Brief Definitive Report

Selective Induction of Monocyte and not Neutrophil-attracting Chemokines after Influenza A Virus Infection

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Summary

It is characteristic for virus infections that monocytes/macrophages and lymphocytes infiltrate infected tissue while neutrophils are absent. To understand the mechanisms selectively attracting mononuclear cells in viral diseases, we examined in an influenza A virus model the expression and regulation of chemokines as candidate molecules responsible for the immigration of leukocytes into inflamed tissue. After influenza A virus infection of human monocytes, a rapid expression of the mononuclear cell attracting CC-chemokine genes MIP-1 , MCP-1, and RANTES occurred which was followed by the release of chemokine proteins. In striking contrast to CC-chemokines, the expression of the prototype neutrophil CXC-chemoattractants IL-8 and GRO-α was completely suppressed after influenza A infection. The release of other neutrophil chemotactic factors was excluded by microchemotaxis assays. These results suggest that the virus-specific induction of mononuclear cell-attracting chemokines accounts for the preferential influx of mononuclear leukocytes into virus-infected tissue.

A hallmark of tissue inflammation is the recruitment, immigration and activation of leukocytes. Gradients of chemotactic factors direct transendothelial migration and movement through the extracellular matrix (1). Most viral diseases are characterized by the development of a specific infiltration consisting predominantly of mononuclear leukocytes while neutrophils are absent as long as no complicating bacterial superinfection occurs. Previous reports show that exposure of monocytes or macrophages to virus results in the release of various proinflammatory cytokines (2–5). Along this line, we demonstrated that an infection of monocytes with influenza A or coxsackie B3 virus induced TNF-α, IL-1, and IL-6 production (6–9). However, the induction of these proinflammatory cytokines cannot explain the development of characteristic mononuclear leukocyte infiltrations in virally infected tissue.

In virus infections, little attention has been focused on the chemokine family and a systematic analysis is still missing. Chemokines are potent chemoattractant cytokines (10, 11) and have to be considered as the main candidate molecules responsible for the selective recruitment of distinct leukocyte populations. Members of the CC-chemokine subfamily, such as MIP-1α[1] (macrophage inflammatory protein-1α), MCP-1 (monocyte chemotactic protein-1), and RANTES (regulated upon activation, normal T cell expressed and secreted) preferentially attract monocytes and lymphocytes (12). The CXC-chemokines which contain an ELR-motif preceding the first cysteine, such as IL-8 (interleukin-8) or GRO-α (melanoma growth stimulatory activity) are major neutrophil chemoattractants (13).

As a first step to study the mechanisms responsible for the generation of a typical virus-induced tissue infiltration consisting predominantly of mononuclear cells, we employed influenza A virus to infect human monocytes. Here we report the selective induction of mononuclear cell attracting chemokines while, in striking contrast, the expression of neutrophil attracting CXC-chemokines was completely suppressed.

Materials and Methods

Cell Preparation and Culture. Human monocytes were prepared from the buffy coat of healthy blood donors generously provided by the Dept. of Transfusion Medicine, University of Marburg, Germany. After separation of the mononuclear cells by Ficoll-Hypaque density gradient centrifugation (6), the monocytes were enriched by elutriation to a purity of >90% as determined by nonspecific esterase staining or FACS®-analysis using FITC-labeled anti-CD 14 (Immunotech, Hamburg, Germany). The neutrophils were separated from red blood cells by a 3% dextran sulfate sedimentation and a subsequent hypotonic lysis of the remaining erythrocytes as previously described (14, 15).

Virus Preparation and Infection. Influenza A virus strain A/PR/8 (H1N1) was kindly donated by Dr. H.-D. Klenk (Institute of Virology, Marburg, Germany) and was propagated and purified as previously outlined in detail (6). Infectivity was assessed by a standard plaque assay as cytopathic effect on confluent cultures of

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/09/1191/06 $2.00
Volume 184 September 1996 1191–1196

mycoplasma-free Madin Darby canine kidney II cells with a 0.5% agarose overlay (16). Human monocytes (0.5×10^6/ml) were infected or mock-infected by exposure to 2 MOI (multiplicity of infection) A/PR/8-virus (2 plaque-forming units per cell) for 1 h under serum-free conditions (6, 7, 9). LPS (10 ng/ml) from Escherichia coli strain 0127:B8 (Difco, Detroit, MI) was used as a positive control for chemokine induction. Culture supernatants were collected at various times after infection and stored in aliquots at −70°C. The remaining cells were used for RNA preparation.

