Herpes simplex virus type 2 induces secretion of IL-12 by macrophages through a mechanism involving NF-κB

Lene Malmgaard, Søren R. Paludan, Søren C. Mogensen and Svend Ellermann-Eriksen

Department of Medical Microbiology and Immunology, University of Aarhus, The Bartholin Building, DK-8000 Aarhus C, Denmark

Interleukin (IL)-12 is an important proinflammatory and immunoregulatory cytokine expressed primarily by macrophages. Although IL-12 appears to be essential for clearance of many bacterial and parasitic infections, only little is known about the production and regulation of this cytokine during viral infections. In this study we have shown that infection of mouse macrophages with herpes simplex virus type 2 (HSV-2) induces secretion of the p40 subunit of IL-12, and this induction was synergistically enhanced by interferon (IFN)-γ. The production of IL-12 p40 was accompanied by production of bioactive IL-12 p70, since HSV-2-induced IFN-γ secretion was blocked by neutralizing antibodies against IL-12. The IL-12-inducing effect of HSV-2 was abrogated when virus infectivity was destroyed by heat or UV irradiation, indicating that a functional viral genome is required and that interaction of viral glycoproteins with cellular receptors is not sufficient. Production of IL-12 p40 was transcriptionally regulated and required de novo protein synthesis. Although IFN-α, IL-1β and tumour necrosis factor-α marginally influenced IL-12 production, they did not seem to constitute the endogenous factor(s) responsible for the effect of the virus infection. HSV-2 infection induced nuclear-binding activity to the κB halfsite of the IL-12 p40 promoter, and inhibitors of nuclear factor (NF)-κB activation significantly reduced IL-12 p40 production in infected cells. Collectively our data show that HSV-2 infection of murine macrophages induces production of IL-12 through a mechanism requiring intermediary synthesis of viral or host proteins and involving activation of NF-κB.

Introduction

Macrophages and natural killer (NK) cells play a central role in resistance to and recovery from many virus infections (Biron et al., 1999; Carr et al., 1997; Heise & Virgin, 1995; Mogensen, 1984). Interleukin (IL)-12 is a pro-inflammatory cytokine produced by macrophages and other antigen-presenting cells during infections with various intracellular pathogens, including some viruses (D’Andrea et al., 1992; Gazzinelli et al., 1994; Heufler et al., 1996; Hsieh et al., 1993; Orange & Biron, 1996a). IL-12 has the ability to induce production of interferon (IFN)-γ and cytopotoxicity of NK and T cells (Kobayashi et al., 1989; Stern et al., 1990) and also to regulate the T cell response in the direction of a cellular (Th1) immune response (Hsieh et al., 1993; Heinzel et al., 1993). Thus, IL-12 is important for induction and maintenance of a suitable immune response, capable of eliminating the pathogen (Hsieh et al., 1993; Heinzel et al., 1993).

The importance of IL-12 production is well characterized for infections in mice with intracellular bacteria like Listeria monocytogenes (Hsieh et al., 1993; Tripp et al., 1994) and intracellular parasites like Toxoplasma gondii (Gazzinelli et al., 1994). These pathogens induce production of IL-12 which in turn is responsible for the early production of IFN-γ by NK cells. Neutralization of IL-12 during such infections results in increased susceptibility and higher microbial burden, which shows that IL-12 is important for resistance against these intracellular bacteria and parasites. Much less is known about the production and function of IL-12 during virus infections, even though these pathogens also have an obligate intracellular habit. It has been best documented for infections with murine cytomegalovirus (MCMV) and influenza A virus that IL-12 is indeed produced during a virus infection and is responsible for the early production of IFN-γ and primary control of the infection (Orange & Biron, 1996a, b; Carr et al., 1999;
Monteiro et al., 1998). On the other hand, IL-12 is not produced during infection with lymphocytic choriomeningitis virus (LCMV) (Orange & Biron, 1996), and despite detection of IL-12 p40 mRNA during infection with murine hepatitis virus (MHV) (Schijns et al., 1996, 1998, Coutelier et al., 1995), IL-12 knock-out mice exhibit an unaltered resistance against this infection (Schijns et al., 1998). During an ocular herpes simplex virus (HSV)-1 infection of BALB/c mice, IL-12 p40 mRNA and protein was detected in lysates of cornea and local lymph nodes, and mRNA was detected in spleen and peritoneal cells infected in vitro (Kanangat et al., 1996). Furthermore IL-12 p40 mRNA has also been detected in mice infected with lactate dehydrogenase elevating virus and adenovirus (Coutelier et al., 1995).

