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MURAMYL PEPTIDES CONFER HEPATOPROTECTION AGAINST MURINE VIRAL HEPATITIS

K. NOEL MASIHI,* HANS KRÖGER,* WERNER LANGE* and LOUIS CHEDID†

*Robert Koch Institute, Federal Health Office, D-1000 Berlin 65, Federal Republic of Germany;
†University of South Florida College of Medicine, Department of Pharmacology and Therapeutics, Tampa, Florida, U.S.A.

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Abstract — The hepatoprotection induced by synthetic muramyl peptides was investigated using a model of lethal murine mouse hepatitis MHV-3 virus infection. MDP and a nonpyrogenic analog, Murametide, inhibited the steep elevation of serum transaminases induced by MHV-3 irrespective of whether the immunomodulators were administered before or after the infection. A significant proportion of MDP or Murametide-treated animals, in contrast to controls, survived the MHV-3 infection. The histopathological examination of the liver revealed marked necrosis of the hepatic parenchymal cells and infiltration of the inflammatory cells in controls but not in MDP-treated animals.

Infections due to hepatitis viruses and *Mycobacterium tuberculosis* are endemic in Southeast Asia and amongst refugees from that region. A study of immune responses to both infections in Indochinese refugees showed a significant association in the reactivity to purified tuberculin protein derivative (PPD) and the presence of hepatitis Be antigen (McGlynn, Lustbader & London, 1985). Persons having a positive PPD skin test tended to be HBeAg negative suggesting that *Mycobacterium tuberculosis* infection may affect the outcome of viral hepatitis.

Mouse hepatitis virus type 3 (MHV-3) belongs to the group of coronaviruses. Parenteral administration of MHV-3 to susceptible mice causes fatal hepatic necrosis culminating in death within a matter of few days. Hepatic necrosis liberates several enzymes that are usually present intracellularly within the liver into the blood circulation. The elevation of serum transaminases is an important biochemical manifestation of human and murine viral hepatitis and can be used diagnostically as a marker of liver damage. Measurements of serum alanine aminotransferase in Indochinese refugees showed normal transaminase levels in PPD-positive persons compared with PPD-negative individuals (McGlynn *et al.*, 1985). It would be of considerable interest if the protection against virus-mediated liver damage associated with tuberculosis infection could be duplicated by immunomodulators of mycobacterial origin.

Mycobacteria contain on their cell walls, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), a small glycopeptide which represents the minimal structure essential for bacterial adjuvanticity. Synthetic MDP and its analogs are endowed with multifarious properties including the stimulation of nonspecific resistance against viral pathogens (Chedid, 1988).

Already in 1980 it was reported that MDP, in combination with trehalose dimycolate, could induce resistance against influenza virus infection (Masihi, Brehmer, Lange & Ribi, 1980, 1983). Several MDP analogs like 6-*O*-acyl, ubiquinone (Masihi, Brehmer, Azuma, Lange & Müller, 1984a), seryl and aminobutyryl (Masihi, Brehmer, Lange, Ribi & Schwartzman, 1984b) conferred long-term resistance against aerogenic influenza virus in combination with trehalose dimycolate. Subsequently, MDP and analogs were shown to induce protection against various strains of influenza (Dietrich, Hochkeppel & Lukas, 1986), herpes simplex virus (Dietrich *et al.*, 1986; Koff, Showalter, Hampar & Fidler, 1985), vaccinia virus (Ikeda, Negishi & Nishimura, 1985), sendai virus (Yamamura, Ishihara, Hamada, Yamamoto & Azuma, 1986) and in combination with an interferon-inducer, against Semliki Forest virus (George, Jain, Gupta & Anand, 1986). MDP can also protect rat hepatocytes against the *in vitro* toxic

effects of acrolein, chloroform and carbon tetrachloride and decrease serum transaminases (Farghali, Machková, Kameiniková, Janků & Mašek, 1984). In the present study, the effect of MDP and a nonpyrogenic analog, Murametide, on biochemical and other parameters was investigated using a model of lethal murine MHV-3 virus infection.

EXPERIMENTAL PROCEDURES

Animals

Five to six-week old NMRI mice were purchased from Zentralinstitut für Versuchstiere, Hannover, F.R.G.

