Virus-Cell Interactions Regulating Induction of Tumor Necrosis Factor Alpha Production in Macrophages Infected with Herpes Simplex Virus

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Received 28 March 2001/Accepted 30 July 2001

Macrophages respond to virus infections by rapidly secreting proinflammatory cytokines, which play an important role in the first line of defense. Tumor necrosis factor alpha (TNF-α) is one of the major macrophage-produced cytokines. In this study we have investigated the virus-cell interactions responsible for induction of TNF-α expression in herpes simplex virus (HSV)-infected macrophages. Both HSV type 1 (HSV-1) and HSV-2 induced TNF-α expression in macrophages activated with gamma interferon (IFN-γ). This induction was to some extent sensitive to UV treatment of the virus. Virus particles unable to enter the cells displayed reduced capacity to stimulate TNF-α expression but retained a significant portion which was abolished by HSV-specific antibodies. Recombinant HSV-1 glycoprotein D was able to trigger TNF-α secretion in concert with IFN-γ. Sugar moieties of HSV glycoproteins have been reported to be involved in induction of IFN-α but did not contribute to TNF-α expression in macrophages. Moreover, the entry-dependent portion of the TNF-α induction was investigated with HSV-1 mutants and found to be independent of the tegument proteins VP16 and UL13 and partly dependent on nuclear translocation of the viral DNA. Finally, we found that macrophages expressing an inactive mutant of the double-stranded RNA (dsRNA)-activated protein kinase (PKR) produced less TNF-α in response to infectious HSV infection than the empty-vector control cell line but displayed the same responsiveness to UV-inactivated virus. These results indicate that HSV induces TNF-α expression in macrophages through mechanisms involving (i) viral glycoproteins, (ii) early postentry events occurring prior to nuclear translocation of viral DNA, and (iii) viral dsRNA-PKR.

Herpes simplex virus (HSV) is an enveloped DNA virus that infects through contact with mucosal membranes. Following the initial HSV replication in epithelial cells, the virus is transported to the ganglia where a latent infection is established (31). A primary HSV infection is normally associated with a vigorous host response, including production of a range of cytokines and chemokines (26, 34, 36). Cytokines play an important role in the immune response to HSV infections. In particular, tumor necrosis factor alpha (TNF-α), which is primarily produced by macrophages, is known to be central for control of virus replication (13, 27). However, TNF-α and TNF-α-induced products are also involved in the immunopathology often associated with HSV infections (2, 10).

The virus-derived entities responsible for induction of TNF-α expression have been characterized for a number of viruses (reviewed in reference 22). For instance, the virion surface proteins gp350 and gp120 of Epstein-Barr virus and human immunodeficiency virus type 1 (HIV-1), respectively, stimulate expression of TNF-α (3, 8). Apart from surface glycoproteins other viral proteins primarily with an intracellular location such as the hepatitis B virus (HBV) protein X (HBx), the human T lymphotropic leukemia virus type 1 Tax protein and the HIV-1 Tat protein directly interact with the intracellular signaling machinery, thus leading to TNF-α expression (7, 15). Some viruses are endowed with several components able to stimulate production of specific cytokines. For instance, HIV-1 encodes four different proteins able to induce interleukin 6 (IL-6) expression (3, 24, 29, 33), and the two HBV proteins HBx and HBV core antigen both trigger expression of TNF-α (15, 37). Hence, induction of a specific cytokine by virus infection may involve several viral components. For HSV little is known about the viral factors that bring about cytokine production. One study has demonstrated that the glycoprotein D (gD), which is responsible for interaction with the herpes virus entry mediators A, B, and C, is capable of stimulating alpha interferon (IFN-α) expression (4). Others showed that infection of a permissive murine epithelial cell line with HSV-1 triggered IL-6 secretion, which was sensitive to UV inactivation of the virus (12). Studies from our laboratory have shown that the ability of HSV-2 to induce secretion of the IL-12/IL-23 subunit p40 in murine macrophages is also sensitive to UV and occurs through a mechanism involving the transcription factor nuclear factor κB (17).

