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Inducing Activity of Respiratory Syncytial Virus on the Production of Macrophage Inflammatory Protein-2 in Murine Macrophages and Airway-lining Cells

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Summary. **We investigated the macrophage inflammatory protein-2 (MIP-2)-producing activity of respiratory syncytial virus (RSV) in a murine macrophage cell line, RAW 264.7 cells, and airway-lining cells by the application of recently developed enzyme-linked immunosorbent assay (ELISA) system. The production of MIP-2 increased significantly in both cells by the infections of RSV in a dose-dependent manner. In addition to ELISA, MIP-2 production could be detected in RSV-infected airway-lining cells by immunocytochemical study. The heat-inactivated virus also enhanced MIP-2 production to comparable levels of the infections with the active virus. Time-related production showed a trend for a biphasic pattern, suggesting that this phenomenon might be due to the combined effects of the direct MIP-2-inducing activity of RSV and an indirect one via the initial production of other cytokines inducible for MIP-2 production. These data indicate that RSV, either in active or inactive form, has the potential to stimulate MIP-2 production in a cultured macrophage cell line and airway-lining cells.**

Key words—respiratory syncytial virus, macrophage inflammatory protein-2, interleukin-8, macrophages, airway-lining cells.

INTRODUCTION

Respiratory syncytial virus (RSV) is the major cause of acute lower respiratory tract illnesses such as broncheolitis and pneumonia in infants and young children, and can cause severe, even fatal, infections in the elderly.[1] Several studies have indicated that RSV is released initially from upper respiratory tract infections and then reaches the bronchoalveolar region, where viruses induce inflammation of the airway epithelium accompanied by peribronchiolar infiltrations of neutrophils.[1,2] It is well known that the accumulation of neutrophils is an important characteristic of inflammation and modulates various inflammatory reactions.[3] Thus, the influx of neutrophils into infected sites is considered to play a significant role on the pathogenesis of RSV infection.

Recently, a novel chemotactic cytokine, named as interleukin (IL)-8, has been found in the conditioned medium (CM) of various cells including monocytes/macrophages, fibroblasts, endothelial cells and epithelial cells in response to stimulation with lipopolysaccharide (LPS) as well as several inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF).[4,5] Recent studies have revealed that the migration of neutrophils is closely related with the enhanced release of interleukin-8 in response to influenza virus infection.[6,7] In RSV infection models using cultured human cells, several studies have demonstrated that the proinflammatory process is due to IL-8 production by airway-lining cells and macrophages.[2,8,9] On the other hand, animal models for RSV infection have been developed[10,11] notably the murine model. Nevertheless, the production of macrophage inflammatory protein-2 (MIP-2), a murine functional equivalent of human IL-8,[12] has not yet been studied in response to RSV infections because of the lack of an MIP-2 assay system. In light of this situation, we have recently developed an enzyme-linked immunosorbent assay (ELISA) system for the detection of MIP-2,[13] providing an opportunity for studying this issue. In this

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study, we focused our attention on clarifying whether RSV has the potential to induce MIP-2 production in murine macrophage RAW 264.7 cells, which were used initially for the discovery of MIP-2,[12] and airway-lining cells in a tracheal organ culture.

MATERIALS AND METHODS

Cell and tracheal organ cultures

Hep-2 cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's MEM (DMEM) supplemented with 10% FBS.[13] Tracheas were obtained from pathogen-free outbred ICR mice (female, 6 weeks of age) under ether anesthesia, and cultured in serum-free DMEM.

Virus

RSV A2 strain, which was kindly supplied by Dr. Watanabe (Rational Drug Design Laboratories, Fukushima, Japan), was propagated in a confluent monolayer of Hep-2 cells in a maintenance medium (MEM supplemented with 2% FBS).[1] When the cytopathic effect reached 80% the cultures were processed to three cycles of freezing and thawing. After centrifugation at 2,000×g for 10 min, the culture supernatant was collected and stored in a small portion at −84°C as a virus stock solution. Virus titers were determined as plaque forming units (PFU) on Hep-2 cells as described previously.[1] RSV was inactivated at 56°C for 2 h according to the method described by Becker et al.[2] The efficiency of inactivation was confirmed by the absence of plaque formation on Hep-2 cells.

RSV infection

RAW 264.7 cells were inoculated into a 96-well microplate at a density of 1×10^5 cells/well and cultured overnight at 37°C. Thereafter, the cells were washed once with serum-free media and exposed for 3 h at 37°C to 20 μl of virus solution at a multiplicity of infection (MOI) of 1 to 0.01 PFU/cell. After virus adsorption, 200 μl of serum-free medium was added (0 h) and further cultured for additional time periods as indicated. As a control, uninfected cells were cultured in the presence or absence of 1 μg/ml of lipopolysaccharide (LPS).[13] For the infection of tracheal organ cultures with RSV, the trachea (approximately 5 mm long) was divided into two pieces by a longitudinal section to facilitate the virus infection and then cultured in the virus-containing media in a 96-well microplate as above.