Chemotaxis Assay. Cell migration was assessed in quadruplicate using a 48-well microchemotaxis chamber (Neuro Probe, Bethesda, MD) (17). 25 μl of the culture supernatants were placed into the lower compartment. 50 ng/ml recombinant MCP-1 or IL-8 (IC Chemikalien, Ismaning, Germany) were used as controls to assess monocyte and neutrophil chemotaxis, respectively. The upper compartment was filled with 50 μl of a freshly prepared monocyte or neutrophil cell suspension (2×10^6/ml). Both compartments were separated by polycarbonate filters (Costar, Bodenheim, Germany). After incubation at 37°C for 90 min the filter was removed, fixed in methanol, and stained with hematoxylin (Sigma, Munich, Germany). The number of migrated monocytes per well was densitometrically evaluated on stained filters by a computer-assisted imaging system (Vilber Lourmat, distributed by Fröbel, Wasserburg, Germany). The number of neutrophils attracted into the lower compartment was quantitated enzymatically by determining β-glucuronidase activity after lysis of the cells (conversion of *p*-nitrophenyl-β-D-glucuronide; Sigma, Munich, Germany).

Determination of Chemokines. Chemokine levels were determined by specific sandwich enzyme-linked immunosorbant assays (ELISA) developed in our laboratory (18). Briefly, 96-well microtiter plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated with a monoclonal antibody for IL-8 (IC Chemikalien, Ismaning, Germany), MCP-1, GRO-α, RANTES (all from R&D Systems, Wiesbaden, Germany), or MIP-1α (Promega, distributed by Serva, Heidelberg, Germany). After the sample a polyclonal antibody of goat or rabbit origin was added and incubated at RT for another hour. All polyclonal antibodies were from R&D Systems, except that for IL-8, which was purchased from IC Chemikalien. Detection was performed with a peroxidase-conjugated third antibody (Dianova, Hamburg, Germany) and subsequent conversion of OPD (o-phenylenediaminedihydrochloride)-substrate.

RNA Preparation and Northern Blot Analysis. Total RNA was prepared by a modified guanidine thiocyanate method as previously described in detail (19). 10 μg total RNA was denatured by glyoxal/DMSO treatment and separated on 1% agarose gels. The RNA was capillary blotted by 20 × SSC to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). After UV cross-linking, hybridization was performed under continuous rotation in a hybridization oven (Biometra, Göttingen, Germany). As already reported in detail (19), the membranes were hybridized with Digoxygenin (DIG)-labeled antisense riboprobes overnight under highly stringent conditions in 50% formamide at 68°C. Bound DIG-labeled riboprobes were visualized non-radioactively using the DIG nucleic acid detection Kit (Boehringer Mannheim) and CDP-Star chemiluminescence substrate (Tropix, Bedford, MA, distributed by Serva, Heidelberg, Germany).

Generation and Labeling of the Riboprobes. 300–400-bp-long probes corresponding to human MIP-1 , MCP-1, RANTES, GRO-α, and IL-8, were generated by RT-PCR and subsequent cloning of the respective PCR-products. 1 μg total RNA from LPS-stimulated human monocytes was oligo-dT-primed and reverse transcribed with Superscript II reverse transcriptase (Life Technologies, Eggenstein, Germany). The cDNA was amplified by specific forward and reverse primers containing artificial restriction sites at their 5′-ends by SuperTaq DNA-polymerase (Stehelin, Basel, Switzerland). The amplified DNA was gel-purified, digested with BamHI and EcoRI and site-directed cloned into the respective sites of pBluescript SK- (Stratagene, LaJolla, CA). The specificity of the inserts was confirmed by sequencing. DIG-labeled sense and antisense riboprobes were generated with T3- or T7-RNA polymerase by the DIG-RNA Labeling Kit (Boehringer Mannheim). Labeling efficiency was examined by dot blot analysis.

