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Induction of Macrophage Procoagulant Activity by Murine Hepatitis Virus Strain 3: Role of Tyrosine Phosphorylation

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The induction of a unique macrophage procoagulant molecule by murine hepatitis virus strain 3 correlates with the severity of viral hepatitis. The role of tyrosine phosphorylation in the signalling pathway leading to procoagulant expression was studied. Murine hepatitis virus strain 3 initiated a rapid increase in phosphotyrosine accumulation. Tyrosine kinase inhibition precluded this increase and abrogated expression of the virus-induced procoagulant mouse fibrinogen-like protein (musfiblp) gene. These findings suggest that manipulation of this signalling pathway in vivo might represent a novel approach to treating this disease.

Studies using a model of viral hepatitis induced by infection with murine hepatitis virus strain 3 (MHV-3) have provided significant insight into the mechanisms underlying the pathogenesis of this disease and have suggested novel approaches to therapy (7). Fulminant hepatitis induced by this virus is characterized by the presence of sinusoidal thrombosis and associated hepatocellular necrosis. Several lines of evidence support the concept that virus-induced macrophage-mediated fibrin deposition (i.e., procoagulant activity [PCA]) plays a central role in the disease process. First, the degree of hepatocellular necrosis following infection correlates well with the induction of macrophage PCA (12, 21). While macrophages from susceptible mouse strains (BALB/cJ) infected with MHV-3 exhibit a marked increase in PCA, those recovered from resistant mice (A/J) fail to do so. Second, the administration of exogenous prostaglandin E_2 completely abrogates viral induction of macrophage PCA both in vitro and in vivo and prevents the development of fulminant hepatitis (1, 8). Finally, pretreatment with an antibody which neutralizes the macrophage PCA induced by MHV-3 prevents sinusoidal fibrin deposition, hepatocellular necrosis, and mortality in infected mice (23). When considered in aggregate, these observations provide strong support for a causal relationship between MHV-3-induced PCA and the development of fulminant hepatitis in this model.

MHV-3-induced PCA is unique among the family of macrophage procoagulants by virtue of its direct prothrombin cleaving activity (28). Recent cloning of the gene has identified it as a mouse fibrinogen-like protein (musfiblp) (26). The cellular mechanisms underlying the induction of this protein are presently being defined. Detailed studies by Holmes and colleagues have characterized the MHV receptor as a 110- to 120-kDa glycoprotein in the carcinoembryonic antigen family of glycoproteins (9, 14). The expression of this receptor in human and hamster cell lines confers susceptibility to MHV infection. However, subsequent events in the signalling pathway(s) leading to the expression of musfiblp in MHV-infected macrophages have not been clearly elucidated.

Recent studies have reported that the induction of tyrosine phosphorylation in macrophages by a variety of stimuli, including lipopolysaccharide (LPS), zymosan, and immunoglobulin G, is a central signalling event leading to the generation of macrophage products (16, 18, 27, 31). The cellular receptors for many of the ligands (e.g., CD14 and the FcII receptor) do not fall into the family of receptor tyrosine kinases but rather have been shown to associate with and activate nonreceptor tyrosine kinases following their ligation (4). The MHV receptor similarly has a short cytoplasmic tail lacking domains capable of tyrosine kinase activity (14), although a splice variant of this receptor with a long cytoplasmic tail has been reported (13). These studies were performed to examine the ability of MHV-3 to induce tyrosine phosphorylation in macrophages and to investigate its role in the expression of musfiblp in MHV-3-stimulated cells. The data demonstrate that MHV-3 is able to induce rapid phosphotyrosine accumulation in these cells and that tyrosine kinase inhibitors prevent viral stimulation of PCA, both at the functional level and at the level of gene expression.

