A) and MHV-3 (Fig. 4B). In response to LPS, nearly equivalent increases in the production of IL-1 were detected in macrophages from A/J and BALB/cJ mice ($P > 0.15$ at all time points). An increase in IL-1 was detected at 2 h following stimulation with LPS, reaching maximum levels by 4 h and then slowly declining to near baseline levels by 24 h (Fig. 4A).

Following stimulation with 10^5 PFU of MHV-3, equivalent IL-1 responses were seen in macrophages from BALB/cJ and A/J mice at 4 h p.i., but by 8 h and at all time points thereafter to 24 h, the production of IL-1 by BALB/cJ macrophages in response to MHV-3 stimulation was greater than that seen in macrophages from A/J mice. For both strains of mice, IL-1 production in response to MHV-3 was less than that in response to LPS ($P < 0.005$) (Fig. 4B). By Northern analysis, in response to LPS, mRNA was detected at 2 h in both BALB/cJ and A/J macrophages and persisted to 8 h (Fig. 5A). In response to MHV-3, mRNA levels were less than those induced by LPS but could be detected by 2 h and persisted for 12 h in macrophages from BALB/cJ mice, whereas in A/J macrophages, IL-1 mRNA peaked at 2 h and thereafter diminished, correlating with functional IL-1 activity (Fig. 5B).

LTB_4. The time course for the production of LTB_4 in BALB/cJ and A/J macrophages in response to LPS is shown in Fig. 6A. A steady time-dependent increase in LTB_4 was observed in macrophages from susceptible mice, attaining a maximum observed value at 24 h poststimulation (13.2 ± 1.4 pg/ml). In contrast, no increase in LTB_4 was seen in macrophages from resistant mice at any time point studied to 24 h.

The time course for the induction of LTB_4 in peritoneal macrophages in BALB/cJ and A/J macrophages infected with MHV-3 is shown in Fig. 6B. A steady increase in LTB_4 was noted in macrophages from susceptible BALB/cJ mice, with peak levels at 24 h (25.2 ± 2.4 pg/ml). Similar to the lack of response to LPS, no significant production of LTB_4 above the basal level of 1.5 ± 1.0 pg/ml was demonstrated in A/J macrophages following MHV-3 infection.

Induction of immunosuppressive molecules by MHV-3. (i) TGF-β. TGF-β is a multifunctional modulator of cell activity (56). It has a broad spectrum of action on several cell types and is known to be a powerful inhibitor of cell proliferation and cell function (29). Within the immune system, TGF-β functions