Results

Differential Chemoattraction of Leukocytes by Factors Released from Influenza A Virus-Infected Monocytes. To identify the leukocyte populations that are responsive to virus-induced chemotactic factors, we infected human monocytes with influenza A virus for 16 h and screened the culture supernatants for chemotactic activities. We found that freshly elutriated monocytes were strongly attracted by factors released from influenza A virus-infected cells (Fig. 1, *left panel*) while neutrophils remained unaffected. The chemotactic response of neutrophils to virally induced supernatants did not differ from the untreated control medium (Fig. 1, *right panel*). As expected, both monocytes and neutrophils were strongly chemoattracted by factors released from LPS-stimulated monocytes, however, a concomitant influenza A virus infection significantly reduced the LPS-induced neutrophil chemotactic activity (Fig. 1, *right col-*

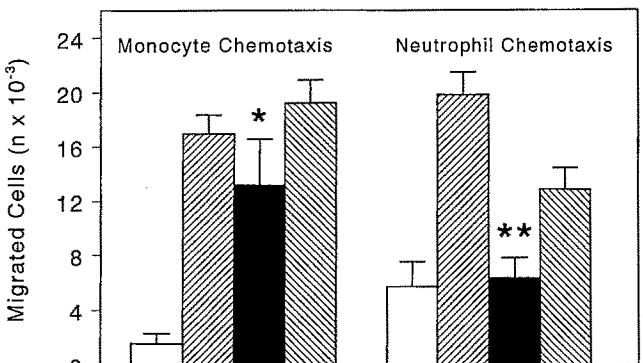

Figure 1. Differential monocyte and neutrophil chemotaxis after influenza A infection. 0.5×10^6 human monocytes were stimulated with LPS (10 ng/ml), infected with influenza A virus (2 MOI) or treated with a combination thereof as indicated. The supernatants were harvested after 16 h and analyzed for monocyte- or neutrophil-specific chemotactic activities in a microchemotaxis assay. The number of migrated cells was determined densitometrically (monocytes) or enzymatically (neutrophils) as described in Materials and Methods. Values represent the mean ± SD of identically prepared quadruplicate cultures. *Significantly different from the unstimulated control ($P < 0.001$). **No statistically significant difference between infected and unstimulated cells.

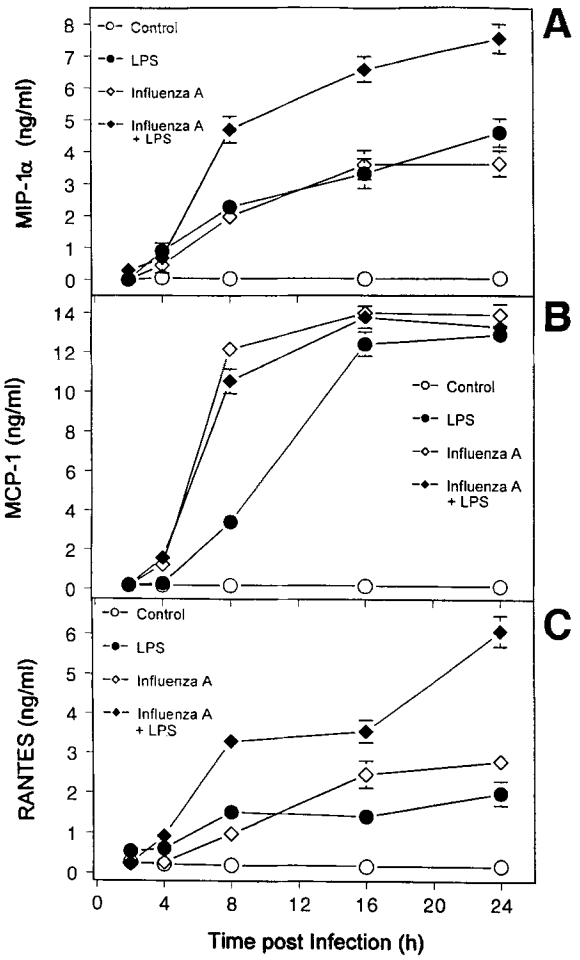

Figure 2. Kinetics of CC-chemokine production by human monocytes. 0.5×10^6 cells were either left untreated, stimulated with LPS (10 ng/ml), infected with influenza A virus (2 MOI) or treated with a combination of influenza A and LPS. The release of MIP-1α (*A*), MCP-1 (*B*) or RANTES (*C*) was determined by specific ELISAs at the indicated time periods. Values represent the mean ± SD of three identically prepared cultures.