IL-12 is a heterodimeric protein composed of the two subunits p35 and p40 (Kobayashi et al., 1989), encoded by separate genes. Expression of the biologically active heterodimer p70 is associated with a large excess of the monomer p40, whereas p35 only exists as part of the heterodimer (D’Andrea et al., 1992). Production of the p40 monomer is inducible by, e.g., lipopolysaccharide (LPS) via transcriptionally regulated mechanisms requiring de novo protein synthesis (Murphy et al., 1995; Ma et al., 1996a). Moreover, this induction is among other things dependent on interaction between transcription factors of the NF-κB family and an NF-κB halfsite element in the p40 promoter (Murphy et al., 1995). Production of p35 is transcriptionally as well as translationally regulated (Ma et al., 1996a; Babik et al., 1999). Despite the fact that many cell types express p35 mRNA constitutively (D’Andrea et al., 1992), expression of this subunit might be limiting for the production of the heterodimer p70 (Snijders et al., 1996; Babik et al., 1999).

Here we show that infection with HSV-2 induces secretion of IL-12 p40 protein in murine macrophages and production of biologically active IL-12 p70, reflected by the inhibition of HSV-2-induced IFN-γ production by neutralization of IL-12. Furthermore, our data show that this induction requires de novo synthesis of viral and/or cellular intermediary factors, and that induction of IL-12 p40 expression by HSV-2 infection is dependent on NF-κB activation.

Methods

Virus. The high-titre stock of the MS strain of HSV-2 used in this study was produced as previously described (Ellermann-Eriksen, 1993). Briefly, mycoplasma-free Vero cells in Eagle's minimum essential medium with 2% foetal calf serum (FCS; Hyclone), 200 IU/ml penicillin and 200 µg/ml streptomycin were infected at an m.o.i. of 0.01. When the cytopathic effect was nearly complete the cells were freeze-thawed twice, and the supernatant was clarified by centrifugation at 3000 × g for 1 h. The virus was pelleted by ultracentrifugation at 45000 × g for 1 h and resuspended in PBS supplemented with 1% BSA. After three 30 s periods of sonication at 40 W, the virus preparation was aliquoted and stored at −70 °C until use. The virus stock had an infectivity titre of 1.5 × 10^9 p.f.u./ml as determined by plaque assay in Vero cells. Virus was thawed immediately before it was required and used as infective virus, subjected to heat-inactivation at 56 °C for 30 min, or inactivated by UV light for 15 min. The virus was normally used at a final concentration of 3 × 10^8 p.f.u./ml, giving an m.o.i. of 6 for J774A.1 cells and 2 for mouse peritoneal cells.

Mice. Inbred, specific pathogen-free BALB/cABOM mice were obtained from Bomhollgaard Animal Breeding and Research Centre. Female mice were used at the age of 8–12 weeks, but for individual experiments only mice born within a single week were used.

Cell cultures. The murine macrophage cell line J774A.1 (ATCC TIB 67) was grown in Dulbecco’s modified Eagle’s medium with 1% Glutamax I (Life Technologies), supplemented with 5% LPS-free FCS, 200 IU/ml penicillin and 200 µg/ml streptomycin. Elicited peritoneal cells from BALB/c mice were obtained as previously described by injection of 2–0 ml 10% thioglycollate intraperitoneally 4 days before harvest of the cells by lavage of the peritoneum with cold PBS, pH 7.4, supplemented with 2% FCS and 200 IU/ml heparin (Baskin et al., 1997). After washing the cells were collected and grown in RPMI 1640 medium (BioWhittaker) supplemented with 10 mM glutamine, 2 mM HEPES and FCS and antibiotics as above.