In this study we have investigated the mechanisms of TNF-α induction by HSV in macrophages. We show that HSV-1 and HSV-2 induce modest production of TNF-α in resting macrophages and strongly stimulate TNF-α production in IFN-γ-treated macrophages. Our data suggest that the mechanisms involved include interaction of gD with a cellular receptor, early postentry events, and activation of the double-stranded RNA (dsRNA)-activated protein kinase by viral RNA.

MATERIALS AND METHODS

Reagents. The recombinant murine cytokines used were TNF-α (Genzyme) and IFN-γ (PharMingen). Recombinant HSV-1 gD and HSV-2 gG were ob-
FIG. 1. TNF-α production in resting and IFN-γ-treated macrophages infected with infectious and UV-inactivated HSV. (A) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with 3 × 10⁵ PFU (multiplicity of infection [MOI], 0.6) of HSV-2 per ml. After the indicated time points posttreatment, supernatants were harvested and analyzed for TNF-α bioactivity. ○, IFN-γ; ●, HSV-2; ▼, IFN-γ + HSV-2. (B) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with the following amounts of HSV-1 (KOS): 3 × 10⁵ PFU/ml (MOI, 0.006), 3 × 10⁶ PFU/ml (MOI, 0.06), 3 × 10⁷ PFU/ml (MOI, 0.6), and 3 × 10⁸ PFU/ml (MOI, 6). (C) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and 3 × 10⁵ PFU (MOI, 0.6) of the three HSV-1 strains HFEM, KOS, and 17+ per ml. (D) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with 3 × 10⁵ PFU (MOI, 0.6) of HSV-2 (black bars) or an equivalent amount of UV-inactivated virus (hatched bars) per ml. For panels B, C and D, supernatants were harvested 8 h postinfection and analyzed for TNF-α bioactivity. Results are shown as means of two cultures ± standard errors of the means. Similar results were obtained in at least three independent experiments.
FIG. 2. Effects of heparin and formaldehyde inactivation on viral replication and TNF-α production. (A) Confluent Vero cells were infected with increasing amounts of HSV-2 (1 × 10^5, 3 × 10^5, 1 × 10^6, and 3 × 10^6 PFU) in the presence and absence of 100 μg of heparin per ml. After 48 h of infection, the cells were stained and plaques were counted. (B) HSV-2 was inactivated by incubation with 3.5% formaldehyde for 24 h at 4°C or, as a control, treated with PBS. The virus was titrated on confluent Vero cells. After 48 h of infection, the cells were stained and plaques were counted. (C) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with 3 × 10^5 PFU (MOI, 0.6) of HSV-2 per ml in the presence and absence of 100 μg of heparin per ml. (D) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with 3 × 10^5 PFU (MOI, 0.6) of HSV-2 per ml or equal amounts of formaldehyde-inactivated virus. Supernatants were harvested 8 h later and analyzed for TNF-α bioactivity. Results are shown as means of two cultures ± standard errors of the means. Similar results were obtained in two independent experiments.

Western blotting. Virus particles and recombinant proteins were denatured in sample buffer (140 mM Tris-HCl [pH 8.5], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 1 mM EDTA, 0.01% Serva Blue G250, 0.06% phenol red) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by blotting onto a polyvinylidene difluoride membrane and blocking for 1 h in Tris-buffered saline (10 mM Tris, 140 mM NaCl) supplemented with 0.05% Tween 20 and 5% skim milk powder. The anti-gD antibody was added for overnight incubation at 4°C. The membrane was washed 4 × 10 min in washing buffer (Tris-buffered saline plus 0.05% Tween 20) and incubated for 1 h at room temperature with a polyclonal HRP-conjugated antibody against mouse immunoglobulin. The membrane was washed as above and the HRP-conjugated antibody was visualized using enhanced chemiluminescence.

Statistical analysis. The data shown are means ± standard errors of the means. Statistical significance was estimated with Student’s t test for unpaired observations. TNF-α concentrations from repeated experiments were pooled and used for the calculations in order to obtain the largest number of observations available for evaluation of statistical significance. P values of <0.05 were considered significant.