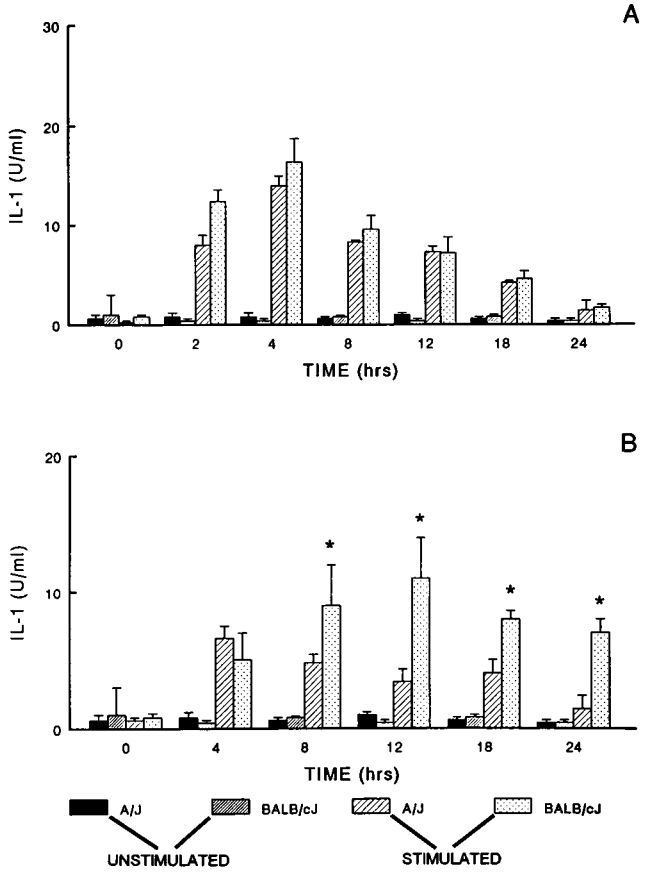

FIG. 4. Induction of IL-1 by LPS and MHV-3. One million macrophages from A/J or BALB/cJ mice were stimulated with LPS at 10 μg/ml (A) or MHV-3 at an MOI of 0.1 (B) for the times shown. IL-1 was measured in the supernatants by a thymocyte proliferation assay. Results represent the mean ± standard deviation for three separate experiments done in triplicate. (* indicates $P < 0.05$ compared with stimulated A/J macrophages.)

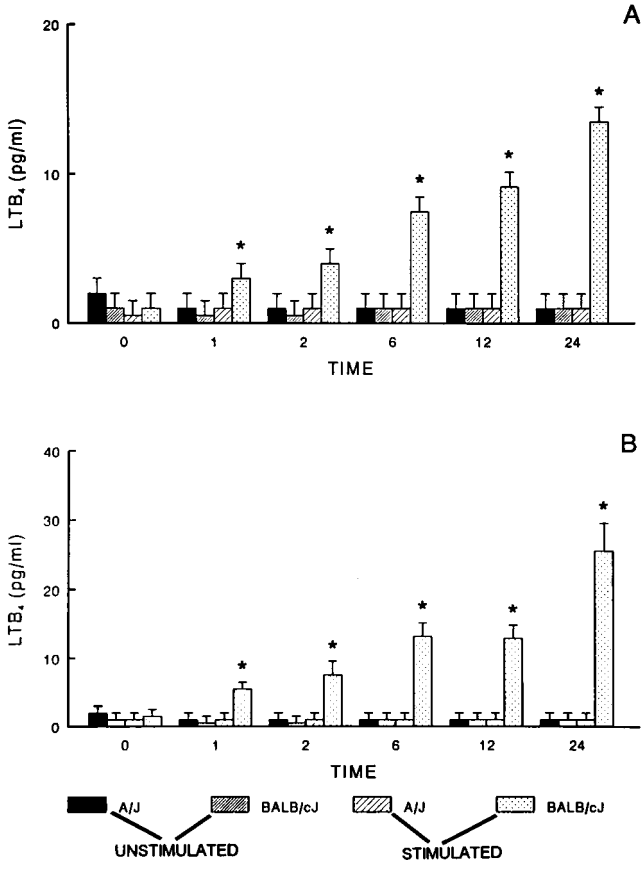

FIG. 6. Induction of LTB_4 by LPS and MHV-3. One million macrophages from A/J or BALB/cJ mice were stimulated with LPS at 10 μg/ml (A) or MHV-3 at an MOI of 0.1 (B) for the times (in hours) shown. LTB_4 was measured in the supernatants by a competitive inhibition RIA as described in Materials and Methods. Results represent the mean ± standard deviation for three separate experiments done in triplicate. (* indicates $P < 0.05$ compared with stimulated A/J macrophages.)

primarily as an immunosuppressive cytokine inhibiting both cellular and humoral immunity (29). We therefore studied whether increased production of this cytokine might account for the lack of responsiveness to MHV-3 infection. Macrophages from resistant (A/J) and susceptible (BALB/cJ) mice produced comparable amounts of TGF-β in response to LPS stimulation over a 24-h period (Fig. 7A). An increase in TGF-β could be detected within 1 h of stimulation, reaching maximal levels at 18 h and decreasing by 24 h. Following MHV-3 infection, TGF-β could be detected in macrophages from BALB/cJ mice within 1 h of infection, reaching maximal levels by 6 h and declining to 50% of peak values over the next 18 h (Fig. 7B). The TGF-β response to MHV-3 was greater than the response to LPS at all time points. In contrast, macrophages from A/J mice failed to produce TGF-β above basal levels in response to MHV-3 at all time points studied to 24 h (Fig. 7B).