For induction of cytokines cells were seeded in 96-well tissue culture plates to give a final concentration of 5 × 10^5 and 1.5 × 10^6 cells/ml in 200 µl RPMI medium and left to settle for 2 h and overnight for J774A.1 cells and peritoneal cells, respectively. After infection or treatment with cytokines for 24 h at 37 °C in a humidified atmosphere with 5% CO₂, the supernatants were harvested for ELISA.

For isolation of total RNA J774A.1 cells were seeded in 10 cm² tissue culture plates at a density of 2.5 × 10^6 cells per plate and allowed to settle for 2 h. The cultures were stimulated and infected 5 h before RNA was extracted.

Cytokines, antibodies and reagents. Recombinant murine cytokines were used at the following concentrations: IL-12 p40 (PharMingen), 3–9–200 pg/ml; IFN-γ for ELISA (R&D), 3–9–200 pg/ml; IFN-γ for stimulation of cells (PharMingen), 100 IU/ml (1.6 ng/ml); IFN-α/β (PBL Biomedical Laboratories, cat. no. 12100-1), 1000 IU/ml (2.32 ng/ml); TNF-α (Genzyme), 500 IU/ml (12.4 ng/ml); IL-1β (R&D) 15 IU/ml (75 ng/ml). The following neutralizing antibodies against murine cytokines were used: monoclonal rat anti IL-12 p40/p70 (clone C17.1, PharMingen), 100 NU/ml (10 µg/ml), and a corresponding purified rat IgG2a (PharMingen); polyclonal sheep anti IFN-α/β (PBL Biomedical Laboratories), 1000 NU/ml; polyclonal goat anti IL-1α/IL-1β (R&D) 100 NU/ml; polyclonal rabbit anti-TNF-α (Genzyme), 500 NU/ml. Antibodies against murine cytokines for ELISAs: monoclonal rat anti IL-12 p40/p70 (clone C15.6, PharMingen); biotin-labelled monoclonal rat anti IL-12 p40/p70 (clone C17.1, PharMingen); rat anti-IFN-γ (R&D), biotin-labelled goat anti-IFN-γ (R&D).

N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), N-acetyl-l-cysteine (NAC), pyrrolidine dithiocarbamate (PDTC) and cycloheximide were purchased from Sigma. Trizol was from Gibco. Oligo(dT)15 primer, Expand reverse transcriptase, deoxynucleotide triphosphates and poly(dI-C) were from Boehringer Mannheim. Taq 2000 DNA polymerase and T4 polynucleotide kinase were purchased from Stratagene. The DNA oligonucleotides were provided by DNA Technology.

RNA extraction and RT–PCR. RNA was extracted with Trizol, following the recommendations of the manufacturer. Briefly, Trizol and chloroform were added and the phases were separated by centrifugation. RNA was pelleted by addition of 2-propanol and centrifugation. Finally, the RNA pellet was washed with ethanol and redissolved in RNase-free water. Using oligo(dT)15 as primer, the RNA (1–2 µg per reaction) was subjected to reverse transcription with Expand Reverse Transcriptase according to the manufacturer’s recommendations. To amplify specific
cDNA the following primers were used for the PCR reactions: IL-12 p40, 5'-CCA CTC ACA TCT GCT CCT CCA CAA 3' (sense), 5'-CAG TTC AAT GGG CAG GGT CTC CTC 3' (antisense); β-actin, 5'-CCA ACC GTG AAA AGA TGA CC 3' (sense), 5'-GCA GTA ATC TCC TCT TGC ATC C 3' (antisense). The products spanned 336 bp (IL-12 p40) and 616 bp (β-actin), respectively. For PCR amplification of cDNA 35 cycles and an annealing temperature of 55 °C were used for both p40 and β-actin.

