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Human Gamma Interferon and Tumor Necrosis Factor Exert a Synergistic Blockade on the Replication of Herpes Simplex Virus

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The replication of herpes simplex virus type 1 (HSV-1) is not inhibited in either HeLa or HEp-2 cells treated with human alpha interferon (HuIFN-α), particularly when high multiplicities of infection are used. However, HuIFN-γ partially inhibits HSV-1 translation in HEp-2 cells infected at low multiplicities. Under these conditions, the transcription of genes α22, TK, and γ0 is greatly diminished. The combined addition of human tumor necrosis factor (TNF) and HuIFN-γ to HEp-2 cells exerts a synergistic inhibition of HSV-1 translation. Cells treated with both cytokines continue synthesizing cellular proteins, even 20 h after HSV-1 infection. As little as 10 U of IFN-γ per ml blocked HSV-1 DNA replication, provided that TNF was also present in the medium. Analyses of HSV-1 gene transcription suggest that the action of both TNF and IFN-γ blocked a step that comes at or prior to early HSV-1 gene expression. This early step in HSV-1 replication inhibited by TNF and IFN-γ occurs after virus attachment and entry into cells, since the internalization of radioactive HSV-1 virion particles was not blocked by the presence of the two cytokines. Therefore, we conclude that the synergistic action of TNF plus IFN-γ affects a step in HSV-1 replication that comes after virus entry but before or at the transcription of immediate-early genes.

The response of animal viruses to the inhibitory effects of interferon (IFN) greatly depends on a number of variables. The IFN species used to block virus replication is an important factor, since some viruses, adenoviruses for example, are inhibited by IFN-γ, but not by IFN-α (14). The virus-cell system is another factor of interest, because not all animal viruses studied are equally sensitive to IFN (11, 19). In general, viruses containing RNA molecules as the genome are more prone to be inhibited by IFN than DNA-containing viruses (11, 19). The replication of vesicular stomatitis virus or poliovirus is very sensitive to all IFN species assayed in the different cell types used (11, 19). On the other hand, the translation of reovirus type 3 is not inhibited by human alpha interferon (HuIFN-α) in HeLa cells, but it is blocked by murine alpha-beta interferon (MuIFN-α-β) in mouse cells (9, 18). The resistance of reovirus replication to IFN is not due to reversion of the antiviral state (9). This behavior contrasts with that of most DNA-containing animal viruses studied (8, 22). Vaccinia virus and adenoviruses owe their resistance to IFN to the fact that they are able to revert the antiviral state established in cells treated with IFN (8, 22). For herpesviruses the situation is more complex, and the response to IFN largely depends on the system under study. Herpes simplex virus type 1 (HSV-1) replicates well in HeLa cells treated with HuIFN-α; neither DNA replication nor viral protein synthesis is inhibited in these cells (4, 17), but the virions made in cells treated with IFN-α are unable to replicate (17). Chatterjee et al. (4) found a reduction in the amount of glycoproteins D and B of HSV-1 and a drastic inhibition of the release of mature extracellular particles. More recently, it was found that the transcription of HSV-1 early genes was inhibited in HeLa cells treated with very high doses (4,000 U/ml) of HuIFN-αA and infected at low multiplicity (20). The use of another cell line, Chang cells, shows that high doses (100 to 1,000 U/ml) of HuIFN-α or HuIFN-β do block the growth of HSV-1. Consequently, no viral proteins appear late in infection when Chang cells are treated with 300 or 1,000 U of HuIFN-β per ml (3). HSV-1 replication is sensitive to IFN in mouse and human macrophages (5, 6, 15, 25). Thus, primary cultures of splenic mouse macrophages treated with MuIFN-α-β do not express immediate-early genes of HSV-1 (15). Treatment of macrophages with IFN-γ in combination with IFN-α or IFN-β resulted in a synergistic inhibition of HSV-1 growth (5). Therefore, the behavior of HSV-1 in human or mouse cells treated with a variety of IFN preparations is different.