RESULTS

HSV-1 and HSV-2 induce secretion of TNF-α in IFN-γ-treated macrophages. We have previously shown that HSV-2 infection of macrophages induces secretion of TNF-α (9) and that IFN-γ synergistically enhances the response (26). The data in Fig. 1A confirm these findings and show that TNF-α production is detectable in the culture supernatants of the...
macrophage-like cell line RAW 264.7 already 2 h after infection and reaches a maximum after 8 h. Mock infection did not induce appreciable amounts of TNF-α in resting or IFN-γ-treated macrophages (data not shown). HSV-1 was also able to induce TNF-α expression in macrophages through cooperation with IFN-γ (Fig. 1B). Moreover, by using three HSV-1 strains with different degrees of virulence (HFEM < KOS < 17+) we found that the capacity to induce TNF-α did not correlate with virus virulence (Fig. 1C).

In separate experiments it has been observed that treatment of RAW 264.7 macrophages with HSV-2 does lead to viral entry and cellular accumulation of immediate-early, early, and late transcripts and proteins (J. Melchjorsen and S. R. Paldan, unpublished data). To assess the requirement for a functional viral genome in TNF-α induction, we tested the ability of UV-irradiated virus to induce TNF-α secretion. The results show that although such treatment significantly reduced the TNF-α-inducing capacity of the virus, UV irradiation was unable to abolish HSV-2-induced TNF-α production (Fig. 1D). In experiments not presented here we further found that induction of TNF-α by UV-inactivated virus followed the same kinetics as infectious HSV-2. Similar results were obtained in murine peritoneal cells with both HSV-1 and HSV-2 (data not shown). The remaining potential to bring about TNF-α secretion was not due to incomplete inactivation of the virus since UV-treated virus was unable to induce immediate-early viral gene transcription and to replicate in Vero cells (data not shown).

**Inhibition of viral entry reduces—but does not abolish—the ability of HSV to induce TNF-α secretion.** The initial step in HSV infections is the interaction between the viral glycoprotein gC and heparan sulfates on the cell surface. Consistently, free heparin has been shown to prevent viral entry presumably by engaging gC molecules on the virus particle (11). In agreement with this we observed that the presence of 100 μg of heparin per ml in the growth medium totally prevented 3 × 10⁵ PFU of HSV-2 from forming plaques in Vero cells (Fig. 2A). Thus, in the presence of saturating amounts of heparin, the interaction between gC and heparan sulfates cannot occur while other viral glycoproteins may still interact with cellular surface proteins. At the level of TNF-α production we found that although heparin reduced the ability of HSV-2 to induce TNF-α secretion by more than 70%, a significant proportion of the TNF-α-inducing potential was retained (Fig. 2C). As in the absence of heparin, a strong synergy between the virus and IFN-γ was observed. The remaining TNF-α-inducing potential of the virus could not be ascribed to nonspecific effects since it was totally inhibited by anti-HSV antibodies. In contrast to TNF-α, induction of interleukin 12 (IL-12) p40 and IL-6 by HSV-2 was fully inhibited by heparin (data not shown).

We also examined the TNF-α-inducing potential of formaldehyde-treated virus, which can bind to but not fuse with the cellular membrane. Although formaldehyde treatment completely inactivated the virus, as assessed by replication in Vero cells (Fig. 2B), it did not entirely prevent TNF-α production (Fig. 2D). Approximately 30% of the full TNF-α-inducing capacity was conserved, and this portion could be inhibited if the cells treated with the inactivated virus also received anti-HSV antibodies.