Administration of MDP

MDP and its analog, Murametide, were synthesized by P. Lefrancier, Institut Choay, Paris, France (Lefrancier, Derrien, Jamet, Choay, Lederer, Audibert, Parant, Parant & Chedid, 1982). Desired amounts of muramyl peptides were dissolved in pyrogen-free physiological saline. All substances were administered by the intraperitoneal (i.p.) route.

MHV-3 infection

MHV-3 was passaged i.p. in young NMRI mice. Livers were removed 3 days after the infection and homogenized in 3 ml of medium/liver using a tissue grinder. Supernatant obtained after centrifugation was diluted and further passaged in mouse L-cells. Marked cytopathic effects could be observed in tissue cultures of L-cells using supernatant dilutions of 10^{-1} to 10^{-3}. Three-day old cultures infected with 1:100 dilution of the supernate were frozen and thawed three times. The supernate obtained after centrifugation was stored in liquid nitrogen. Various dilutions were injected i.p. into mice for the determination of lethal dose. Half a milliliter of 1:750 dilution injected i.p. consistently gave LD_{100} in 6-week old NMRI mice and was used for all experiments.

Determination of serum glutamate oxalate aminotransferase (GOT) and glutamate pyruvate aminotransferase (GPT) levels

The GOT and GPT enzyme activities present in nonhemolytic sera collected at different intervals were determined using the standard method (Bergmeyer, 1974). Reagents for the test were purchased from Boehringer Mannheim, F.R.G. The enzyme activity is presented in mU/ml.

Determination of ADPR transferase activity

Nuclei from liver cells were isolated using the technique previously described (Blobel & Potter, 1966). The ADPR transferase activity was measured in the presence of DNAse (Kidwell & Burdette, 1974).

Histology

For histological studies, mice were given saline or 1 mg of MDP 1 h, 24 h, and 48 h after the MHV-3 infection. Livers were removed on day 3 after the viral infection and fixed in 10% formalin. Histological sections were stained with hematoxylin and eosin.

RESULTS

Effect on MDP pretreatment on MHV-3 infection

Thirty animals were administered a single dose of 300 μg of MDP by the i.p. route. The effect of MDP itself on liver enzymes was determined in a group of 10 pretreated mice. Twenty mice from the MDP-pretreated group and 20 normal mice injected with saline were infected i.p. with MHV-3 24 h after the MDP administration. Sera were collected everyday for four days. The results of enzyme activities are presented in Fig. 1. Serum GOT and GPT activities were not induced after the administration of MDP alone. In contrast, the MHV-3 infection induced

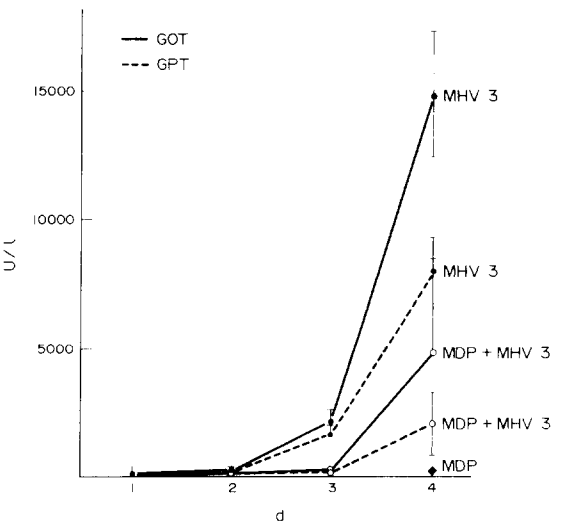

Fig. 1. Serum GOT (———) and GPT (---------) activities of mice after MHV-3 infection (●), after pretreatment with 300 μg of MDP followed 24 h later by infection (○), and after similar administration of MDP alone (◆).