Specific-pathogen-free female Swiss Webster mice were obtained from Taconic Laboratories. Peritoneal macrophages were harvested by lavage, with 10 ml of sterile Hanks balanced salt solution, 4 days following intraperitoneal injection of 2 ml of thioglycolate medium. Lavage fluids were pooled and erythrocytes were lysed by brief hypotonic shock. The cell population was then diluted to 10^6 cells per ml in RPMI 1640 containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) and aliquoted into polypropylene tissue culture tubes. This technique generated a cell suspension with a viability in excess of 95%, as assessed by trypan blue exclusion, and a cell population of 80 to 90% macrophages, as assessed by nonspecific esterase staining, Wright's staining, and transmission electron microscopy. 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 (21). For studies using dead virus, killing was accomplished by UV irradiation for 15 min (UVG-11 ultraviolet lamp; Ultra-Violet Products Inc.).

Cells were stimulated at 37°C in 5% CO_2 in the presence or absence of MHV-3 at a multiplicity of infection (MOI) of 2.5:1. In some experiments, cells were pretreated with either genistein (Calbiochem; 10 μg/ml for 1 h), herbimycin A (Calbiochem; 1 μg/ml for 2 h), or tyrphostin 51 (Biomol; 1 μg/ml for 1 h) prior to stimulation. Control cells were exposed to the

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dimethyl sulfoxide vehicle, 0.1%, during the preincubation period. At the end of the incubation period, cells were sedimented by centrifugation at 300 × *g* for 10 min, resuspended in RPMI 1640, and frozen at −70°C for the measurement of PCA. For phosphotyrosine detection, cells were rapidly sedimented and pellets were immediately solubilized in boiling Laemmli sample buffer.

Tyrosine phosphorylation was determined by immunoblotting with antiphosphotyrosine antibodies (10, 17). Following electrophoresis on 10% polyacrylamide gels, the samples were blotted onto Immobilon with the Bio-Rad Mini Trans-Blot system for 1.25 h at 100 V. The blot was incubated with 10 ml of blocking solution and then exposed to a 1/1,000 dilution of a polyclonal antiphosphotyrosine antibody (Transduction Laboratories) for 2 h while shaking at room temperature. The blot was then washed four times with antibody buffer solution and incubated with a 1/5,000 dilution of goat anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Amersham). The blots were washed and quantitated by an enhanced chemiluminescence detection system (Amersham) (32). PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of normal citrated human plasma in a one-stage clotting assay (22). An 80 μl sample of macrophage cell lysates obtained by freeze-thawing was added to 80 μl of citrated normal human platelet-poor plasma, and then 80 μl of 25 mM $CaCl_2$ was added to initiate the reaction. The time taken for the appearance of a fibrin gel at 37°C with gentle agitation was recorded. Clotting times were converted to milliunits of PCA by comparison with clotting times of a rabbit brain thromboplastin standard in which 36 mg (dry weight) per ml was assigned a value of 100,000 mU of PCA. The induction of PCA from a baseline of 1,030 mU/10^6 macrophages to 4,800 mU/10^6 macrophages in cells stimulated by MHV-3 alone represented a shortening of the clotting time from 50 to 37 s. The assay was used over the range of 10 to 10,000 mU of PCA, this range being linear with normal plasma substrate. Previous studies have shown that PCA induced with MHV-3 has prothrombinase-like activities (28).

The induction of musfiblp mRNA was assessed by Northern (RNA) blot analysis with the 1.3-kb cDNA probe for musfiblp (26). Briefly, 5 × 10^6 cells were pelleted, and total RNA was extracted by the method of Chomczynski and Sacchi (6). After electrophoresis, RNA was transferred to Immobilon and hybridized with a randomly ^{32}P-labelled cDNA probe for murine musfiblp (26). Comparable RNA loading among lanes was ensured by probing with a cDNA probe for rat α-tubulin (19).

All solutions were tested for endotoxin contamination by the standard *Limulus* amoebocyte lysate assay (Association of Cape Cod, Woods Hole, Mass.) and were found to contain <0.1 ng of endotoxin per ml, which constituted the lower limit of the test. Statistics were calculated by a one-way analysis of variance and Neumann-Keuls test for comparison among groups. Data are expressed as the mean and standard error of *n* observations.