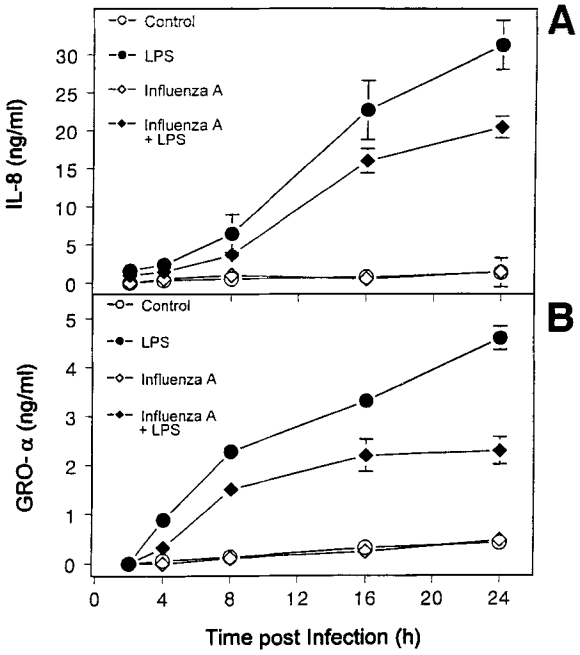

Figure 3. Lack of CXC-chemokine release after influenza A infection. The experiments were performed as described in Fig. 2. IL-8 (*A*) or GRO-α (*B*) production was determined by specific ELISAs at the indicated time periods. Values represent the mean ± SD of three identically performed experiments.

umn). Antibodies to the CC-chemokines MIP-1α and MCP-1 added to the influenza-induced supernatants, markedly reduced but did not completely abrogate the monocyte-specific chemotactic response, which indicates that additional mononuclear cell attracting chemotactic factors were released after influenza A infection. To distinguish between chemotaxis as directional migration and chemokinesis as random cell migration, we added equal concentrations of virus-induced supernatants to both sides of the polycarbonate filter. Under these conditions, the migration of monocytes was arrested indicating that a chemotaxis and not random chemokinesis had taken place.

Kinetics of CC-Chemokine Induction. The finding that monocytes selectively migrated in response to factors released from virus-infected cells was further analyzed by studying the kinetics of MIP-1α, MCP-1, and RANTES release after influenza A of infection of human monocytes for 2–24 h. We found a rapid and high release of the monocyte and lymphocyte chemoattractants MIP-1α and MCP-1 (Fig. 2, *A* and *B*), and a rather delayed and moderate, but still significant RANTES production (Fig. 2 *C*). Already 4 h after infection, elevated MCP-1 and MIP-1α levels were detected which reached a maximum around 16 h later, while the onset of RANTES release started between 8 and 12 h after infection. The stimulation of human monocytes with LPS led to a similar CC-chemokine production and was additive to influenza A infection.

Failure of CXC-Chemokine Production after Virus Infection. The lacking generation of neutrophil attracting activity after influenza A virus infection (Fig. 1) was further substantiated by a kinetic analysis of IL-8 and GRO-α induction. In contrast to the marked release of CC-chemokines, no significant production of both neutrophil chemoattractants IL-8 and GRO-α was found at any time of the kinetic analysis between 2 and 24 h after infection (Fig. 3, *A* and *B*). LPS was used as a positive control stimulus and expectedly led to the release of high levels of both CXC-chemokines which, however, were significantly reduced when the producer cells were simultaneously infected with influenza A virus. The induction of an IL-8-inhibitor was excluded since influenza A virus-induced supernatants did not inhibit the production of IL-8 upon stimulation with LPS.

