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Contribution of Tumor Necrosis Factor α and Interleukin-1 α on the Production of Macrophage Inflammatory Protein-2 in Response to Respiratory Syncytial Virus Infection in a Murine Macrophage Cell Line, RAW264.7

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The production of several inflammatory cytokines, such as murine macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor (TNF), and interleukin (IL)-1, was investigated in response to respiratory syncytial virus (RSV) infection in a murine macrophage cell line, RAW264.7, with special reference to mutual relation of their productions. The kinetics of MIP-2 production showed a trend for a biphasic pattern, that is, MIP-2 levels became detectable from 2 h postinfection (p.i.) and increased markedly until 8 h p.i. Thereafter, this level fell to the same level until 16 h p.i. and then increased again. TNF α was also detectable at 2 h p.i. and then increased sharply until 8 h p.i., when the peak level attained. Compared with the levels of MIP-2 and TNF α, that of IL-1 α/β, especially IL-1 β, was lower (ng versus pg/ml order). The presence of anti-TNF α or anti-IL-1 α antibody did not influence the early phase of MIP-2 production but significantly inhibited the late phase, suggesting that MIP-2 is induced by the combined effects of RSV infection via direct induction and indirectly after initial induction of TNF α and IL-1 α productions. Although RSV-infected RAW264.7 cells had no alteration in viability compared with mock-infected control, these data demonstrate that RSV is a potent inducer of inflammatory cytokines by direct induction and indirectly via the initial production of other cytokines. ***J. Med. Virol. 53:145–149, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: respiratory syncytial virus; macrophage inflammatory protein-2; macrophage; tumor necrosis factor; interleukin-1

INTRODUCTION

Respiratory syncytial virus (RSV), a member of *Paramyxoviridae*, is the most common cause of lower respiratory infection in infants and young children, and is closely associated with significant mortality in children with cardiopulmonary diseases or immunodeficiency [Denny et al., 1986]. RSV is released initially from upper respiratory tract infections and then reaches the bronchoalveolar region, where RSV induces inflammation accompanied by peribronchiolar infiltrations of neutrophils [Becker et al., 1991; Franke-Ullmann et al., 1995]. It is well known that the accumulation of neutrophils is an important characteristic of inflammation by modulating various inflammatory reactions [Gallin and Wright, 1987]. Thus the influx of neutrophils into infected sites is considered to play a significant role on the pathogenesis of RSV infection.

Recently, novel chemotactic cytokines, called the chemokine family, have been found in the conditioned medium (CM) of various cells in response to stimulation with lipopolysaccharide (LPS) as well as several

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inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) [Matsushima and Oppenheim, 1989; Watanabe et al., 1989; Deforge et al., 1992]. Human IL-8 and rat CINC are the representative chemokines. Several investigators have reported that RSV has the potential to induce IL-8 production in cultured human cells such as pulmonary epithelial cells [Arnold et al., 1994], bronchial epithelial cells [Noah and Becker, 1993], granulocytes [Arnold et al., 1994], alveolar macrophages [Becker et al., 1991], and nasal epithelium cells [Becker et al., 1993]. Although they also pointed out that RSV induced other inflammatory cytokines such as IL-1 α/β, TNF, and IL-6, they made no reference to the contribution of IL-1 and TNF, which are IL-8–inducible cytokines, on the production of IL-8.

In addition to the human IL-8 described above, we recently demonstrated that RSV induces effectively murine macrophage inflammatory protein-2 (MIP-2), a representative murine chemokine, in a murine macrophage cell line, RAW264.7 cells [Ochiai et al., 1996b]. However, the mechanisms on MIP-2–inducing activity of RSV have not yet been completely understood. The present study was undertaken to clarify whether RSV induces MIP-2 production directly or indirectly via the initial production of other cytokines inducible for MIP-2 production.