The search for different cytokine combinations which are more effective in blocking HSV-1 replication in human cells may lead to an improvement of the available chemotherapy against this important human pathogen. In this regard, a new cytokine known as tumor necrosis factor (TNF) has shown some promise, used either alone or in combination with different IFNs against a number of animal viruses (13, 30). TNF is a cytokine with pleiotropic effects (1, 21). TNF is a 17,000-dalton polypeptide synthesized by macrophages in response to several agents such as lipopolysaccharide or tubercle bacilli (1, 21). This protein is best known for its antitumor effects in vivo and for its in vitro cytotoxicity to a number of transformed cell lines (1, 21). In addition, TNF also stimulates the growth of normal fibroblasts (26, 28), inhibits the synthesis of lipoprotein lipase (1), and activates polymorphonuclear leukocytes (23). Recently, an antiviral activity of TNF was described (13, 30). TNF alone shows an antiviral effect in several cell lines against a number of animal viruses, such as vesicular stomatitis virus, encephalomyocarditis virus, adenovirus type 2, or HSV-2 (13, 30). More interestingly, the antiviral effects of TNF are enhanced synergistically by IFN-γ (30). These results prompted an investigation to clarify the action of TNF and IFN-γ against HSV-1 in human fibroblasts, and the results are presented here.

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MATERIALS AND METHODS

Cells and viruses. HEp-2 and Vero cells were grown in petri dishes (Nunc, Roskilde, Denmark) containing 10 ml of Dulbecco modified Eagle medium (E4D) supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.) and incubated at 37°C in a 5% CO_2 atmosphere.

HSV-1 was grown on Vero cells in E4D medium supplemented with 2% calf serum (E4D2). The fraction obtained after removal of cell debris by low-speed centrifugation was used as the source of virus. Virus preparations were titrated by plaque assay.

Plasmids. The 2-kilobase (kb) *Pst*I (α22 gene), 2.4-kb *Eco*RI (TK gene), and 3-kb *Hin*dIII (γ0 gene) restriction fragments obtained from HSV-1 genome digestion and cloned in pBR322, pACYC184, and pACYC177, respectively, were generously provided by E. Tabarés (Madrid, Spain).

IFNs and TNF. Human lymphoblastoid interferon, HuIFN-(Ly)-α (1.7×10^6 U/ml), was a generous gift from N. Finter (Wellcome Research Laboratories, Beckenham, England). Recombinant human gamma interferon, rHuIFN-γ (2×10^7 U/ml), and recombinant human tumor necrosis factor, rHuTNF-α (6×10^7 U/mg), were generously provided by G. R. Adolf (Ernst-Boehringer-Institut für Arzneimittel-Forschung). Human fibroblast interferon, HuIFN-β (5×10^4 U/ml), was from Lee Biomolecular Research Laboratories (San Diego, Calif.).

Conditions of infection. HEp-2 cells, grown in 96-well, 60- or 100-mm-diameter dishes, were infected with HSV-1 at the multiplicity of infection (MOI) indicated in each experiment in the Results section. After 1 h of incubation at 37°C, the medium was removed, and E4D2 was added. The time of virus addition was considered −1 h, and zero time was taken to be the time when the virus was removed. IFN and TNF treatment was done 20 to 24 h before infection at the concentration described in the figure legends, in E4D2. Medium with IFN and TNF was removed, and the infection was done as described above.

Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. HEp-2 cells grown in 96-well dishes were incubated with 50 μl of methionine-free medium and 1.25 μCi of [^{35}S]methionine (1.45 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 1 or 2 h at the time after infection indicated in each experiment. Cell monolayers were dissolved in 50 μl of sample buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue). Samples were heated at 90°C for 5 min, and 10 μl was applied to a 15% polyacrylamide gel and run overnight at 100 V/20 cm. Fluorography was carried out in 20% (wt/vol) 2,5-diphenyloxazole in dimethyl sulfoxide. The gels were finally dried and exposed with Kodak films (10).