Figure 1 shows the time course of phosphotyrosine accumulation in macrophages exposed to MHV-3 (MOI, 2.5). MHV-3 induced a time-dependent increase in phosphorylated tyrosine residues starting as early as 1 min. Bands were observed at molecular masses of 33, 38, 41, 44, 49, 65, 86, and 91 kDa. Previous studies had shown that this antibody was specific for phosphotyrosine residues rather than for phosphoserine or phosphothreonine residues (17). Two inhibitors of tyrosine kinase activity were used to confirm that the increase represented a rise in kinase activity. As shown in Fig. 2, both genistein and herbimycin significantly reduced the level of

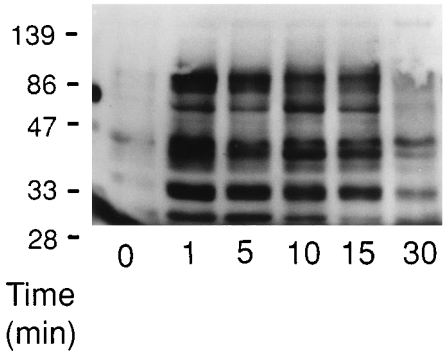

FIG. 1. Induction of tyrosine phosphorylation by MHV-3 in murine peritoneal macrophages. Cells (10^6/ml) were incubated with MHV-3 (2.5 × 10^6 PFU) for various times, rapidly sedimented, and resuspended in boiling Laemmli buffer. The phosphorylation of tyrosine residues was evaluated as described in the text. Time zero was the point immediately following the addition of virus to cells. The data shown are representative of three separate similar studies.

phosphotyrosine accumulation observed at 10 min without affecting cell viability (>90% by trypan blue exclusion). Inhibition was observed at the 5-min time point and as late as 120 min (data not shown).

To determine the functional significance of tyrosine phosphorylation in response to MHV-3, the effect of tyrosine kinase inhibition on macrophage PCA was studied. Figure 3 shows the time course of the effect of herbimycin on MHV-3-induced PCA. In MHV-3-stimulated cells, an increase in PCA was observed by 3 h, with a marked rise occurring after 6 h. At all time points studied, herbimycin markedly reduced MHV-3-induced PCA. Baseline PCA remained stable over the 9-h incubation period and was reduced slightly by the inclusion of herbimycin. Further, herbimycin inhibited PCA expression over a range of MOIs examined (data not shown). To support the concept that this effect was due to the inhibition of tyrosine

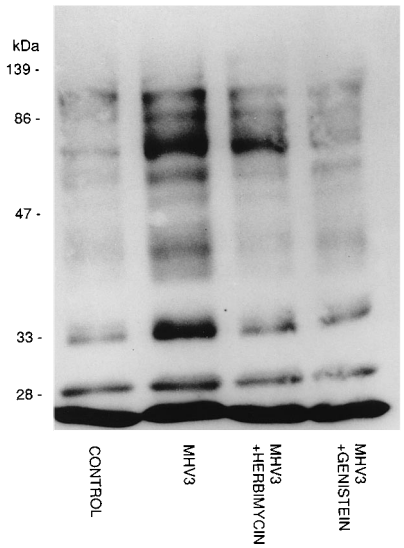

FIG. 2. Effect of tyrosine kinase inhibitors on MHV-3-induced tyrosine phosphorylation. Cells (10^6/ml) were preincubated with genistein (10 μg/ml for 1 h) or herbimycin (1 μg/ml for 2 h) and then treated with MHV-3 for 10 min. Cells were rapidly sedimented and resuspended in boiling Laemmli buffer. The phosphorylation of tyrosine residues was evaluated as described in the text. The data show a representative study of three similar independent studies. Molecular mass markers are shown at the left.