To analyze the requirement for viral entry in TNF-α induction more thoroughly, we used an HSV-1 mutant lacking the glycoprotein gL. This HSV glycoprotein, which forms heterodimers with gH, is essential for fusion of the virus envelope with the cellular membrane (30, 35). Thus, HSV particles deficient in gL are able to interact with cellular-surface heparan sulfates and proteins and to adhere to the cells but fail to fuse with the cellular membrane (32). We confirmed these previous observations using Vero cells (Fig. 3A). As to the ability of these viruses to influence TNF-α expression in RAW 264.7 cells, we found that gL86 virus grown in Vero cells (gL86/Vero) failed to induce appreciable amounts of TNF-α in resting or IFN-γ-containing macrophages (Fig. 3B). However, when the number of gL86 virus particles that could not be ascribed to nonspecific effects since it was totally inhibited by anti-HSV antibodies. In contrast to TNF-α, induction of interleukin 12 (IL-12) p40 and IL-6 by HSV-2 was fully inhibited by heparin (data not shown).

![FIG. 3. Replication and induction of TNF-α by gL-deficient HSV-1.](http://jvi.asm.org/)

(A) Confluent 79VB4 and Vero cells were infected with increasing amounts of gL-rescued gL86 (gL86/79VB4) and gL86 (gL86/Vero), respectively. After 48 h of infection, the cells were stained and plaques were counted. Antigen levels of the two virus preparations were compared by Western blotting using a specific gD-directed monoclonal antibody. (B) RAW 264.7 cells were treated with 100 IU of IFN-γ per ml and infected with 3 × 10⁵ PFU (MOI, 0.6) of gL86/79VB4 or equivalent amounts of gL86/Vero per ml. Supernatants were harvested 8 h later and analyzed for TNF-α bioactivity. Results are shown as means of duplicate cultures ± standard errors of the means. Similar results were obtained in three independent experiments.
Recombinant gD was present in both unglycosylated and vari-
and subjected to Western blotting. We found that whereas
in independent experiments.

The level of gD in the sample was estimated by comparison with the recombinant gD standard. Similar results were obtained in two
onto an SDS-PAGE together with increasing amounts of recombinant gD and subjected to Western blotting using a monoclonal antibody against
gD. The level of gD in the sample was estimated by comparison with the recombinant gD standard. Similar results were obtained in two
experiments.

Vero), and hence lacking gL, displayed reduced ability to in-
duce TNF-α production (Fig. 3B) compared to the virus grown in
gL-expressing Vero cells (gL86/79VB4). However, and in
parallel with the results shown in Fig. 2, inhibition of entry did not
totally abolish TNF-α production. The presence of anti-
HSV antibodies completely abolished induction of TNF-α by
gL86/Vero. Collectively these results show that viral entry is not
required for induction of TNF-α secretion by macrophages but also demonstrate that postentry events are required for
maximal TNF-α production.

Recombinant gD induces TNF-α secretion in macrophages. It has previously been shown that HSV-1 gD is able to trigger
secretion of IFN-α (4), and signaling cellular receptors for gD
are known (18, 23). Given the observation that HSV-1 and
HSV-2 can induce TNF-α production independent of entry, we
wanted to investigate how gD influenced TNF-α production.
To test this, RAW 264.7 cells were cultured in the presence
and absence of IFN-γ and recombinant gD and supernatants
were assayed for TNF-α bioactivity. As seen in Fig. 4A, gD
treatment alone led to a modest increase in TNF-α levels but
significantly induced TNF-α secretion if given together with
IFN-γ. Three hundred nanograms per ml of gD was sufficient
to stimulate TNF-α production in IFN-γ-treated cells, and
maximal levels of TNF-α were reached with around 3 μg of gD
per ml (Fig. 4B). Another HSV glycoprotein, gG-2, did not
trigger TNF-α production in RAW 264.7 macrophage-like
cells. When examining the ability of recombinant gD to induce
TNF-α production in peritoneal macrophages, we observed
that these cells responded to the glycoprotein in a manner
similar to that of RAW 264.7 cells (data not shown).