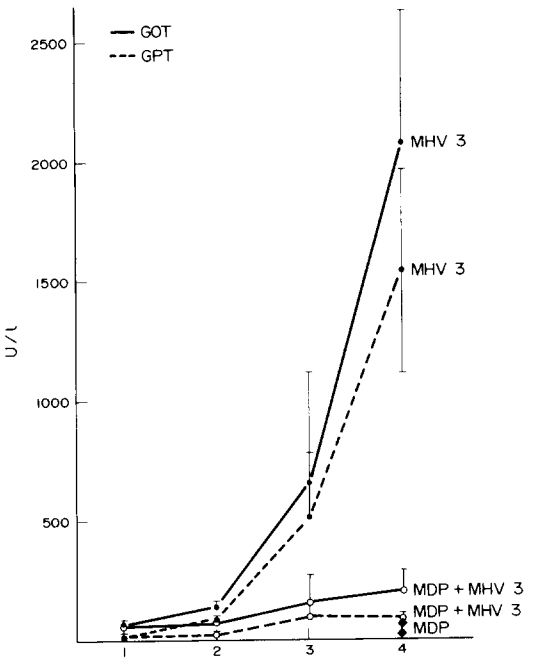

Fig. 2. Serum GOT (———) and GPT (---------) activities of mice after MHV-3 infection (●), after treatment with 100 μg of MDP at 1, 24, 48 and 72 h following infection (○), and after similar administration of MDP alone (◆).

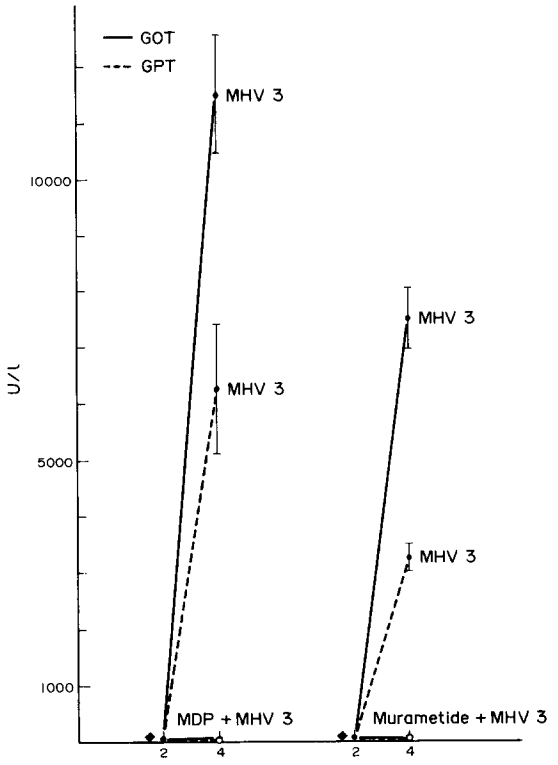

Fig. 3. Serum GOT (———) and GPT (---------) activities of mice after MHV-3 infection (●), after treatment with 1 mg of MDP or Murametide at 1, 24, 48 h following infection (○), and after similar administration of MDP alone or Murametide alone (◆).

increased GOT and GPT activities on day 3 and the enzyme levels were elevated even further on day 4, a time period when many of the animals were dying. Pretreatment with MDP greatly reduced the rise in GOT and GPT levels observed after the MHV-3 infection.

Effect of administering MDP after MHV-3 infection

Sixteen mice were given 100 μg of MDP 1 h, 24 h, 48 h and 72 h after the MHV-3 infection. Another group of 16 mice was similarly treated with MDP but did not receive the viral infection. A third group of 16 mice received MHV-3 infection only. Sera were collected every day for 4 days after the infection. Results presented in Fig. 2 show that the multiple administration of MDP alone did not affect the liver transaminases. MDP given after the MHV-3 infection could inhibit the induction of serum GOT and GPT (Fig. 2).

The effect of MDP or its potent nonpyrogenic analog murametide was investigated at a higher dosage. Twenty-eight animals each were given 1 mg of MDP or 1 mg of Murametide 1 h, 24 h, and 48 h after the MHV-3 infection. Eight animals were each similarly treated with MDP or Murametide but did not receive the viral infection. Another group of 28 animals served as virus controls. One milligram of MDP given three times after the MHV-3 infection inhibited the GOT and the GPT activities on day 4 whereas high levels of these enzymes were induced in

Table 1. Effect of MDP treatment on ADPR transferase in liver cell nuclei of MHV-3 infected mice.

Treatment	Hours after infection 3	24	48
Saline	8058	9210	9937
MHV-3	14105	13337	8975
MHV-3 + MDP	12751	13429	9005

Mice were infected intraperitoneally with MHV-3 virus and treated with MDP (1 mg, i.p.) at 1, 22, and 42 h post-infection.
*counts/min per mg DNA.