**Results**

**HSV-2 infection induces production of IL-12 p40 and bioactive IL-12 in macrophages**

In preliminary studies we compared the murine macrophage-like cell line J774A.1, and thioglycollate-activated BALB/c peritoneal macrophages. As seen in Fig. 1, HSV-2 infection induced secretion of IL-12 p40 in both cell types. Furthermore IFN-γ, which alone could not induce IL-12 p40, synergistically enhanced the IL-12 production induced by HSV-2 infection in both types of cells (2P < 0.002 for both cell types). Thus J774A.1 cells, which are derived from BALB/c mice, appear to be reasonably representative of primary macrophages, at least as far as IL-12 p40 production is concerned.

To verify that production of IL-12 p40 was indeed associated with accumulation of bioactive IL-12 p70 we examined whether IL-12 neutralization affected HSV-2-induced IFN-γ production. Peritoneal cells were treated with neutralizing IL-12 antibodies and infected with HSV-2 for 24 h. Infection mediated a strong induction of IFN-γ secretion, which was not inhibited by control antibodies. Specific IL-12 antibodies, however, inhibited IFN-γ production in a dose-dependent manner (Fig. 2). These results show that production of the p40 monomer is associated with accumulation of bioactive IL-12.

**Kinetics and dose-response of HSV-2-induced IL-12 p40 production**

In J774A.1 cells HSV-2 infection induced IL-12 p40 production in a dose-dependent manner from 10−2 to 106 P.f.u./ml (m.o.i. from 0.2 to 6; Fig. 3). Above this concentration the cytopathic effect of virus replication profoundly affected the production of IL-12 p40. Kinetic studies in J774A.1 cells showed that the levels of IL-12 p40 in the supernatant increased from 8 h after infection and reached a maximum approximately 24 h after infection. Experiments extending to 48 h after infection showed that the levels remain high throughout this period (data not shown). Furthermore, cotreatment with IFN-γ and HSV-2 did not alter the kinetics of IL-12 p40 production, but merely increased the levels produced (data not shown).

**Induction of IL-12 p40 by HSV-2 and IFN-γ is dependent on replication-competent virus, is transcriptionally regulated and requires de novo protein synthesis**

To examine if IL-12 p40 expression was dependent on virus replication or whether a viral surface protein was sufficient to...
L. Malmgaard and others

Fig. 1. Production of IL-12 p40 in cultures of murine peritoneal cells and a macrophage cell line. Cells from the macrophage cell line J774A.1 and thioglycollate-elicited peritoneal cells, both obtained from BALB/c mice, were treated with 100 U/ml IFN-γ and infected with 3 × 10^6 p.f.u./ml HSV-2 (m.o.i. for J774A.1 cells = 6 and for peritoneal cells = 2). Supernatants from duplicate cultures were harvested after 24 h and analysed for IL-12 p40 by ELISA. The results are expressed as means ± SEM. Similar results were obtained in three separate experiments.

Fig. 2. Effect of neutralizing antibodies against IL-12 on HSV-2-induced IFN-γ production. BALB/c peritoneal cells were treated with a neutralizing IL-12 p40/p70 antibody (α-IL-12) or a control antibody (c-Ab) in concentrations of 1 or 10 µg/ml and infected with 3 × 10^6 p.f.u./ml HSV-2 (m.o.i. = 2). Supernatants from duplicate cultures were harvested after 24 h and analysed for IFN-γ by ELISA. The results are expressed as means ± SEM. Similar results were obtained in five separate experiments.

induce IL-12 production we tested if inactivated virus preparations were as active as infectious virus in this respect. As shown in Fig. 4, neither heat- nor UV-inactivated HSV-2 could induce IL-12 p40 production even after cotreatment with IFN-γ. The production of IL-12 p40 could only be induced by replication-competent virus particles.

It is well known that induction of IL-12 p40 production by LPS is transcriptionally regulated (Ma et al., 1996 b; Murphy et al., 1995). We examined whether this was also the case when macrophages were infected with HSV-2 and/or treated with IFN-γ. By RT–PCR performed on RNA from J774A.1 cells we showed that a low basal level of IL-12 p40 mRNA is present in untreated cells. HSV-2 infection, but not IFN-γ treatment, marginally augmented the accumulation of IL-12 p40 mRNA. Furthermore, the combined virus infection and cytokine treatment increased the level of specific mRNA considerably (Fig. 5a). Thus IL-12 p40 production in response to HSV-2 infection, both in normal cells and in cells treated with IFN-γ, is transcriptionally regulated.
Infected macrophages. Cells were pretreated with 20
1000 IU
the three cytokines.