MATERIALS AND METHODS

Cells and Virus

Hep-2 cells were cultured in Eagles's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Murine macrophage RAW 264.7 cells were obtained from American Type Culture Collection and cultured in Dullbecco's MEM (DMEM) supplemented with 10% of FBS. RSV (A2 strain), kindly supplied from Dr. Watanabe (Rational Drug Design Laboratories, Fukushima, Japan), was propagated in a confluent monolayer of Hep-2 cells in maintenance medium (MEM with 2% of FBS). When the cytopathogenic effect (CPE) reached 80%, the cultures were processed to three cycles of freezing and thawing. After centrifugation at 2000 × 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 unit (PFU) on Hep-2 cells as described previously [Murphy et al., 1990; Frankle-Ullmann et al., 1995]. The culture supernatant from uninfected Hep-2 cells was prepared as above and used for a mockinfection control.

Cytokine Production in the Presence or Absence of Anti-TNF α and Anti-IL-1 α Antibodies in Response to RSV Infection

Infection and culture conditions have been described previously [Ochiai et al., 1996]. Briefly, a confluent monolayer of RAW 264.7 cells in a 96-well plate was washed once with DMEM and then infected with 25 μl of virus solution giving a multiplicity of infection (MOI) of 3 PFU/cell for 60 min at 37°C. After virus adsorption, 75 μl of serum-free DMEM was added (0 h) and further cultured. Mock-infected cells were processed similarly. In some experiments, infected cells were cultured in the presence of either rabbit anti-TNF α neutralizing antibody or hamster anti-IL-1 α monoclonal neutralizing antibody (Genzyme, Cambridge, MA) to examine the influence of TNF α and IL-1 α on the MIP-2 production. The CM was collected at the indicated times postinfection (p.i.) and clarified by centrifugation at 2000 × g for 10 min to compare cytokine levels by enzyme-linked immunosorbent assay (ELISA) as described below.

Cytokine, Cell Viability, and Virus Yield Assays

Murine TNF α, IL-1 α, and IL-1 β in the CM were quantified by ELISA kits purchased from Genzyme (Cambridge, MA). The level of murine MIP-2 in the CM was also assayed by ELISA, as described previously [Ochiai et al., 1996a]. In brief, rabbit anti-murine MIP-2 antibody and biotinylated anti-murine 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 before terminating the reaction with 2M H_2SO_4. The absorbance was measured at an optical density (OD) of 490 nm on a microplate reader (Bio-Rad). Purified cytokines, which were supplied in the kits (TNF α and IL-1 α/β) or prepared in our laboratory (MIP-2), were used for standardization of each cytokine. Triplicate cultures were used for each experimental point for statistical analysis. Cell viability of RSV- and mock-infected RAW264.7 cells were examined by trypan blue exclusion test. Virus yields in the collected CM were determined by plaque assay as above.

Statistical Analysis

The results were expressed as the mean ± standard deviation (SD). The differences were examined by Bonferroni's least significant difference test.

RESULTS

Kinetics of Cytokine Production in RSV-Infected RAW264.7 Cells

The levels of MIP-2, IL-1 α/β, and TNF α in RAW264.7 cells after infection with RSV (MOI 3) were monitored for a 24-h timecourse (Fig. 1). Compared with cytokine levels at 0 h p.i., the levels of all these cytokines became detectable at 2 h. Thereafter, the time-related production was, however, quite different by each cytokine. TNF α levels were increased rapidly toward 8 h. Thereafter, the peak level was reached at 8 h (4.0 ± 0.04 ng/ml) and was decreased to one third of the peak at 12 h and then maintained to almost same levels. Although the levels of IL-1 α were lower compared with those of TNF α, these values increased gradually toward 20 h (118.4 ± 8.8 pg/ml). The levels of IL-1 β were extremely low throughout the experiment (at maximum 30 pg/ml). In sharp contrast to the kinetics of TNF α and IL-1 α showing monophasic pattern,