To assess if the levels of recombinant gD required to induce
TNF-α secretion corresponded to the quantity present on 3 ×
10^5 PFU of HSV-1, this amount of virus was loaded onto an
SDS-PAGE together with known amounts of recombinant gD
and subjected to Western blotting. We found that whereas
recombinant gD was present in both unglycosylated and vari-
ous glycosylated forms, the gD in the virus particle appeared to
be uniformly glycosylated as assessed from the mobility in the
gel. Moreover, the experiment shows that 3 × 10^5 PFU of
HSV-1 contain approximately 0.5 μg of gD (Fig. 4C) and thus
shows that the amounts of recombinant gD capable of inducing
TNF-α are comparable to the levels present on 3 × 10^5 PFU
of HSV-1.

Induction of TNF-α by HSV is independent of glycosylation of the virus. Since previous work from other laboratories has
shown that HSV-1-induced IFN-α expression by dendritic cells is
inhibited by the presence of free sugars in the growth me-
dium and by neutralizing antibodies against the mannose re-
ceptor (20), we wanted to investigate if sugar moieties also
were involved in stimulation of TNF-α production in
macrophages. When 50 mM D-mannose was present in the growth
medium we did observe significant reduction of TNF-α pro-
duction (Fig. 5D) (P = 0.003), whereas the same amount of
N-acetylglucosamine did not affect the induction of TNF-α by
HSV (Fig. 5E). The free sugars did not affect viral replication
(Fig. 5A and B).

However, since mannose (at least in the doses used in our
study) was mildly toxic to the RAW 264.7 cells, we also
assessed the involvement of glycoprotein sugar moieties in
TNF-α induction by stripping the virus for sugars prior to
infection. Incubation of the virus with PNGase for 3 h in-
creased the mobility of gD on SDS-PAGE, showing that the
treatment did deglycosylate the virus (data not shown). How-
ever, the infectivity was unaltered, as was the TNF-α-inducing
potential (Fig. 5C and F). These results indicate that HSV
infection of macrophages induces TNF-α expression indepen-
dently of the glycosylation status of the virus.

Induction of TNF-α is independent of the transactivating
potential of VP16 and the viral protein kinase UL13 and partly
dependent on nuclear translocation of the HSV genome. Since
full induction of TNF-α secretion requires viral entry we
wanted to investigate whether the virion-associated transcrip-
tion factor VP16, either directly or indirectly via stimulation of immediate-early gene expression, was involved in activation of TNF-α expression. For this purpose we used the VP16 mutant in1814, which is unable to associate with cellular transcriptional coactivators (1). When RAW 264.7 cells were infected with this virus or the VP16-rescued virus (in1814R) we found an unaltered ability to induce TNF-α (Fig. 6A). In fact, we observed that although in1814R was severalfold more infectious than in1814, the TNF-α-inducing capacity correlated with virus quantity (gD levels) rather than the plaque-forming potential (data not shown). Likewise, when examining the ability of the UL13 mutant R7356 to induce TNF-α, we also found no defect relative to the wild-type virus (Fig. 6B).

To further characterize the part of the TNF-α-inducing signal that was dependent on viral entry, we used the HSV-1 mutant tsB7, which is unable to release DNA into the nucleus at 39.5°C (6). This allowed us to dissect the involvement of specific events occurring after viral entry. We found that wild-type HFEM was capable of inducing TNF-α expression at 39.5°C (Fig. 6C). Interestingly, although the tsB7 mutant also induced TNF-α expression, this occurred at significantly lower levels than those observed for the wild-type virus (P = 0.001). At 37°C the abilities of the two viruses to induce TNF-α expression were indistinguishable (data not shown). Thus, VP16 and UL13 are not central players in HSV-induced TNF-α secretion in macrophages, which involves virus-cell interactions occurring both before and after translocation of the viral genome to the nucleus.