As mean concentrations (pg
duplicate cultures were harvested after 24 h and analysed for IL-12 p40 by ELISA. The results are expressed
J774A.1 cells, treated with 100 U/ml IFN-

Effect of cycloheximide on IL-12 p40 mRNA accumulation in HSV-2-

Fig. 5. (a) Accumulation of IL-12 p40 mRNA in HSV-2-infected
macrophages. J774A.1 cells were treated with 100 U/ml IFN-γ and
infected with 3 × 10^6 p.f.u./ml HSV-2. Total cellular RNA was extracted
after 4 h and reverse transcription was performed with oligo(dT)_15
primers. PCR amplification was done with primers specific for IL-12 p40
and β-actin. Similar results were obtained in four separate experiments.

(b) Effect of cycloheximide on IL-12 p40 mRNA accumulation in HSV-2-
infected macrophages. Cells were pretreated with 20 μg/ml cycloheximide
(CHX) 1 h before treatment with IFN-γ and infection with HSV-2 as above.
After 4 h total cellular RNA was extracted and RT–PCR was performed as
above on serial 3-fold or 10-fold dilutions of cDNA as indicated. Similar
results were obtained in two separate experiments.

Table 1. Effect of stimulation with recombinant cytokines and inhibition of endogenously
produced cytokines on IL-12 p40 production in macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medium</th>
<th>IFN-γ</th>
<th>HSV-2</th>
<th>IFN-γ + HSV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>515 ± 0.5</td>
<td>42.3 ± 0.0</td>
<td>115.6 ± 7.6</td>
<td>375.6 ± 44.0</td>
</tr>
<tr>
<td>IFN-α</td>
<td>46.2 ± 1.6</td>
<td>11.2 ± 0.9</td>
<td>174.3 ± 47.6</td>
<td>272.6 ± 25.7</td>
</tr>
<tr>
<td>Anti-IFN-α/β</td>
<td>35.1 ± 1.2</td>
<td>21.2 ± 1.6</td>
<td>89.2 ± 1.2</td>
<td>366.4 ± 27.0</td>
</tr>
<tr>
<td></td>
<td>19.2 ± 0.4</td>
<td>11.0 ± 0.4</td>
<td>96.3 ± 7.0</td>
<td>174.1 ± 18.6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>35.5 ± 4.0</td>
<td>45.3 ± 5.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>15.3 ± 1.7</td>
<td>ND</td>
<td>122.8 ± 8.5</td>
<td>231.0 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>28.5 ± 2.0</td>
<td>15.2 ± 0.3</td>
<td>174.7 ± 9.9</td>
<td>350.6 ± 13.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>34.8 ± 3.4</td>
<td>23.8 ± 1.8</td>
<td>170.3 ± 9.5</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-IL-1α/β</td>
<td>28.2 ± 1.1</td>
<td>22.5 ± 0.3</td>
<td>203.0 ± 15.3</td>
<td>527.2 ± 9.6</td>
</tr>
</tbody>
</table>

The requirement for de novo protein synthesis as an
intermediary event in IL-12 p40 expression is still controversial
(Aste-Amezaga et al., 1998; Hayes et al., 1995). We therefore
performed semiquantitative RT–PCR analysis of RNA from
HSV-2-infected and IFN-γ-treated J774A.1 cells, cultured with
or without the presence of cycloheximide. As seen from Fig.
5(b) cycloheximide did not influence the constitutive expression
of IL-12 p40 mRNA, whereas it completely abrogated
the strong synergistic action brought about by HSV-2 and
IFN-γ. It thus appears that the synergistic induction of IL-12
p40 expression is dependent on de novo protein synthesis, a
conclusion which is also supported by the slow kinetics of the
induction of this cytokine subunit as well as the inability of
UV-inactivated HSV-2 to induce expression.