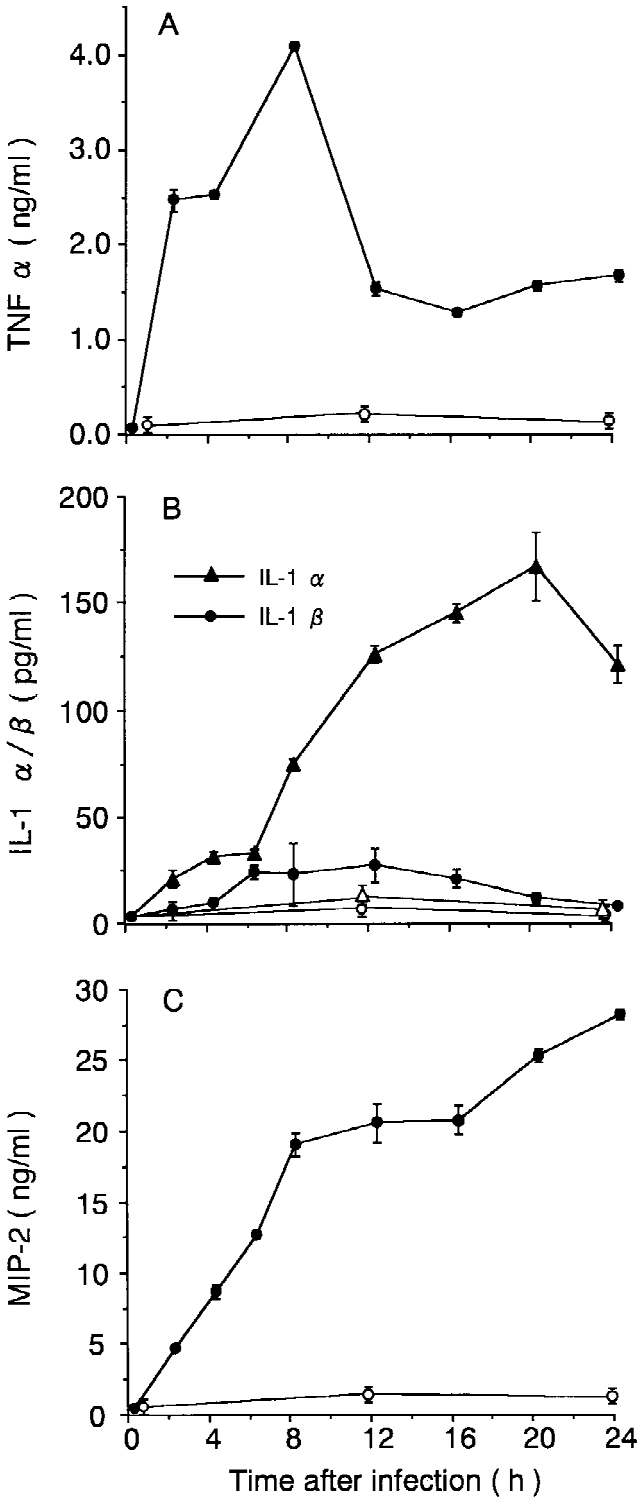

Fig. 1. Kinetics of cytokine production in RSV-infected RAW264.7 cells. The cells in a 96-well plate were infected (closed symbol) or mock-infected (open symbol) with RSV at an MOI of 3 and cultured at 37°C as described in Materials and Methods. At the indicated time p.i., the CM was collected to assay the concentrations of TNF α **(A),** IL-1 α/β **(B),** and MIP-2 **(C)** by ELISA. Data are shown as the mean ± SD (thin bar) of 3 determinations.