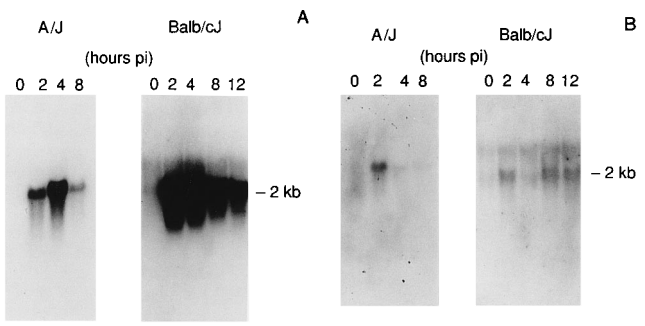

FIG. 5. IL-1-α mRNA expression in macrophages stimulated with MHV-3 or LPS. Ten micrograms of total RNA extracted from BALB/cJ and A/J macrophages which had been stimulated with 10 μg of LPS per ml (A) or 10^5 PFU of MHV-3 (B) was added to each lane and hybridized with an IL-1-α cDNA as described in Materials and Methods. An α-tubulin cDNA was used to ensure equal loading of all lanes (data not shown).

(ii) PGE_2. PGE_2 is an immunosuppressive prostanoid known to downregulate the production of a variety of inflammatory molecules, including TNF and IL-1, and PCA (55). We therefore tested the hypothesis that an increased production of this molecule in A/J mice might be responsible for the lack of response of macrophages from these mice to MHV-3.

The time course for induction of PGE_2 production following MHV-3 infection was more rapid in macrophages from resistant A/J mice than in those from susceptible BALB/cJ mice (1 h versus 2 h), and at 2 h p.i., the amount produced was greater (5×10^{-13} M compared with 1×10^{-13} M; $P < 0.03$) (Fig. 8A). However, by 6 h p.i., there were no significant differences in PGE_2 production although maximum levels of PGE_2 production by macrophages from BALB/cJ mice (10^{-12} M) oc-

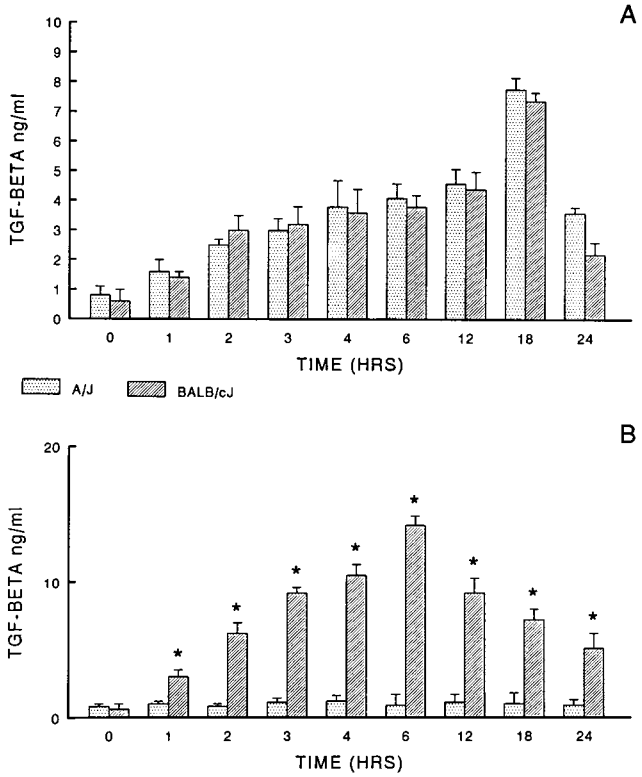

FIG. 7. Induction of TGF-β by LPS and MHV-3. One million A/J or BALB/cJ macrophages were stimulated with LPS at 10 μg/ml (A) or MHV-3 at an MOI of 0.1 (B) for the times shown. TGF-β was measured in the supernatants by the inhibition of the growth of the mink lung epithelial cell line Mv1Lu. Results represent the mean ± standard deviation for three experiments. (* indicates $P < 0.05$ when compared with stimulated A/J macrophages.)