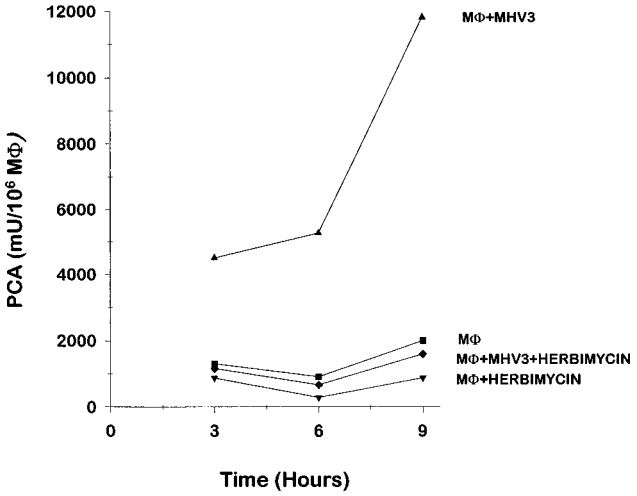

FIG. 3. Time course of the effect of herbimycin on MHV-3-induced PCA. Cells (10^6/ml) were preincubated with herbimycin (1 μg/ml for 2 h) or vehicle and then exposed to MHV-3 for various times. At the indicated times, cells were pelleted, resuspended in RPMI 1640, and frozen at −70°C for later PCA assay. The data shown are the means of duplicate samples in a single experiment and are representative of four independent studies. MΦ, macrophages.

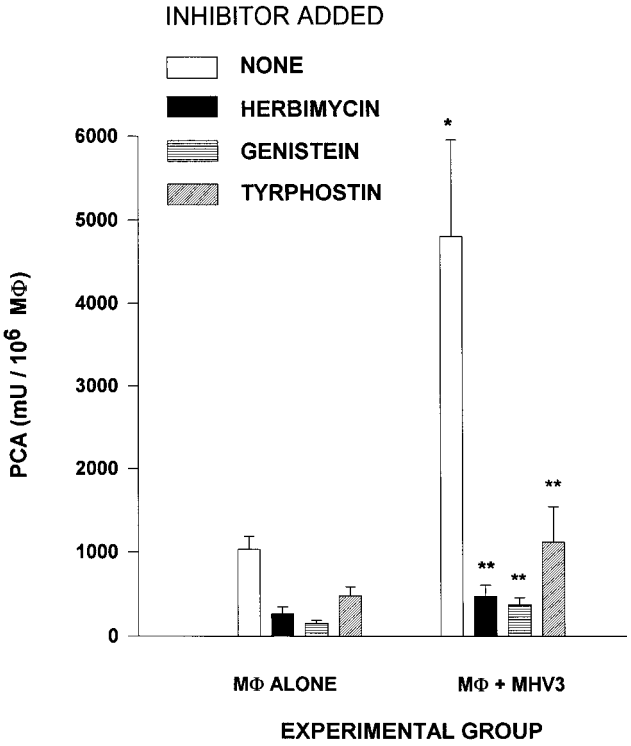

FIG. 4. The effect of different tyrosine kinase inhibitors on MHV-3-induced PCA. Cells (10^6/ml) were stimulated with MHV-3 (MOI, 2.5) in the presence or absence of herbimycin (1 μg/ml with a 2-h preincubation), genistein (10 μg/ml for a 1-h preincubation), tyrphostin 51 (25 μg/ml with a 2-h preincubation), or vehicle and incubated for 6 h. Cells were then pelleted, resuspended in RPMI 1640, and frozen at −70°C for later PCA assay. The data shown are the means of 3 to 8 independent studies, each performed in duplicate. MΦ, macrophages; *, $P < 0.01$ versus macrophages alone; **, $P < 0.01$ versus MHV-3 alone.

phosphorylation rather than to a nonspecific effect of herbimycin, two other inhibitors of tyrosine kinase activity with different mechanisms of action were used (2, 20). As illustrated in Fig. 4, both genistein and tyrphostin, in addition to herbimycin, reduced MHV-3-induced PCA. Further, since dead virus do not induce PCA expression (29), we examined their effect on phosphotyrosine accumulation. As illustrated in Fig. 5, UV-irradiated virus exhibited a markedly reduced ability to induce tyrosine phosphorylation compared with that of live virus. Parallel studies indicated that these viral particles were unable to induce functional PCA (data not shown). Considered together, these data are consistent with a role for tyrosine phosphorylation in MHV-induced PCA.