RNA isolation and analysis. HEp-2 cells were grown in 100-mm dishes and infected at 5 PFU per cell. At the times indicated, cells were lysed in a buffer containing 10 mM Tris hydrochloride (pH 7.8), 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40. After nuclei were removed by centrifugation at $2,000 \times g$ for 5 min, supernatants were mixed with an equal volume of buffer containing 20 mM Tris hydrochloride (pH 7.8), 350 mM NaCl, 20 mM EDTA, and 1% sodium dodecyl sulfate. Samples were extracted three times with a mixture of phenol and chloroform (24:1, vol/vol), and the RNA was precipitated with ethanol (7, 12). Dot blot analysis with ^{32}P-labeled nick-translated HSV-1 TK DNA, α22 DNA, and γ0 DNA was performed as previously described (27).

DNA isolation and analysis. HEp-2 cells were grown in 60-mm dishes and infected with HSV-1 at 30 PFU/ml. At 16 h postinfection (p.i.), cells were collected, and DNA was isolated essentially as described previously (2). DNA was immobilized onto nitrocellulose membranes with a microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.) and subjected to hybridization to nick-translated TK DNA.

Preparation of radiolabeled viral particles. HEp-2 cells grown in 100-mm dishes and infected with 10 PFU of HSV-1 per cell were labeled with 50 μCi of [^{35}S]methionine per ml from 10 to 24 h p.i. Cells were scraped, and the fraction obtained after removal of cell debris was centrifuged over a 30% dextran cushion at 40,000 rpm for 3 h at 4°C in a 65 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellet was suspended in Dulbecco modified Eagle medium and used as radiolabeled virions to measure virus entry.

Virus entry. The entry of virus into cells was measured by estimating the trichloroacetic acid-precipitable radioactivity in the cell monolayer at different times after infection with radiolabeled virus (see above) at 37°C. The values obtained at 4°C were taken as a measure of virus adsorption. At the indicated times, the medium was removed and cell monolayers on 24-well dishes were washed with phosphate-buffered saline–Dulbecco modified Eagle medium and 0.0025% trypsin–0.08% EDTA in Dulbecco modified Eagle medium, precipitated with 5% trichloroacetic acid, washed with ethanol, solubilized with 0.1% NaOH–1% sodium dodecyl sulfate, and counted in an Intertechnique scintillation spectrometer.

RESULTS

Effects of different IFNs on HSV-1 replication. Previous experiments indicated that HSV-1 was able to replicate and synthesize proteins in human fibroblasts (HeLa cells) treated with IFN-α (4, 17). To analyze the sensitivity of HSV-1 replication to HuIFN-α, HuIFN-β, and HuIFN-γ, HEp-2 cells were treated with a high concentration (400 U/ml) of different human IFNs and were infected with HSV-1 at several multiplicities. As found in previous studies, the synthesis of HSV-1 proteins is very resistant to treatment with HuIFN-α (Fig. 1). This phenomenon is dependent upon the MOI used. When cells are infected at low MOI, the expression of some viral proteins is blocked. The increase in MOI overcomes the inhibition of HSV-1 protein synthesis in cells treated with IFN-α. It is surprising to find that IFN-β behaves as IFN-γ and that both IFNs show a higher activity against HSV-1 than does IFN-α. At low MOI most of the HSV-1 proteins are not evident when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas at high MOI, viral protein synthesis is similar to that of control cells not treated with IFN. These results reinforce the idea that HSV-1 replication is quite resistant to IFN, particularly to IFN-α, as far as translation is concerned.