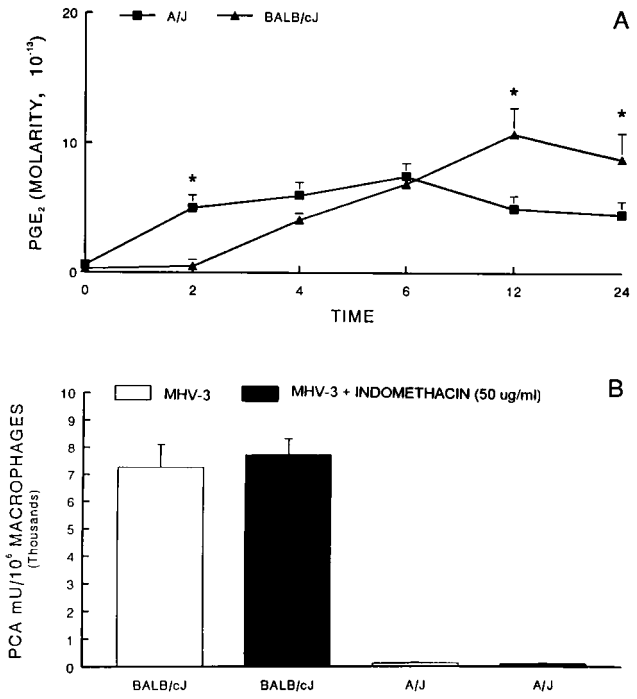

FIG. 8. (A) Production of PGE_2 following MHV-3 stimulation. One million A/J or BALB/cJ macrophages were stimulated with MHV-3 at an MOI of 0.1 for the times shown, and PGE_2 levels in the supernatants were measured by RIA. Results represent the mean ± standard deviation for three separate experiments done in triplicate. (* indicates $P < 0.05$.) (B) Effect of indomethacin on MHV-3-stimulated PCA production. One million A/J or BALB/cJ macrophages were preincubated in medium with or without indomethacin at a concentration of 50 μg/ml for 1 h and then stimulated with MHV-3 at an MOI of 0.1. The cells were harvested after the times shown, and PCA was measured in a one-stage clotting assay as described in Materials and Methods. Results represent the mean ± standard deviation for three separate experiments done in duplicate.

curred later than in macrophages from A/J mice (12 h versus 6 h).

To determine whether endogenously produced PGE_2 was capable of modulating induction of PCA by MHV-3-stimulated macrophages from BALB/cJ or A/J mice, indomethacin (50 μg/ml) was added during stimulation of macrophages with MHV-3. This concentration had previously been shown to inhibit PGE_2 release (data not shown). Figure 8B shows that indomethacin was unable to restore PCA production by macrophages from A/J mice infected with MHV-3, nor did it alter PCA production by MHV-3-stimulated macrophages from BALB/cJ mice.

DISCUSSION

Although the mechanism of susceptibility and resistance to MHV-3 is poorly understood, previous work has implicated the macrophage as a contributor to resistance to MHV-3 (5, 23, 53). Initial studies have suggested that differences in the rate of MHV replication in macrophages from resistant and susceptible animals accounted for host resistance or susceptibility (5, 23). However, subsequent work has failed to reproduce these differences in patterns of viral replication (31, 41). In the present study, there were no significant differences in viral replication in macrophages from resistant and susceptible animals, supporting the concept that additional factors may be involved in the differential response of these mice to infection with MHV-3.