The induction of PCA by MHV-3 has been shown to require viable organisms and is roughly proportional to the size of the initial viral inoculum (29). Since tyrosine kinase inhibitors have been shown to inhibit the replication of some viruses (15), viability studies were performed to rule out the possibility that the inhibitors acted by this mechanism. At concentrations used to inhibit PCA induction, none of the inhibitors altered the viability or growth kinetics of MHV-3 (data not shown).

MHV-3-induced PCA has previously been shown to exhibit direct prothrombin cleaving activity as its mechanism for initiating the coagulation cascade (28). The cDNA has recently been cloned, and the MHV-3-induced PCA has been identified as mouse fibrinogen-like protein (26). To determine the effect of tyrosine kinase inhibition on musfiblp gene expression, Northern blot analysis was used (Fig. 6). While there was no constitutive expression of the musfiblp gene in macrophages, incubation with virus caused a marked increase by 5 h, as previously reported (26). Genistein completely prevented the MHV-stimulated rise in the level of mRNA transcripts. Probing with the cDNA for α-tubulin indicated comparable loading among lanes. The apparent slight reduction in the size of the α-tubulin mRNA in the genistein groups was not a consistent finding.

The present studies demonstrate a role for tyrosine phosphorylation in the signalling pathway leading to the induction of macrophage PCA by MHV-3. The virus induced a rapid rise in phosphotyrosine accumulation, which peaked at 10 to 15 min and was prevented by specific tyrosine kinase inhibitors. These inhibitors were also able to abrogate MHV-3-stimulated functional PCA as well as the expression of the gene encoding the unique MHV-3-induced prothrombinase, musfiblp. This effect was not due to altered cell viability, nor was viral viability or replication affected. Finally, the observation that dead virus showed a markedly reduced ability to induce phosphotyrosine accumulation compared with that of live virus provides a correlation between tyrosine phosphorylation and the stimulation of PCA expression. The mechanism whereby dead virus binds to receptors on macrophages but does not induce tyrosine phosphorylation or PCA expression remains unclear. We speculate that UV irradiation might alter viral coat proteins so that the interactions required for binding to the receptor persist, while those necessary for cell activation may have been lost or altered.

Recent studies have shown that endotoxin-stimulated tyrosine phosphorylation participates in macrophage activation leading to the expression of various macrophage products (18, 31). Several lines of evidence suggest that endotoxin contamination was not responsible for the observations described in these studies. First, all solutions including the viral inoculum were essentially endotoxin free as determined by the *Limulus* lysate assay. Second, the assays were performed in serum-free medium. Since LPS-stimulated tyrosine phosphorylation at low endotoxin concentrations is mediated via the CD14 receptor (30), the absence of serum (and thus any source of LPS-bind-

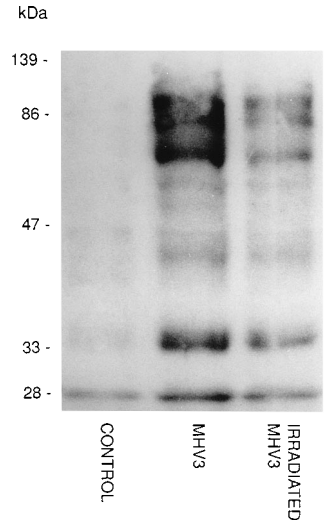

FIG. 5. Induction of tyrosine phosphorylation by live and UV-irradiated MHV-3 in murine peritoneal macrophages. Cells (10^6/ml) were incubated with live MHV-3 (2.5×10^6 PFU) or UV-irradiated virus (2.5×10^6 nonviable viral particles) for 10 min, rapidly sedimented, and resuspended in boiling Laemmli buffer. The phosphorylation of tyrosine residues was evaluated as described in the text. The data shown are representative of three separate similar studies.