Differential Chemokine Gene Expression in Influenza A of Infected Cells. To elucidate the underlying molecular mechanisms upregulating CC-chemokine production and suppressing neutrophil chemoattractants, we studied chemokine gene expression by Northern blot analysis at 2, 4, 8, 16, and 24 h

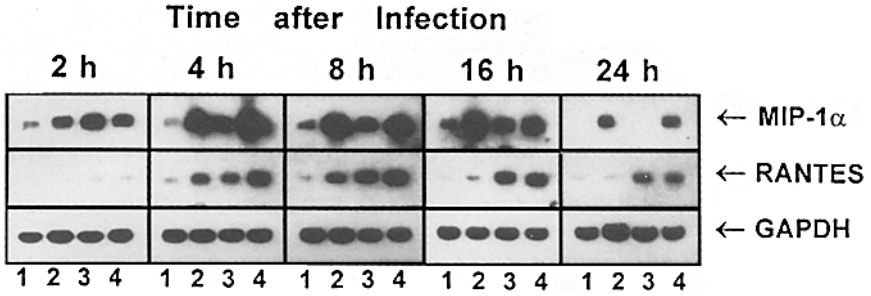

Figure 4. Time course of CC-chemokine gene expression in response to LPS and infection with influenza A virus. 10^7 monocytes remained either untreated (lanes *1*), were exposed to 10 ng/ml LPS (lanes *2*), 2 MOI influenza A (lanes *3*) or a combination of LPS and virus (lanes *4*). At the indicated times after infection, 10 μg of total RNA was analyzed for MIP-1α, RANTES, and GAPDH expression by DIG-labeled riboprobes. A representative analysis out of five is shown.

after infection. We found a rapidly inducible MIP-1α mRNA accumulation after 2 h with maximum levels at 4–8 h after influenza A virus infection (Fig. 4, *upper lane*). Virus-induced MCP-1 expression was found to develop similarly with maximum mRNA levels between 4 and 8 h after infection (data not shown). The onset of RANTES gene expression occurred more slowly with peak mRNA levels at 8 h after infection (Fig. 4, *middle lane*). LPS as a strong inducer of chemokine gene expression increased chemokine mRNAs to rather similar levels.

Gene expression of the neutrophil attracting chemokines IL-8 and GRO-α was found to totally differ from CC-chemokines. Both, IL-8 and GRO-α mRNA were rapidly downregulated or completely disappeared after infection with influenza A virus (Fig. 5). The constitutive IL-8 and GRO-α gene expressions occasionally found in preactivated monocytes, an example of which is shown in Fig. 5 (donor 2), was completely switched off by virus infection. In addition, the strong LPS-induced IL-8 and GRO-α expression was substantially suppressed by influenza A virus (Fig. 5). Hybridization with sense-riboprobes did not reveal any specific signal (not shown).

Discussion

Mononuclear leukocytes play an important role in antiviral host defense and the recruitment of monocytes and lymphocytes by extravasation into the infected tissue is a crucial event in virus-induced inflammatory reactions. Neutrophils are characteristically absent as long as no complicating bacterial superinfection occurs.

We previously reported the induction of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 by infection with influenza A virus in human monocytes and murine macrophages (6–9). Since these cytokines lack chemotactic properties, they are not directly responsible for the generation of mononuclear infiltrates after virus infection. In our present study we identified a selective induction of mononuclear cell attracting chemokines (Fig. 2) while, in striking contrast, neutrophil attracting chemokines were not released after virus infection (Fig. 3). Apart from the specifically examined neutrophil attracting chemokines IL-8 and GRO-α, chemotaxis assays indicated that other neutrophil chemotactic activities, even as yet unidentified, were also lacking or, when LPS-induced, strongly reduced after an influenza A infection (Fig. 1). Our gene expression data obtained by Northern blot analyses essentially support this differential response: On the one hand we found a strong induction of mRNA coding for mononuclear cell attracting chemokines (Fig. 4), on the other hand a complete suppression of the IL-8 and GRO-α genes after influenza A virus infection (Fig. 5). A priming step, essential for a strong release of proinflammatory cytokines such as TNF-α (6, 7), was not required for the induction of a maximal CC-chemokine response in human monocytes after infection with influenza A virus.