MHV-3 stimulation of macrophages from susceptible BALB/cJ mice resulted in a rapid production of IL-1, TNF, TGF-β, LTB_4, and the procoagulant musfiblp, whereas macrophages from resistant A/J mice either did not produce these mediators or produced them in lesser amounts and for a shorter duration. Although a multiplicity of infection (MOI) of 0.1 was used for most experiments shown, similar results were obtained with an MOI of 10 (not shown). Thus, we believe that the inflammatory mediators detected following MHV-3 infection of macrophages originate from infected cells, although activation of adjacent uninfected macrophages cannot be excluded. The observation that macrophages from A/J mice were able to produce IL-1, TNF, and TGF-β in response to LPS implies that the differences in signaling must be specific to MHV-3 rather than a global nonresponsiveness of these macrophages. It is unclear why A/J macrophages did not produce LTB_4 in response to either LPS or MHV-3. Possible explanations include inhibition of 5-lipoxygenase activity through a yet unclear mechanism or a defect in the production of a membrane protein termed the 5-lipoxygenase-activating protein, which is required for LTB_4 synthesis in response to ionophore stimulation (20, 44).

Kinetic differences in the production and subsequent downregulation of inflammatory mediator levels in response to MHV-3 were observed. The differences may be due to (i) differences in induction of mRNA transcription in macrophages from susceptible and resistant mice, which may in turn be related to differences in signal transduction or to mediator interactions; (ii) differential impairment of host cell mRNA

translation by MHV-3 (27); and (iii) differential rates of degradation of mRNA and functional protein.

Evaluation of the mRNA transcripts for musfiblp indicated that the levels paralleled the expression of PCA activity in A/J and BALB/cJ mice. Three different musfiblp-specific RNA species were detected in MHV-3-infected macrophages, with the predominant species being approximately 4 kb in length. Small amounts of larger RNAs, approximately 6.5 and 7.5 kb in length, were also observed. The 4-kb RNA corresponds in size to transcripts of musfiblp originally described in cytotoxic T lymphocytes and is thought to represent a fully spliced mature mRNA (30). The less abundant larger RNA species may correspond to either unspliced or alternatively spliced transcripts or possibly polyadenylation at an alternative downstream polyadenylation site. The maximum level and duration of expression of the predominant 4-kb mRNA transcripts from the macrophages from BALB/cJ mice was significantly greater than those observed in macrophages from A/J mice, suggesting differences in the signaling pathways in response to MHV-3 in A/J mice compared with BALB/cJ mice. Alternatively, the differences in the level of transcripts may be related to different degrees of mRNA stability in the two strains. Hilton et al. (27) have reported that MHV infection of fibroblasts may destabilize at least some host mRNAs at late times of infection; this effect could contribute to the decline of musfiblp mRNAs that we observe 12 h p.i. However, no functional PCA was produced in A/J mouse-derived macrophages. Previous studies with a monoclonal antibody against the prothrombinase (3D4.3) have also failed to demonstrate PCA expression in MHV-3-stimulated A/J macrophages (22), suggesting that the musfiblp transcripts detected in A/J macrophages are either not translated or not properly modified posttranslation. Our previous observation that PGE_2 reduces functional PCA in MHV-3-stimulated BALB/cJ macrophages by a posttranscriptional mechanism supports the notion that protein modification is required for expression of PCA (12). When considered together, these findings suggest that differences in signaling as well as possibly transcription, translation, and posttranslational modification may contribute to the marked differences in musfiblp production to MHV-3 stimulation in resistant and susceptible mice.

The finding that the nature and pattern of the procoagulants induced by LPS and MHV-3 were different was not totally unexpected. First, in previous studies as well as the present study, analysis using factor-deficient plasmas has consistently shown that the PCA induced by LPS is dependent on factor VII for its activity, whereas the MHV-3-induced PCA is dependent only on factor II for its activity (13, 36). Second, monoclonal antibodies produced against the MHV-3-induced procoagulant recognize the MHV-3-induced PCA but do not react with LPS-induced PCA or with human or rabbit thromboplastin (37). Third, in this study, Northern analysis using a murine TF cDNA and a cDNA to musfiblp showed that LPS stimulation of macrophages from both A/J and BALB/cJ mice augments the level of transcripts for TF but not musfiblp, whereas MHV-3 stimulation of macrophages from these same strains of mice causes transcription of musfiblp and not TF. Induction of TF in macrophages has also been shown to be enhanced by $CD3^+$ $CD4^+$ $CD8^-$ TH1 cells (21), whereas we have recently shown that musfiblp production by macrophages following MHV-3 infection is inhibited by $CD3^+$ $CD4^+$ TH1 cells (11). Finally, recent data have demonstrated the presence of a 56-bp LPS response element within the TF promoter that confers LPS responsiveness (39). On initial scanning of the 5′ promoter region of musfiblp, this sequence is not present, possibly explaining the inability of LPS to induce musfiblp transcription.