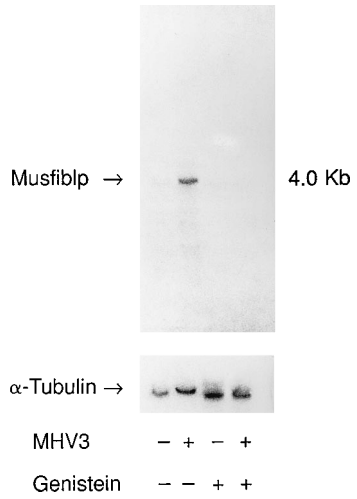

FIG. 6. Northern blot analysis of the effect of genistein on the level of musfiblp mRNA transcripts. Cells were incubated with or without genistein (10 μg/ml with a 2-h preincubation) in the presence or absence of MHV-3 (MOI, 2.5) for 5 h. RNA was extracted and Northern blot analysis was performed with the cDNA probe for musfiblp. The blots were then stripped and reprobed with the cDNA for α-tubulin to ensure comparable RNA loading. The blot shown is representative of three independent studies.

ing protein) would preclude cell stimulation through this pathway, even if minute amounts of endotoxin were present. Finally, the Northern blot analysis indicated the expression of the musfiblp gene following exposure to the virus, and its level was reduced by treatment with genistein. Endotoxin does not induce this gene, and thus, its expression must be attributed to the presence of the virus.

The mechanisms underlying the induction of tyrosine phosphorylation in response to virus and the precise role of the phosphorylated proteins in cell activation were not investigated in these studies. The observation that the increase in phosphotyrosine residues was transient and preceded a rise in PCA expression supports the notion that tyrosine phosphorylation participates early in the signalling cascade leading to gene expression. This pattern is consistent with that observed for other macrophage stimuli such as LPS and zymosan (27, 31). The receptor for MHV-3 is a 110-kDa glycoprotein which is a member of the murine carcinoembryonic antigen family. Its short intracellular domain precludes its ability to function as a receptor tyrosine kinase, thus suggesting the possible involvement of associated nonreceptor protein tyrosine kinases. Further, the lack of a tyrosine residue within the proposed cytoplasmic domain suggests that the MHV receptor is also not a substrate for tyrosine phosphorylation. However, splice variants of the receptor with a long cytoplasmic tail containing tyrosine residues have been reported to serve as MHV receptors (3, 13). Phosphorylation of one of these carcinoembryonic antigen-related glycoproteins may have participated directly in the signalling pathways, as has been reported for cell-CAM 105 (11). In our studies, a protein with a molecular mass of ~65 kDa was seen to be tyrosine phosphorylated in the virus-treated cells. Several *src* family tyrosine kinases known to be present in macrophages (e.g., *hck*, *fgr*, and *src*) lie close to this mass and thus may be substrate proteins (5). The present studies also showed rapid phosphorylation of two proteins (41 and 44 kDa) whose molecular masses correspond to those reported for two distinct mitogen-activated protein kinases in macrophages (31). These kinases are felt to play an important role in cell signalling in a variety of cell types (24). Further studies are needed to precisely define the identity of the various substrate proteins and their participation in MHV-3-induced tyrosine phosphorylation and activation of musfiblp gene expression.

In summary, the data reported herein indicate a role for tyrosine phosphorylation in the signalling pathway leading to the induction of the musfiblp gene and expression of the unique procoagulant in MHV-3-stimulated macrophages. Defects in this cascade may contribute in part to the resistance of certain mouse strains to MHV-3. A recent report has documented the ability of tyrosine kinase inhibition to abrogate lethality in a murine endotoxemia model (25). This effect correlated with the ability of the inhibitor to abrogate the endotoxin-stimulated release of tumor necrosis factor. Since the expression of macrophage PCA correlates with lethality associated with MHV-3-induced viral hepatitis, the present studies suggest that modulation of PCA expression with inhibitors of tyrosine phosphorylation might represent a novel approach to the treatment of this disease.

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