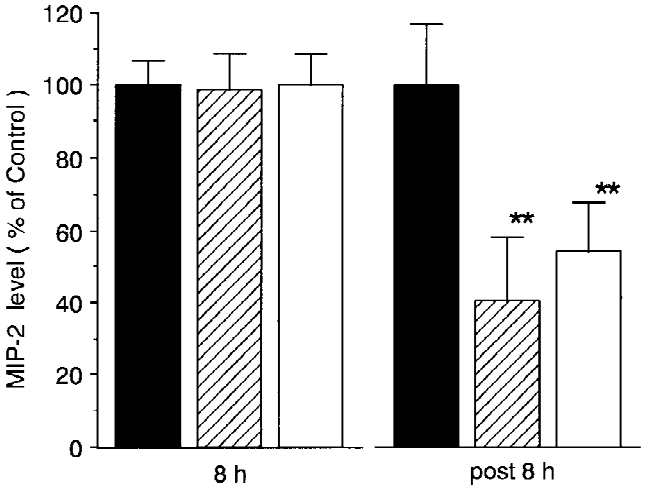

Fig. 2. Influence of neutralizing antibodies against TNF α and IL-1 α on the MIP-2 production in RSV-infected RAW264.7 cells. The infected cells were cultured in the absence of antibody as a control (solid bar), or in the presence of either anti-IL-1 α (light oblique bar) or anti-TNF α (open bar) antibody. Infection and culture conditions were the same as described in the legend for Figure 1. At 8 h p.i. (left panel) and 24 h p.i. (right panel), the CM of each experimental group was collected to assay MIP-2 concentrations by ELISA. In the left panel, MIP-2 levels were directly expressed as percent values in which control MIP-2 level at 8 h p.i. was 100. In the right panel, $MIP\text{-}2_{24\text{-}8}$ (increasing rate during 16 h from 8 h to 24 h) was initially calculated in each experimental group by the following formula; $MIP\text{-}2_{24\text{-}8} = MIP\text{-}2_{24} - MIP\text{-}2_{8}$, where $MIP\text{-}2_{24}$ and $MIP\text{-}2_{8}$ are MIP-2 concentrations at 24 h and 8 h, respectively. Thereafter, these $MIP\text{-}2_{24\text{-}8}$ levels were expressed by percent values as above. Data are shown as the mean (thick bar) ± SD (thin bar) of 3 determinations. Double asterisks indicate a significant difference from control (solid bar) with a *P* value of <0.01.

that of MIP-2 showed a trend for a biphasic pattern, that is, MIP-2 levels increased during the first 8 h (20 ng/ml), fell to almost the same levels toward 16 h, and then increased again to 30 ng/ml until 24 h. In a mock infection control, no significant production was observed in either cytokine throughout the experiments, eliminating the possibility that Hep-2 derived factors involve in these phenomena.

Influence of Anti-TNF α and Anti-IL-1 α Neutralizing Antibodies on MIP-2 Production in Response to RSV Infection

Because TNF and IL-1 are well-known inducers for MIP-2 production [Watanabe et al., 1989], it was studied whether these cytokines contribute on the biphasic production of MIP-2. To examine this issue directly, the MIP-2 levels were compared in the infected cells cultured in the presence and absence of either anti-TNF α or anti-IL-1 α neutralizing antibody at doses of 50 μg/ml. As shown in Figure 2, MIP-2 levels at 8 h p.i. showed no significant difference among experimental groups (15.4 to 15.6 ng/ml and 15.6 ng/ml for the presence and absence of antibodies, respectively). In sharp contrast to the MIP-2 levels at the relatively early phase corresponding to the first response of MIP-2 production, those at the late phase, such as 24 h p.i., were different among experimental groups. The MIP-2 level

TABLE I. Viability of Mock-Infected and RSV-Infected RAW264.7 Cells

Time in culture (h)	Viable cells (%)[a]	
	Mock-infection	RSV-infection
8	95.8	96.5
24	96.2	96.4
48	93.7	94.2

[a]Viable cells were examined by trypan blue test.

in the absence of antibody (control) was increased continuously to 27.5 ng/ml, whereas the increase rate of MIP-2 during the following 16 h (from 8 h to 24 h p.i.) was limited to 40.3% and 54.6% of the control by the presence of anti-IL-1 α and anti-TNF α antibodies, respectively. By the presence of antibody, the level of TNF α was reduced to 31% of that in antibody-free control culture at 8 h p.i., when the level of TNF α attained its peak. The level of IL-1 α was also reduced to 15% of the control by the presence of antibody, when compared at 16 h p.i., when IL-1 α was prosperously released in antibody-free culture. The inhibitory effect of each antibody at a dose of 5 μg/ml was negligible. These data suggest that the initial induction of TNF and IL-1 are responsible in part for the late phase of MIP-2 production.