The dsRNA-activated protein kinase PKR participates in TNF-α expression in HSV-infected macrophages. Given the partial sensitivity of TNF-α induction by HSV to UV irradiation of the virus we wanted to investigate if the dsRNA-activated protein kinase (PKR) played a role in TNF-α induction by HSV. Since accumulation of viral mRNA during virus replication is sensitive to UV irradiation and PKR is a potent stimulator of proinflammatory signaling, we speculated that this kinase could potentially contribute to the cellular signaling machinery leading to TNF-α expression. To test this we used a previously described RAW-derived cell line (16) overexpressing a PKR mutant (M7) lacking the first RNA-binding domain of the protein. As a control we used a cell line (RAW-pBKCMV) stably transfected with the empty pBK vector. This cell line responded to HSV-1 and HSV-2 infection alone with a remarkably high production of TNF-α, which was enhanced by cotreatment with IFN-γ (Fig. 7A and data not shown). RAW-PKR-M7 was also capable of producing TNF-α after HSV-1 and HSV-2 infection but at significantly lower levels (P = 0.01).
and infected with 3/H11003 and 3/H9253 treated with 100 IU of IFN-
R7356 or wild-type F strain per ml. (C) Cells preheated to 39.5
°C. (A to C) After 8 h of infection, supernatants were
incubated at 39.5°C. (A) Cells were treated with 100 IU of IFN-
/H9253 HSV-1 mutants with defects in VP16 and UL13 or nuclear transloca-
tion of viral DNA. (A) Cells were treated with 100 IU of IFN-
/H9253 in 1814 or the rescued virus in 1814R per ml. (B) The cells were treated
with IFN-γ as above and infected with 3 × 10^5 PFU (MOI, 0.6) of
R7356 or wild-type F strain per ml. (C) Cells preheated to 39.5°C were
infected with 3 × 10^5 PFU (MOI, 0.6) of tsB7 or wild-type HFEM per ml. The cells were further
incubated at 39.5°C. (A to C) After 8 h of infection, supernatants were
harvested and analyzed for TNF-α bioactivity. Results are shown as
means of duplicate cultures ± standard errors of the means. Similar
results were obtained in three independent experiments. *, P < 0.05.

To assess if the role of PKR was dependent on viral dsRNA
we tested how the cell lines responded to UV-inactivated virus.
Interestingly, unlike what was observed after treatment of the
cells with infectious virus, UV-inactivated virus resulted in
comparable levels of TNF-α production in the two cell lines
(Fig. 7B). Finally, we wanted to examine if in fact postentry
events other than PKR contributed to TNF-α production. To
do this we used the PKR mutant cell line used in experiments
illustrated in Fig. 7A and B and the above-described viruses
gL86/Vero and gL86/79VB4 (Fig. 3). As seen from Fig. 7C,
gL86/79VB4 remained a significantly more potent inducer of
TNF-α than was gL86/Vero (P = 0.006) despite lack of a
functional PKR system in the RAW-PKR-M7 cell line. Thus,
these results suggest that signaling through viral dsRNA-acti-
ated PKR plays a role in HSV-induced TNF-α expression in
macrophages and that entry-dependent events other than PKR
are involved.

**DISCUSSION**

Production of cytokines is an essential part of the early
response to viral infection. This induces an antiviral state in
infected cells and recruits cells of the immune system to the
site of infection. Macrophages play an essential role in the
natural immune response to many viruses and among these
HSV (13, 21). One of the macrophage-derived products that
contribute to inhibition of HSV replication is the cytokine
TNF-α (13, 27), which induces a number of antiviral effector
mechanisms, notably production of nitric oxide (5, 26). In this
study we have investigated the virus-cell interactions that trigger
production of TNF-α in murine macrophages.

The ability of HSV infection to stimulate TNF-α expression
to any major extent in RAW 264.7 macrophage-like cells was
dependent on the presence of IFN-γ. Similar findings have
previously been done in another murine macrophage-like cell
line (J774A.1) and in murine peritoneal cells (26). Since IFN-γ
is known to be produced early during HSV infections by NK
cells (19, 25), this observation emphasizes the importance of
interplay between different cells of the immune system in order
to bring about a robust host response.