Endogenous IFN-α/β, TNF-α and IL-1α/β are not
responsible for the induction of IL-12 p40 production

The intermediary proteins required for IL-12 p40
production could be host-derived factors such as transcription
factors or cytokines, viral proteins or both. We have previously
shown that virus-induced TNF-α is responsible for expression
of inducible nitric oxide synthase during an HSV-2 infection
(Baskin et al., 1997), and we therefore wanted to examine if
macrophage-derived cytokines are also involved in induction
of IL-12 p40. IFN-α/β, TNF-α and IL-1 are all produced by
murine macrophages early during infection with HSV-2
(Ellermann-Eriksen et al., 1986; Ellermann-Eriksen, 1993; un-
published results). Their role in IL-12 p40 production was
investigated by stimulation with recombinant cytokines and
by neutralization of endogenously produced cytokines with
specific antibodies. Of the three cytokines examined (Table 1),
L. Malmgaard and others

Fig. 6. Effect of HSV-2 on NF-κB activation in J774A.1 cells. (a) Nuclear proteins from cells treated for 2 h with 100 U/ml IFN-γ, 3 × 10^6 p.f.u./ml HSV-2 and 10 µg/ml LPS were analysed for κB-binding activity by EMSA. A 32P-labelled synthetic oligonucleotide corresponding to the κB halfsite of the murine IL-12 p40 promoter was used as probe. Similar results were obtained in three separate experiments. (b) Effect of competition by identical (κB p40) and mutated (mκB p40) cold κB-probe on protein binding in NF-κB EMSA. Conditions were the same as in (a), except that a 100-fold excess of cold probe was added. Similar results were obtained in three separate experiments.

only TNF-α had an IL-12 p40-inducing effect of its own (2P = 0·01), which was not further enhanced by IFN-γ treatment (2P = 0·23). IFN-α did not significantly inhibit IL-12 p40 production (2P = 0·35), as seen in previous studies (Cousens et al., 1997, 1999), and none of the three cytokines was able to synergize with HSV-2 infection or IFN-γ treatment. The only synergism seen was that between HSV-2 and IFN-γ (2P = 0·03, 0·05 and 0·01 for the three experiments respectively). Addition of neutralizing antibodies against the three cytokines further substantiated that the ability of HSV-2 to induce IL-12 p40 production was not to any major extend dependent on intermediary production of these cytokines.

NF-κB is involved in activation of the IL-12 p40 promoter during infection with HSV-2

The murine IL-12 p40 promoter has been reported to contain an NF-κB halfsite involved in the induction of IL-12 expression by LPS (Murphy et al., 1995). By EMSA performed on nuclear extracts from untreated J774A.1 cells we found that this NF-κB halfsite binds constitutive proteins, resulting in two bands (Fig. 6a). Infection with HSV-2, but not treatment with IFN-γ, induced an additional band (arrow) not observed in uninfected cells. This band was also seen in cells both infected with HSV-2 and treated with IFN-γ and was comparable to a band induced by LPS. The inducible band could be competed away by addition of an excess of cold probe, whereas cold mutated κB-probe was unable to do this (Fig. 6b). Furthermore, the constitutive bands were not competable by the specific probe, whereas the mutated probe removed one of these bands.

The potential functional significance of the NF-κB halfsite was tested by addition of TPCK, a well-characterized inhibitor of NF-κB activation. Treatment with TPCK resulted in a dose-dependent reduction of the HSV-2-induced IL-12 p40 production, with a concentration of 4 µM reducing IL-12 p40 production by 90% during HSV-2 infection (Fig. 7). IFN-γ treatment of HSV-2-infected cells was partly able to compensate for the TPCK-induced reduction. Two other inhibitors of NF-κB activation (NAC and PDTC) with different modes of action gave essentially similar results (data not shown). No apparent toxic effect of the inhibitors could be noticed microscopically in the concentration range used. Collectively, these results indicate a role of NF-κB in the HSV-2-induced activation of the IL-12 p40 promoter.

Discussion

Production of cytokines is an early event during the innate immune response against invading viruses. In addition to functioning as activators and regulators of the innate immune
system, cytokines bridge the unspecific and adaptive immune response, notably by controlling differentiation of naive T lymphocytes (Biron, 1998; Trinchieri, 1995).