To compare the effect of IFN-α and IFN-γ on the transcription of HSV-1 genes, the experiment shown in Fig. 2B was conducted. Total RNA from infected cells treated or not treated with IFN was extracted at 4 and 8 h p.i. and hybridized with probes derived from α22, TK, and γ0 HSV-1 genes (29). Inhibitors of protein synthesis such as cycloheximide block the expression of β and γ genes but not the transcription of the α class (Fig. 2A). On the other hand, inhibitors of viral DNA replication such as phosphonoformate have an inhibitory effect on γ gene expression, but they do not block the transcription of α and β genes. HSV-1 expresses the γ0 and TK genes at control levels in HEp-2

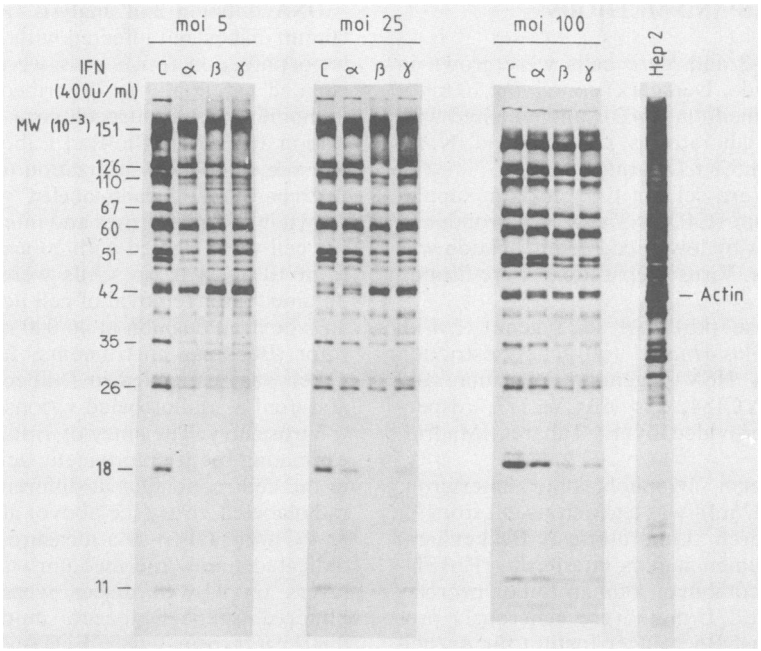

FIG. 1. Effect of IFNs on protein synthesis in HEp-2 cells infected with HSV-1. Cells grown in 96-well dishes were treated with IFNs 20 h before infection at the MOI indicated. Metabolic labeling was carried out for 2 h at 15 h p.i. with [^{35}S]methionine, and proteins were analyzed as described in Materials and Methods. C, Control without IFN. The numbers indicate molecular size in kilodaltons.

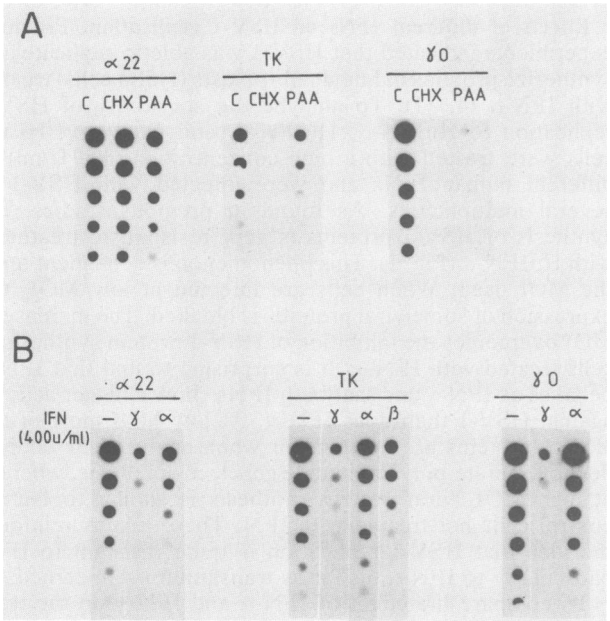

FIG. 2. Effects of different IFNs on the transcription of different HSV-1 genes. Cells were infected with 5 PFU of HSV-1 per cell, and mRNA was extracted at 4 h p.i. for the α22 gene blots and at 8 h p.i. for the TK and γ0 gene blots. (A) Effect of cycloheximide (CHX) (5×10^{-5} M) and phosphonoacetic acid (PAA) (250 μg/ml) on transcription of HSV-1 immediately-early (α22), early (TK), and late (γ0) genes. (B) Effect of IFNs on transcription of HSV-1 genes. Cells grown in 100-mm dishes were treated with IFN 24 h before infection. C, Control; –, control without treatment.