Differences in transcription of IL-1 and TNF were also observed in MHV-3-stimulated macrophages from A/J and BALB/cJ mice. IL-1 and TNF mRNA transcription from macrophages from resistant A/J mice was rapid but decreased by 8 h p.i. In contrast, mRNA transcripts in macrophages from susceptible BALB/cJ mice continued to increase up to 12 h p.i. This is in distinct contrast to the equivalent time course of induction of IL-1 and TNF mRNAs by macrophages from both strains of mice in response to LPS. The apparent inconsistencies between TNF and IL-1 mRNA and protein levels must be interpreted bearing in mind that protein levels were estimated by functional bioassays. Furthermore, the regulation of TNF and IL-1 protein expression is extremely complex and occurs at the levels of transcription (28, 52), mRNA stability (10), and translation (6). For functional activity of IL-1β, cleavage of an inactive precursor by interleukin-converting enzyme is required (58).

A role for TNF-α in liver injury has been suggested with respect to the fulminant hepatitis induced by *Propionobacterium acnes* and galactosamine (47). Both TNF and IL-1 are known to activate endothelial cells to produce TF (7, 48) and may thus contribute to sinusoidal thrombosis following MHV-3 infection by enhancing production of procoagulants by endothelial cells (17, 37, 42). Furthermore, LTB_4 may also contribute to the development of hepatic necrosis by causing vascular permeability changes (59) or by directly damaging hepatocytes as has been described following frog virus 3 infection (26).

One possible mechanism underlying the differential susceptibility to infection and the differential induction of macrophage products might be that A/J mice generate higher levels of immunosuppressive factors in response to MHV-3 than do BALB/cJ mice. TGF-β, a known immunosuppressive cytokine, was found to be produced by macrophages from susceptible but not resistant mice in response to MHV-3 infection. Although the exact role for TGF-β in MHV-3 infection has not been determined, recent experiments in our laboratory have shown that TGF-β primes macrophages from susceptible mice to express increased amounts of PCA in response to MHV-3 stimulation (unpublished data), and thus TGF-β may be a contributing factor to the severity of MHV-3-induced hepatic necrosis. Furthermore, induction of TGF-β by MHV-3 may account for the lack of immune responsiveness which is a known consequence of MHV-3 infection in susceptible mice (16, 19, 33).

Previous studies from our laboratory have shown that exogenous administration of PGE_2 blocks induction of the MHV-3-induced macrophage PCA in vitro and in vivo (12) and prevents fulminant hepatic necrosis in vivo (55). However, the hepatoprotective effects of PGE_2 and inhibition of PCA were observed only at pharmacologic doses (10^{-8} to 10^{-6} M); at less than 10^{-12} M, no inhibition of PCA or hepatoprotection was observed. Although in this study, PGE_2 was produced earlier and in greater amount in A/J macrophages, the amount produced would appear to be insufficient to inhibit induction of PCA and prevent hepatic necrosis. In addition, the failure of indomethacin to affect PCA production in response to MHV-3 suggests that the amount of prostaglandin produced does not regulate macrophage expression of PCA by either an autocrine or a paracrine effect. Considered together, these data suggest that A/J mice are not protected from MHV-3 infection or induction of cytokines by virtue of their ability to generate increased levels of immunosuppressive factors.

In conclusion, the pattern of immediate response genes to

MHV-3 infection in macrophages from susceptible BALB/cJ mice differs markedly from that of the resistant A/J strain. Furthermore, these particular mediators, or indeed other genes regulated in a similar fashion, may be responsible for the synthesis of products that either primarily drive or contribute to the complex pattern of pathology seen in MHV-3 infection as we have previously reported.

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