Viability of RSV- and Mock-Infected RAW 264.7 Cells

As shown in Table 1, viable cells at 8 h p.i. were 96.5% and 95.8% for RSV- and mock-infected cells, respectively. At 24 h and 48 h p.i., these values were 96.4% to 94.2% and 96.2% to 93.7% for RSV- and mock-infected cells, respectively. These data indicate that there is no significant difference in cell viability between RSV- and mock-infected cells at any time point. Furthermore, no CPE was found in RSV-infected cells throughout the experiments, although virus antigen could be detected by immunofluorescence (data not shown). As for virus growth in RAW264.7 cells, output virus titer in the culture supernatant did not exceed that unadsorbed at 24 h p.i. These data suggest that RSV exposure results in no cytolytic infection in RAW 264.7 cells.

DISCUSSION

In human models, several studies have reported that RSV has the potential to stimulate the inflammatory cytokines such as IL-8, TNF, IL-1, and IL-6 in the cultured macrophages and air way-lining cells [Arnold et al., 1994; Becker et al., 1991]. In accordance with these studies, a recent investigation showed that RSV could induce the enhanced production of MIP-2, a murine functional equivalent of human IL-8, in murine macrophage RAW264.7 cells and airway-lining cells [Ochiai et al., 1996]. Based on these findings, we investigated whether RSV could induce TNF and IL-1 productions with special reference to their contribution to MIP-2 production in RAW264.7 cells. It was shown that RSV could induce TNF α and IL-1 α production at a low but recognizable level in RAW264.7 cells (Fig. 1). Indeed, the levels of TNF α and IL-1 α in RSV-infected RAW264.7 cells were approximately 20- and 80-fold, respectively, lower than those in LPS-stimulated RAW264.7 cells, although MIP-2 levels were almost comparable in both cases [Sakai et al., 1997]. However, as shown in an inhibition assay of the antibodies [Fig. 2], the most important factor is that TNF α and IL-1 α could contribute to the second phase of MIP-2 production even at a low level. In addition, the fact that TNF α levels decreased after 8 h (Fig. 1) might indicate that the released TNF interacts again with the cells, resulting in the induction of MIP-2 production responsible for the second phase. Thus MIP-2 production is due to the combined effects of the direct MIP-2–inducing activity of RSV and an indirect effect via the initial productions of TNF α and IL-1 α, resulting in biphasic production of MIP-2. In other experimental systems using LPS-stimulation [Yoshimura et al., 1987] and influenza virus infection [Ochiai et al., 1993], chemokine production seems to be monophasic. In the case of influenza infection, Choi and Jacoby [1992] reported that IL-8 gene expression was specifically enhanced, whereas IL-1 gene expression was not. Thus the biphasic MIP-2 production in RSV-infected RAW264.7 cells is considered to be an unique feature. When comparing MIP-2–inducing activity in RAW264.7 cells among several viruses, MIP-2 level in RSV-infected cells was higher approximately 3-fold than that in influenza virus (A NWS virus) -infected cells [Hirabayashi et al., 1995]. However, Sendai virus (Nagoya strain), a member of *Paramyxoviridae*, did not enhance MIP-2 production in RAW264.7 cells (data not shown). Based on these findings, RSV might be ranked as one of the most potent inducible viruses for MIP-2 production.

In this study, RSV-infected RAW264.7 cells showed no alternation in cell viability compared with mock-infected cells in accordance with a previous report [Panuska et al., 1990]. As for RSV growth in macrophages, several studies have indicated that RSV infection is generally abortive [Ullmann et al., 1995], or if the infection is productive, a extremely long time (more than 480 h) is required for the virus release [Panuska et al., 1990]. In addition, it has been shown that virus yield in RAW264.7 cells is originally lower compared with that in other murine macrophage cell lines [Raschke et al., 1978]. There was no positive evidence to support RSV growth in RAW264.7 cells within a 24-h time course in this study. It should be ruled out that the possibility that the second phase of MIP-2 production is due to the enhanced release of MIP-2 by the CPE of RSV infection.

In conclusion, RSV is the potent inducer of a wider range of inflammatory cytokines which are closely related with another cytokines. Thus these findings also make RSV infection an attractive entity for further studies of the mechanisms that induce inflammatory cytokines. On the other hand, chemokine has been suggested as a key inflammatory mediator in pulmonary diseases including virus infections [Standiford et al.,

1990; Becker et al., 1993]. To study this issue, animal models for RSV infection, notably the murine model, have been developed [Taylor et al., 1984; Bangham et al., 1986].

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