Detection of cytokines by ELISA and immunocytochemistry

MIP-2 levels in the CM were determined by antibody sandwich ELISA as described previously.[13] Briefly, rabbit anti-MIP-2 antibody and biotinylated anti-MIP-2 antibody were used as the capture and second-layer antibodies, respectively. Color development was continued for several minutes by the addition of peroxidase-coupled streptavidin and substrate-chromogen (3,3′-diaminobenzidine tetrahydrochloride; DAB) solution before terminating the reaction with 2 M H_2SO_4. The absorbance was measured at an optical density (OD) of 492 nm on a microplate reader (Bio-Rad). The hyperimmune rabbit anti-MIP-2 serum was prepared by repeated intracutaneous injections with the recombinant MIP-2 emulsified with Freund complete adjuvant (for the initial injection) or an incomplete one (for the booster injections). The levels of IL-1 and TNF were determined by ELISA kits purchased from Genzyme (Cambridge, MA, USA). Three wells were used for each experimental point to calculate the mean±standard error (SE) of the mean. For immunocytochemical staining of airway-lining cells, the trachea was cultured in the presence or absence of RSV for 24 h, and then its stamp preparation was made on the glass slide. After drying at room temperature, the cells were fixed with an ice-cold methanol-acetone mixture (1 : 1 vol./vol.) for 10 min and then reacted with rabbit anti-MIP-2 antibody (1 mg/ml) for 30 min at room temperature. Thereafter, the cells were processed by staining with the LSAB kit (DAKO, Carpinteria, CA, USA), consisting of biotinylated anti-rabbit immunoglobulin, peroxidase-conjugated streptavidin and substrate-chromogen (DAB) solution, according to the instructions provieded by the company. Each reaction was carried out for 15 min at room temperature.

RESULTS

MIP-2 production in RAW 264.7 cells in response to RSV infection

As shown in Fig. 1, a significant increase of MIP-2 level could be detected in LPS-stimulated RAW 264.7 cells which are a representative experimental system for MIP-2 production (27.5 and 0.9 ng/ml for LPS-

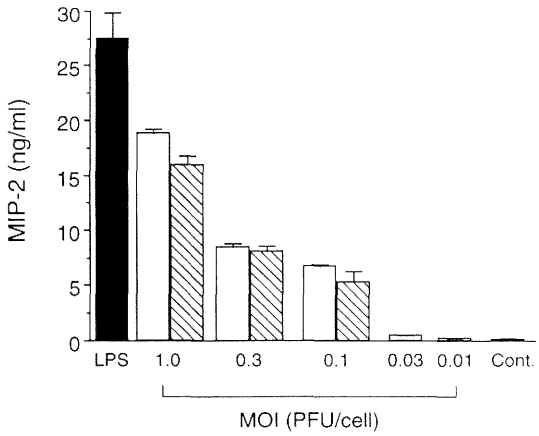

Fig. 1. MIP-2 production in RAW 264.7 cells in response to stimulation with LPS or RSV infection. RAW 264.7 cells in a 96-well plate were cultured at 37°C in the presence of 1 μg/ml of LPS (solid bar). Alternatively, the cells were infected with either active RSV (open bars) or heat-inactivated RSV (hatched bars) at the indicated MOI. As a control, cells were cultured in the absence of stimulators (Cont.). Thereafter, MIP-2 levels in the 24-hour CM were determined by ELISA. Three wells were used for each experimental point to calculate the mean ± SE (thin bars).

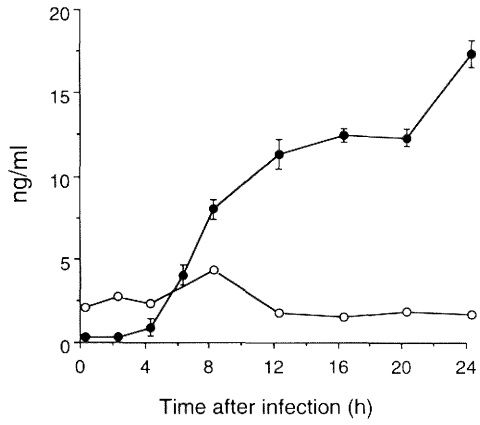

Fig. 2. Time-related MIP-2 and TNF productions in RSV-infected RAW 264.7 cells. RAW 264.7 cells were infected with RSV at an MOI of 1 PFU/cell and then cultured as described in the legend for Fig. 1. Culture supernatants were collected at the indicated time after infection to determine MIP-2 (closed circle) and TNF (open circle) levels by ELISA. Three wells were used for each experimental point to calculate the mean (closed circles) ± SE (thin bars). In the case of TNF, SE was extremely small (0.05 to 0.08) and thereby this value was not shown.