Macrophages represent one of the main sources of early cytokine production during virus infections. For instance, numerous reports have shown that IFN-α/β, TNF-α and IL-1 are rapidly produced by macrophages in response to virus infections (Ellermann-Eriksen et al., 1986; Macatonia et al., 1995; Ellermann-Eriksen, 1993; Sareneva et al., 1998). By contrast, less is known about the production and function of another macrophage-produced cytokine, IL-12, during virus infections.

In this study we have shown that in vitro infection of murine peritoneal cells and the macrophage cell line J774A.1 with HSV-2 induces secretion of IL-12 p40, which is accompanied by secretion of biologically active IL-12 p70. Our experiments did not address the question of whether IL-12 is produced by infected macrophages or by uninfected bystanders. The secretion of IL-12 p40 protein in response to HSV-2 was regulated at the transcriptional level as seen from enhanced IL-12 p40 mRNA accumulation. Similar results have been obtained by Kanangat et al. (1996) during in vitro infection of spleen cells and peritoneal macrophages with HSV-1. In an in vivo infection model with MCMV Orange & Biron (1996a, b) were able to detect IL-12 both in serum of infected mice and in supernatants of spleen cell cultures established from infected mice. Essentially similar results have also been obtained in a murine model of influenza A virus infection (Monteiro et al., 1998).

In addition we found that IFN-γ synergizes with HSV-2 in IL-12 p40 production. This is in agreement with studies using other IL-12-inducing stimuli such as LPS, poly(I-C), heat-killed L. monocytogenes and infection with live L. monocytogenes and Mycobacterium bovis BCG (Skeen et al., 1996; Flesch et al., 1995). The synergistic effect was maximal when IFN-γ and HSV-2 were administered simultaneously and was not further enhanced by priming of the cells with IFN-γ for up to 20 h before virus infection (data not shown). This is in contrast to the effect of IFN-γ pretreatment on IL-12 production induced by other stimuli like LPS and BCG infection (Ma et al., 1996 b; Flesch et al., 1995). Possibly this phenomenon might reflect a general difference in the mechanism of induction executed by bacteria or bacterial products versus a viral infection, requiring a lag time for expression of the inducing signal.

As to the functional implications of IL-12 expression during virus infection our data demonstrate that HSV-2-induced IL-12 is partly responsible for induction of IFN-γ production during infection of macrophages in vitro, since neutralizing antibodies against IL-12 p70 were able to reduce IFN-γ secretion by at least 50%. Similar conclusions were reached by Orange & Biron (1996a, b) and Monteiro et al. (1998) after in vivo infection of mice with MCMV and influenza A virus, respectively.

Infection of peritoneal and J774A.1 cells with UV-irradiated HSV-2 did not result in secretion of IL-12 p40. Similar findings with HSV-1 have been reported by Kanagat et al. (1996). This indicates that factors present in the virus particle, for example surface glycoproteins, are not sufficient to stimulate secretion of IL-12 p40, as has been reported for virus-induced secretion of IFN-α/β (Ellermann-Eriksen, 1993; Ankel et al., 1998). Moreover, the ability of HSV-2 and IFN-γ to activate IL-12 p40 transcription required de novo production of intermediary factors, as shown by sensitivity of the induction to cycloheximide. These factors could be either virus-induced cellular proteins and/or viral proteins. In either case they need to be produced early after infection, since IL-12 p40 mRNA can be detected after just 2 to 4 h (data not shown). In the light of this assumption early virus-induced cytokines represent good candidates for such intermediary factors. By stimulation with IFN-γ, TNF-α and IL-1β and neutralization of endogenously produced cytokines by specific antibodies we concluded that none of the mentioned cytokines seem to play any major role as intermediary factors in virus-induced production of IL-12. Although it has been documented that IFN-α/β is able to inhibit IL-12 production both in vitro and in vivo (Cousens et al., 1997, 1999), we did not observe this effect either when stimulating with recombinant IFN-γ or when neutralizing endogenously produced IFN-α/β during infection with HSV-2. The concentrations of IFN-γ used in the studies of Cousens et al. (1997, 1999) and in our experiments were within the same range. Likewise, differences in the preparations of IFN-γ used seems unlikely to be the explanation for the diverging results. The discrepancy could most likely reflect the different cell cultures in use, since we have used a macrophage cell line for our experiment whereas the other studies were done with mixed cell cultures like spleen leukocytes.