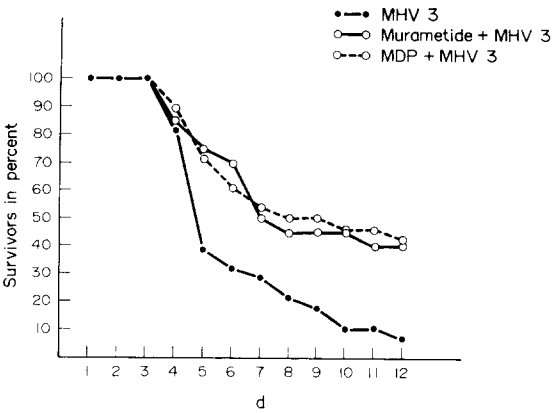

Fig. 4. Effect of survival of mice of 1 mg of MDP (○---------○) or murametide (○———○) administered at 1, 24 and 48 h after MHV-3 infected (●———●).

the virus controls (Fig. 3). Nonpyrogenic Murametide was active and inhibited the GOT and the GPT induction (Fig. 3). Neither MDP alone nor Murametide alone induced liver enzyme activities at this dosage.

The activity of ADPR transferase in the nuclei of liver cells increases after infection with MHV-3 (Table 1). In contrast, treatment with 1 mg of MDP 1 h after infection resulted in a very small reduction of ADPR transferase activity. Additional treatments with MDP at 22 h and 42 h post-infection did not after the ADPR transferase activity at 24 h or 48 h.

One milligram of MDP or Murametide administered three times after the MHV-3 infection conferred significant protection to respectively 42% and 40% of treated mice compared to only 7% survivors in the control group ($<P0.01$) (Fig. 4). Lower doses of MDP given prior to or after the MHV-3 infection did not significantly affect the mortality (data not shown).

Histopathology

Mice infected with MHV-3 developed extensive necrotic lesions around the central artery on day 3 (Fig. 5a). Marked necrosis of the hepatic parenchymal cells was accompanied by infiltration of the inflammatory cells. In contrast, MDP-treated animals exhibited a strong suppression of the hepatic necrosis and a lack of cellular infiltration on day 3 after the MHV-3 infection (Fig. 5b).

DISCUSSION

The results of the present study demonstrate the hepatoprotective activity of synthetic muramyl peptides in viral hepatitis. MDP and Murametide could prevent the steep elevation of serum GOT and GPT transaminase levels induced by MHV-3 infection. The inhibition of both GOT and GPT enzyme activities was observed up to at least 96 h after the MHV-3 infection irrespective of whether MDP was given before or after the infection. The histopathological examination of the liver revealed the marked protection conferred by MDP treatment in contrast to the extensive necrosis of hepatic parenchymal cells in the control animals.

Certain chemicals can cause profound liver damage. The ability of MDP to protect rat hepatocytes against the *in vitro* toxic effects of acrolein, chloroform and carbon tetrachloride has been described. Serum aspartate and alanine transaminases detected 17 h after i.p. carbon tetrachloride administration to rats could also be decreased by intravenous MDP pretreatment (Farghali *et al.*, 1984). The ability to protect the liver against damage by viral or chemical agents may constitute an important property of immunomodulators like MDP.

Nicotinamide adenine dinucleotide (NAD) functions as an important catalytic coenzyme in the oxidation reduction reactions. In addition, NAD also participates in certain biological processes like adenoribosylation as a substrate. The enzyme ADPR transferase cleaves NAD with subsequent adenoribosylation of specific proteins. The NAD-adenoribosylation metabolism is involved in quite a number of processes like DNA repair differentiation, enzyme regulation etc.

The activity of ADPR transferase in the early stages of MHV-3 infection was somewhat lower in MDP-treated animals than in the controls. Further experiments are currently in progress to define the role of NAD metabolism in the damaged liver.

The mechanisms involved in the protection conferred by MDP are not fully elucidated as yet. MDP administration increases the incorporation of radiolabeled palmitic acid into the hepatocyte phospholipids suggesting a stabilizing effect on hepatocytes (Farghali, Machková, Julis, Buchar, Janků & Mašek, 1986). In addition, stimulation of nonspecific resistance mechanisms by MDP may have been responsible, in part, for the protection against MHV-3 infection. The clearance of colloidal carbon is impaired by MHV-3 (Gledhill, Bilbey & Niven, 1965). Since MDP and several of its analogs can enhance the clearance of colloidal carbon particles from the blood by the reticuloendothelial system (Fraser-Smith, Waters & Matthews, 1982) and activate a variety of macrophage functions (Leclere & Chedid, 1982), the MHV-3 induced