stimulated and -unstimulated cells, respectively). These findings indicate that the ELISA system used in this study could specifically detect the MIP-2 response. Thus, this ELISA system was applied to examine MIP-2 levels in response to RSV infection with various MOI (1 to 0.01 PFU/cell). As a result, MIP-2 levels in 24-hour CM of RSV-infected cells increased in an MOI-dependent manner, that is, 18.8, 8.4 and 6.7 ng/ml were detected in the CM by infections with an MOI of 1, 0.3 and 0.1, respectively. However, attempts to increase the effect by the infection with an MOI of 0.03 or 0.01 were negligible. Although virus antigens could be detected in the infected cells with an MOI of 1 PFU/cell by immunofluorescence staining with RSV-specific antibody (ViroStat, Portland, ME, USA). virus growth was not recognized by the plaque assay of 24-hour CM (data not shown). In addition, this effect of the heat-inactivated virus was examined in the infections with various virus doses corresponding to an MOI of 1 to 0.1 in active virus infection. Data demonstrated that the inactivated virus maintained an MIP-2-inducing activity at a level comparable to the infectious virus. LPS was not detected in the virus solution. These data suggest that RSV, either in active or inactive form, has the potential for MIP-2-inducing activity.

Time-related MIP-2 and TNF productions in RSV-infected RAW 264.7 cells

As shown in Fig. 2, MIP-2 levels in RSV-infected cells were comparable to those in uninfected cells during 0 to 4 h, and then increased lineally towards 8 h. Thereafter, increasing rates were retarded at once until 20 h, and then these values increased again towards 24 h, showing a trend for a biphasic pattern. Based on the biphasic prodoction, it was further examined whether RSV could induce the production of IL-1 and TNF, which are well-known inducers for MIP-2 production.[4,5] As shown in Fig. 2, a low but recognizable level of TNF (1.2 ng/ml) was detected at 0 h, but this level increased towards 8 h at which the peak level attained (4 ng/ml). Thereafter, TNF levels decreased to the initial level, indicating that the duration of TNF induction was shorter than that of MIP-2. In addition, the fact that TNF levels decreased after 8 h suggests that the released TNF interacts again with the cells, resulting in the induction of MIP-2 production responsible for the second phase. As for IL-1, enhanced production was not detected (data not shown).

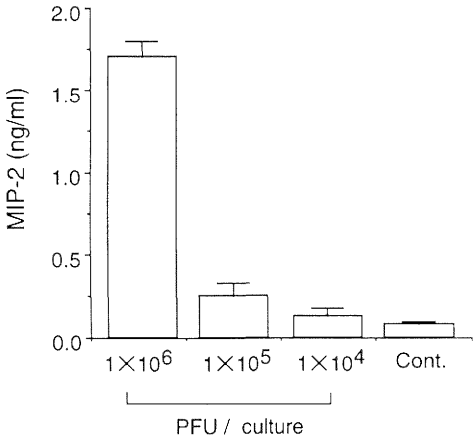

Fig. 3. MIP-2 production in tracheal organ culture in response to RSV infection. A trachea divided into two pieces was cultured at 37°C in the presence of RSV at the indicated titers (PFU/culture). As a control, the organ was cultured in the absence of RSV (Cont.). Thereafter, MIP-2 levels in the 24-hour CM were determined by ELISA. Three wells were used for each experimental point to calculate the mean (open bars) ± SE (thin bars).

Production of MIP-2 in RSV-infected airway-lining cells

Since airway-lining cells are the primary site of RSV infection, production of MIP-2 by the interaction of RSV with these cells was examined. When the trachea was cultured in the absence or presence of various titers of RSV (1×10^4 to 1×10^6 PFU/culture), MIP-2 levels in 24-hour CM were enhanced in a virus titer-dependent manner (Fig. 3). In the presence of 1×10^6 PFU/culture, the MIP level was 1.70 ng/ml.

To clarify the contribution of airway-lining cells for the MIP-2 production in the tracheal organ culture, an immunocytochemical study was carried out. As shown in Fig. 4, stamp preparation of an uninfected trachea was only faintly or not at all stained with the dye. In sharp contrast, stamp preparation of a trachea cultured for 24 h in the presence of RSV (10^6 PFU/culture) was significantly stained with a dense color. This preparation also confirmed that the densely stained cells were actually airway-lining cells, by the presence of villi on their surface. These data suggest that the MIP-2 level increases in response to RSV infection in airway-lining cells as well as RAW 264.7 cells, a macrophage cell line.