Our particular interest in MIP-1α as a major monocyte and lymphocyte chemoattractant in antiviral defense is supported by previous in vivo studies performed in a MIP-1α-deficient mouse model (20). MIP-1α-deficient animals lacked an inflammatory myocardial response to coxsackie virus infection and the mononuclear infiltration developing in the respiratory tract after influenza A infection was substantially reduced. In addition, virus clearance was significantly slower in MIP-1α-deficient mice, indicating that the CC-chemokine MIP-1α represents a major factor not only for the localization of mononuclear cells to the site of a viral infection but possibly also for a direct antiviral activity. Our study extends these findings by demonstrating that other important mononuclear cell attracting chemokines were additionally induced by influenza A virus infection.

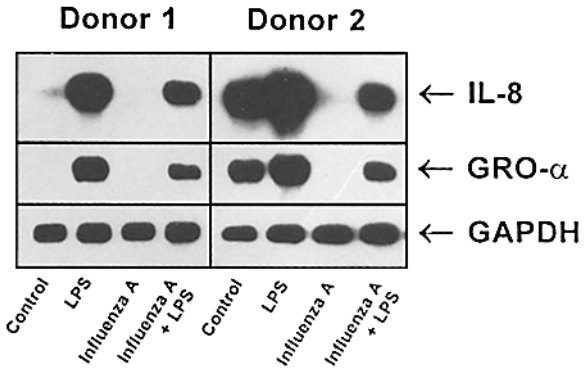

Figure 5. Suppression of CXC chemokine expression by virus infection. Human monocytes were treated as described in Fig. 4 and total RNA was isolated 8 h after infection. Gene expression of IL-8, GRO-α, and GAPDH was investigated by Northern blot analysis with DIG-labeled riboprobes. The results of two independently performed experiments are shown to demonstrate donor-specific variations.

The general importance of the CC-chemokines MIP-1α, MIP-1β, and RANTES has recently been underlined by their apparent anti-HIV activity (21).

The induction of mononuclear leukocyte attracting chemokines after virus infection has been previously reported for MCP-1 and MCP-2 after exposure of monocytes to measles virus (22). Rather contradictory results, however, have been published for the viral inducibility of the neutrophil attracting chemokine IL-8. IL-8 has been reported to be induced by measles virus infection of fibroblasts (23) and by influenza A virus, respiratory syncytial virus (RSV) and rhinovirus in pulmonary epithelial cells or alveolar macrophages (24–26). In contrast, RSV has been shown to suppress IL-8 production by induction of IL-10 (27) and IL-8 gene transcription has been demonstrated to be inhibited by virus-inducible interferon (28).

Our results clearly support the notion that an influenza A virus infection abolishes the production of neutrophil attracting CXC-chemokines and instead selectively induces CC-chemokines. For IL-8 and GRO-α, the complete lack of production could be correlated on the transcriptional level with an entire absence of the coding mRNAs (Fig. 5). The discrepancies to previous reports (23–26) may be explained by the easy inducibility of the IL-8 gene. Even adherence (29) or detachment (30) of cells represent sufficient stimuli for IL-8 production. To avoid in vitro artifacts in this study, we employed elutriation as the method of choice to purify monocytes of lowest preactivation. Even then, monocytes from certain blood donors were occasionally obtained that constitutively expressed high levels of IL-8 mRNA which, however, was strongly downregulated upon influenza A virus infection (Fig. 5, donor 2).

In conclusion, our data offer an explanation for the predominant mononuclear leukocyte infiltration of virally infected tissue on the basis of a selective induction of monocyte and lymphocyte attracting chemokines. These findings indicate an essential role for mononuclear cell attracting chemokines in antiviral immunity. Further expression and promoter analyses are presently in progress to identify distinct virus components, transcription factors as well as *cis*- and *trans*-activating or suppressing virus responsive elements that differentially upregulate or suppress distinct chemokine subfamilies.

We gratefully acknowledge Dr. J.-M. Schröder and Dr. U. Mrowietz, University of Kiel, Germany, for their support to establish the chemotaxis assays, A. Friedetzky for providing us with a GAPDH-insert containing vector, and the Dept. of Transfusion Medicine, University of Marburg, Germany, for buffy coat blood.

This work was supported by grants of the Deutsche Forschungsgemeinschaft (Sp 395/2-1 and Ge 354/8-3).

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Received for publication 13 May 1996 and in revised form 15 July 1996.

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