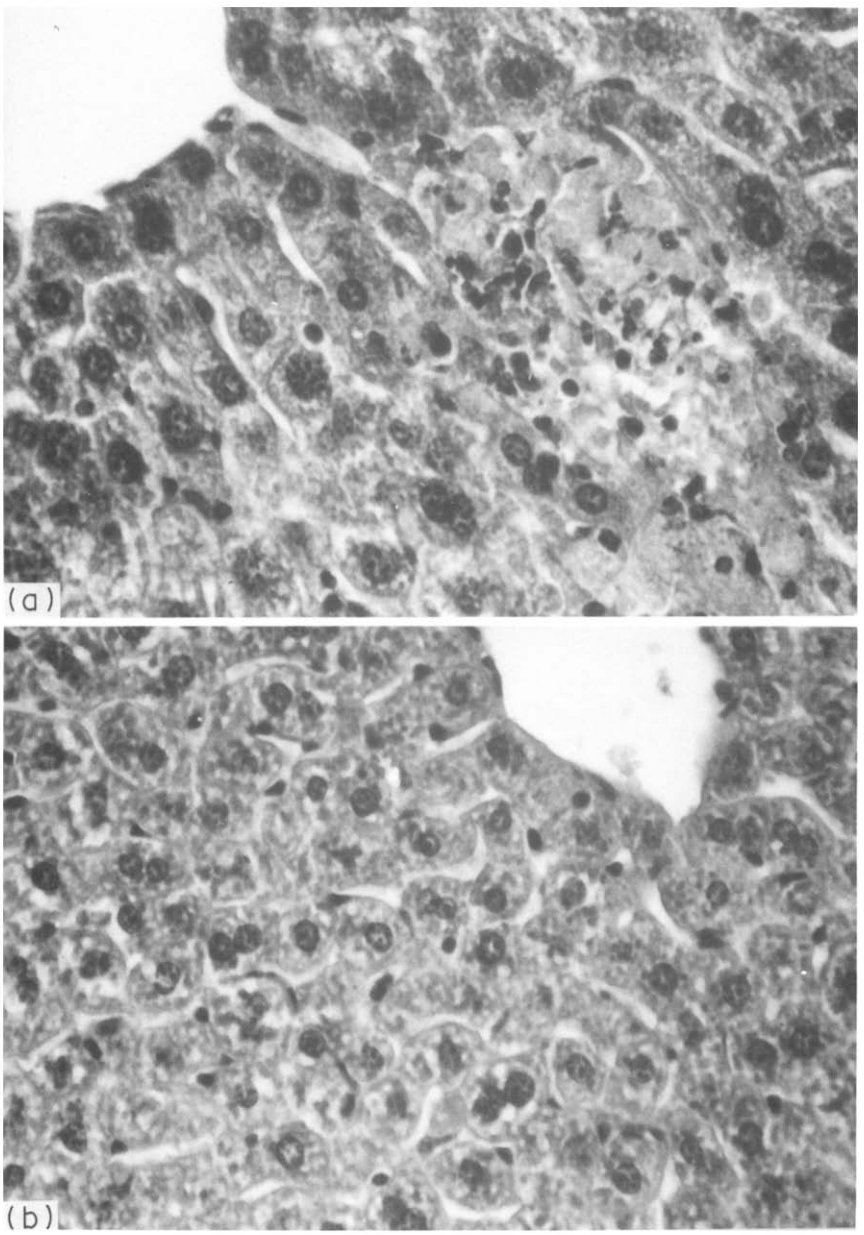

Fig. 5. Liver histopathology of mice infected with MHV-3 (a) and treated with 1 mg of MDP at 1, 24 and 48 h after infection (b).

dysfunction may be balanced by MDP. A protective role for endogenous interferon is suggested by interferon induction in MHV-3 infection in both the resistant and susceptible strains of mice and the finding that prior administration of anti-interferon serum causes accelerated mortality (Virelizier, 1981). Muramyl peptides can augment viral interferon production (Sakuma, Azuma & Yoshida, 1984), stimulate the NK cell activity in the liver (Talmadge, Schneider, Collins, Phillips, Herberman & Wiltrout, 1985) and spleen (Masihi, Lange & Rohde-Schulz, 1987), and activate the cytotoxic properties of murine kupfer cells (Xu & Fidler, 1984).

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