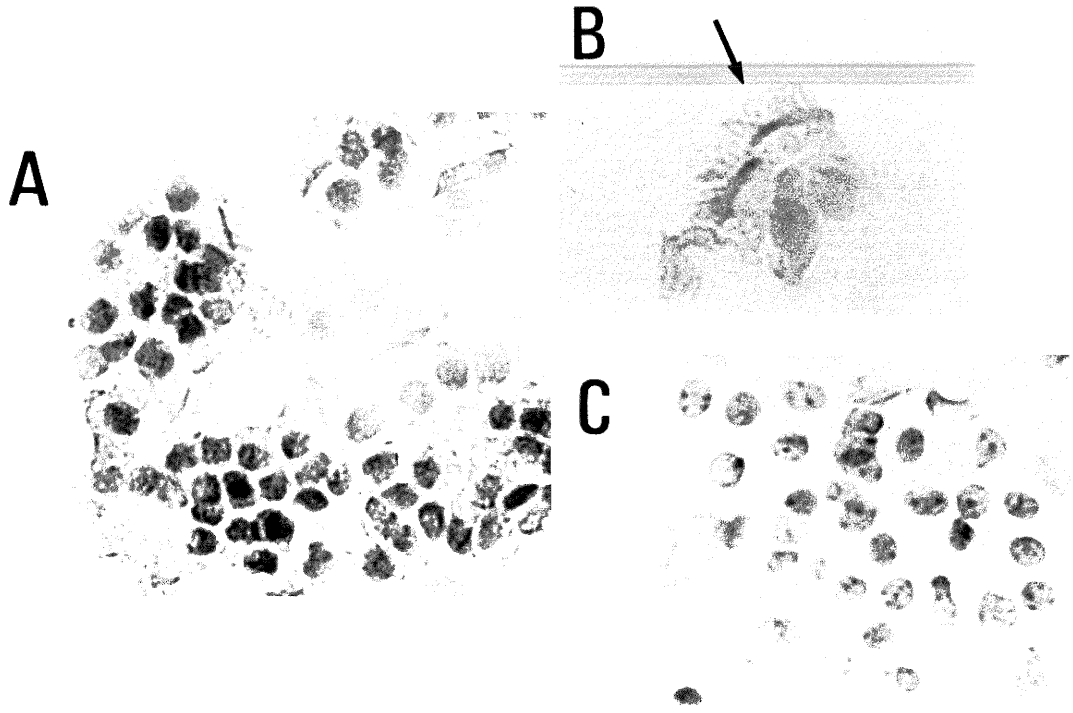

Fig. 4. Detection of MIP-2 in airway-lining cells infected with RSV by immunocytochemical staining. The trachea was cultured in the presence (**A** and **B**) or absence (**C**) of RSV. Twenty-four hour later, airway-lining cells were stamped on glass slides and processed for immunocytochemical staining. Infection and culture conditions have been described in the legend for Fig. 3. Magnification was ×200 for (**A**) and (**C**), or ×600 for (**B**). A small arrow in (**B**) indicates the villi on the cell surface.

DISCUSSION

Several studies have shown that RSV can enhance human IL-8 production.[2,8,14] In addition to these studies, this study demonstrates that RSV has the potential to induce the enhanced production of MIP-2, a murine counterpart of IL-8, in a macrophage cell line, RAW 264.7 cells, and airway-lining cells, in an MOI-dependent manner by the application of recently established ELISA system for MIP-2.[13] Furthermore, an immunocytochemical study confirmed that MIP-2 was actually produced in airway-lining cells by RSV infection. In sharp contrast to the influenza virus,[7] heat-inactivated RSV could maintain MIP-2-inducing activity as shown in this and a previous study.[2] In RAW 264.7 cells, virus antigens could be detected but not virus growth, in accordance with a previous report,[1] suggesting that MIP-2 production might be induced even by an abortive RSV infection as well as a heat-inactivated virus infection.

Time-related MIP-2 production showed a trend for a biphasic pattern in RSV-infected RAW 264.7 cells. It has been shown that MIP-2 production is inducible in response to the stimulation with inflammatory cytokines such as IL-1 and TNF.[4,5] Recent studies also showed that RSV effectively induced TNF production but not IL-1 in human macrophages and granulocytes.[2,15] In the case of RSV-infected RAW 264.7 cells, we have confirmed that TNF production is actually enhanced to 4 ng/ml at 8 h, but IL-1 is not. These findings suggest that the biphasic pattern might be due to the combined effects of RSV stimulation, that is, the direct stimulation of MIP-2 production and indirect one via the initial production of TNF.

Several studies have indicated that the inflammatory response, including the influx of neutrophils, plays an important role on the pathogenesis in influenza virus infections.[7,16,17] However, the pathogenesis of RSV infections has not yet been completely understood. It is considered that the MIP-2-inducing activity of RSV demonstrated in this study will contribute to further studies on this issue using a murine model.

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