For some viruses it has been shown that more than one
mechanism is involved in cytokine induction. HBV is able to
stimulate TNF-α production through both HBx and the HBV
core antigen (15, 37). Likewise, several HIV-1 proteins pro-
mine IL-6 expression (3, 24, 29, 33). We found that the ability
of HSV to induce TNF-α expression is dependent on entry-
dependent events and at least two different postentry events:
one independent of release of viral DNA into the nucleus and
one dependent on PKR and a functional viral genome. These
results are compatible with the model shown in Fig. 8.

The entry-independent TNF-α induction could be mimicked
by recombinant gD. It is possible that the viral glycoprotein
interacts with one of the cellular herpes virus entry mediators,
which in turn mediates intracellular signaling. In this respect it
is interesting that the herpesvirus entry mediator A is a mem-
ber of the TNF receptor superfamily and is known to activate
some of the transcription factors involved in regulation of
TNF-α transcription (18). However, it is also possible that gD
induces TNF-α expression through binding to receptors other
than the entry mediators. In fact, it has been reported that the
ability of gD to stimulate IFN-α secretion can be inhibited with
antibodies directed against the chemokine receptors CCR3
and CXCR4 (4). The concept of immune stimulation by viral
surface proteins is well described in the literature. To illustrate,
gp350 of Epstein-Barr virus and gp120 of HIV-1 stimulate
expression of TNF-α (3, 8), and it was recently reported that the fusion protein of respiratory syncytial virus is recognized by toll-like receptor 4 and CD14 (14).

Concerning the postentry events involved in TNF-α induction, we found that both UV-sensitive and -insensitive mechanisms are involved. The existence of the entry-dependent UV-insensitive response was documented primarily by the observations (i) that the gL-deficient virus particles (gL86/Vero) induced lower levels of TNF-α than did the gL-containing virions (gL86/79VB4) in RAW-PKR-M7 cells, which lack dsRNA-responsive PKR; (ii) that UV-irradiated virus induced comparable amounts of TNF-α in RAW-PKR-M7 and RAW-pBK-CMV cells; and (iii) that tsB7 was a less potent inducer of TNF-α than HFEM at the nonpermissive temperature, under which condition the mutant is unable to release its DNA into the nuclei of infected cells. The exact nature of the virus-cell interaction responsible for this response is not known but is independent of VP16 and UL13. Potential candidates include fusion of the viral envelope with the cell membrane, tegument proteins other than VP16 and UL13, changes in the cytoskeleton caused by transport of the capsid to the nuclear pores, or sensing by the cell of HSV capsids.

The entry-dependent signal sensitive to UV irradiation of virus involved PKR. This conclusion is based on the finding that the PKR mutant cell line RAW-PKR-M7 produced lower amounts of TNF-α than did the control cell line RAW-pBK-CMV in response to infectious virus but similar levels after treatment with UV-inactivated virus. Since PKR is activated by dsRNA (38) and UV irradiation of virus inhibits transcription from the viral genome, these results suggest that this signal is comprised of PKR activated by HSV mRNA produced during the viral life cycle.

In summary, we have shown that the HSV glycoprotein gD is able to induce secretion of TNF-α in macrophages and that postentry events are required for full induction. Our results suggest that macrophages can react to HSV with three levels of TNF-α secretion: (a) a low response that is induced when the...
macrophage detects the virus on the cell surface, (b) a stronger response that is initiated when the virus has entered the cell, and (c) full induction when the infecting virus goes through its replication cycle.

ACKNOWLEDGMENTS

We thank Patricia G. Spear for the gL86 HSV-1 mutant and the 79VB4 cell line, Chris M. Preston for the viruses in1814 and in1814R, and Bernard Roizman for the mutant viruses tsB7 and R7356. We are also indebted to Sytske Wessling-Wester for providing the recombinant HSV-2 gG. The generous donation of the cell lines RAW-pBK-CMV and RAW-pBK-CMV-VP6 by John Aruffo and greatly appreciated. The skillful technical help provided by Birthe Søby and Elin Jakobsen has been invaluable.

This work was supported by grants from the Danish Health Science Research Council (grant number 12-1622), Fonden til Lægevidenskabens Fremme (grant number 00182), and The Leo Research Foundation.

REFERENCES