An alternative possibility would be that early produced viral factors, like products of the immediate early (IE) HSV-2 genes, are responsible for activation of IL-12 p40 expression. The HSV-1 IE proteins ICIP and ICP27 have been reported to be required for sustained activation of NF-κB following infection (Patel et al., 1998). We are currently investigating this possibility. The requirement for de novo protein synthesis is also seen for activation of the IL-12 p40 promoter with other inducers like LPS or Staphylococcus aureus strain Cowan (SAC) (Aste-Amezaga et al., 1998). This observation argues against the direct involvement of HSV-encoded factors and suggests a role for a common cellular signalling pathway in the IL-12 p40 induction by various stimuli.

The dissection of the molecular mechanisms in regulation of the IL-12 p40 promoter by pathogens and by cytokines has not been completed yet. Several promoter elements seem to be involved and activation is probably influenced by complex interactions between different transcription factors. Among these NF-κB has been shown to play a central role. A number of independent studies have identified an NF-κB-responsive element in the murine and human IL-12 p40 promoter and have demonstrated protein-binding to this sequence during in-
duction with bacteria or bacterial products like SAC, heat-killed L. monocytogenes and LPS (Murphy et al., 1995; Plevy et al., 1997; Gri et al., 1998) as well as by a non-bacterial inducer, CD40 ligand (Yoshimoto et al., 1997). By supershift assays some of these proteins were identified as members of the NF-κB/Rel family. Involvement of the NF-κB/Rel family of transcription factors has also been demonstrated functionally by use of inhibitors of NF-κB activation (Kang et al., 1999; D’Ambrosio et al., 1998; Mazzeo et al., 1998; Na et al., 1999). In accordance with these studies we demonstrate virus-induced protein-binding to a sequence identical to the NF-κB element of the IL-12 p40 promoter. Specificity of this binding was confirmed by competition, where an identical cold κB-probe, but not a mutated cold κB-probe, could compete for binding. Furthermore, the functional relevance of the activation of NF-κB was tested by using different inhibitors of the activation. All three inhibitors (TPCK, NAC, PDTC), with different modes of action, effected a significant reduction in the virus-induced induction of IL-12 p40 production.

IFN-γ is an important component of the host defence against an invading virus, for instance by its ability to activate macrophages (Mogensen & Virelizier, 1987). Specifically, IFN-γ is known to play a pivotal role in host defence against HSV infections (Cantin et al., 1999; Parr & Parr, 1999; Yu et al., 1996). Our data show that during in vitro infection HSV-2 is able to induce production of IL-12, which in turn is responsible for part of the IFN-γ secretion. However, production of IL-12 during viral infection does not seem to be a general feature. Experimental murine infection with LCMV and infection of human blood cells with influenza A virus were not associated with secretion of IL-12 (Orange & Biron, 1996a; Sareneva et al., 1998). Furthermore, IL-12 produced endogenously during virus infections has been shown not to be essential for the late IFN-γ production by T cells (Monteiro et al., 1998; Orange & Biron, 1996a). This indicates that other factors like IL-18 and IFN-γ/β are involved in regulation of IFN-γ production (Sareneva et al., 1998; Cousens et al., 1997, 1999). The ability of IL-18 to synergize with both IL-12 and IFN-γ/β for induction of IFN-γ secretion (Sareneva et al., 1998) supports the idea of redundancy between these cytokines in IFN-γ induction.

The skilful technical assistance of Ms Birthe Søby, Ms Elin Jacobsen and Ms Maria Moussavi is greatly acknowledged. This study was supported by grants from the Danish Health Science Research Council (grant number 12-1622) and the Aarhus University Research Foundation (grant number F-2000-Sun-1-89).

References


Received 15 June 2000; Accepted 31 August 2000