cells treated with 400 U of HuIFN-α per ml and infected at low MOI of HSV-1 (Fig. 2B). The synthesis of the α22 gene, however, is partially inhibited by HuIFN-α, suggesting that under these conditions IFN-α also has some inhibitory effect on the expression of early genes. The sensitivity of HSV-1 gene expression to IFN-γ is clearly apparent when the transcription of the three genes is analyzed (Fig. 2). An inhibition of more than eight times in the expression of α22 was observed, and consequently, the expression of the TK and γ0 genes was also blocked. These results suggest that the replication of HSV-1 in human fibroblasts can be partially inhibited by IFN-γ when a low MOI is used. Therefore, HSV-1 in this system somehow resembles the situation in mouse macrophages treated with MuIFN-β. These experiments clearly reinforce the idea that the final outcome of HSV-1 replication in a given cell type depends not only on the species of IFN used but also on other variables, such as the concentration of IFN, the MOI of the experiment, and so on (3, 11, 14, 19).

Synergistic effect of TNF and IFN-γ against HSV-1 replication. The combined addition of human TNF and HuIFN-γ leads to a synergistic inhibition of HSV-1 replication (1, 30). Treatment of HEp-2 cells with TNF alone, even at concentrations of 100 ng/ml, had no effect on HSV-1 protein synthesis (Fig. 3). In agreement with the results shown in Fig. 1 and 2, neither IFN-α nor IFN-γ at low concentrations (10 U/ml) prevented the appearance of HSV-1 late proteins. Strikingly, the combination of TNF and IFN-γ fully suppressed the synthesis of HSV-1 proteins (Fig. 3), indicating a synergistic action. This effect was not observed when IFN-α was added, further suggesting that the activities of IFN-α and IFN-γ against DNA-containing viruses are not similar. Since the MOI used was an important factor determining the resistance or sensitivity of HSV-1 late-protein synthesis to IFN-γ, we analyzed the effect of this factor on HSV-1 translation (Fig. 4). Again, HSV-1 proteins were

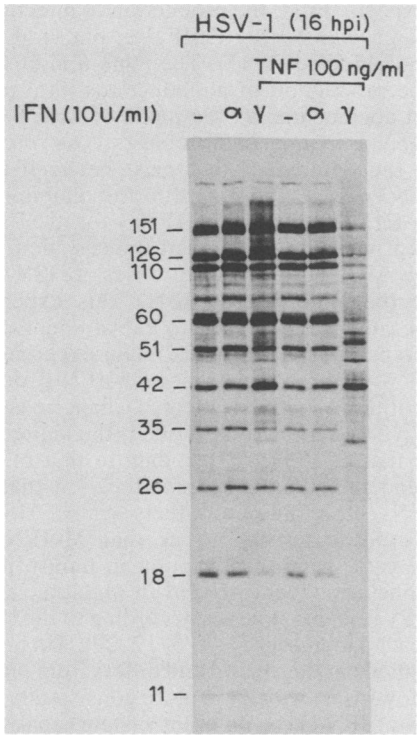

FIG. 3. Effect of IFN-α, IFN-γ, and TNF treatment on protein synthesis in HEp-2 cells infected with HSV-1. Cells were grown in 96-well dishes and treated with TNF (100 ng/ml), IFN-α (10 U/ml), and IFN-γ (10 U/ml) 24 h before infection with 25 PFU per cell. Cells were labeled at 16 to 18 h p.i. with [^{35}P]methionine, and proteins were analyzed as described in Materials and Methods. −, Control without teatment. The numbers indicate molecular size in kilodaltons.

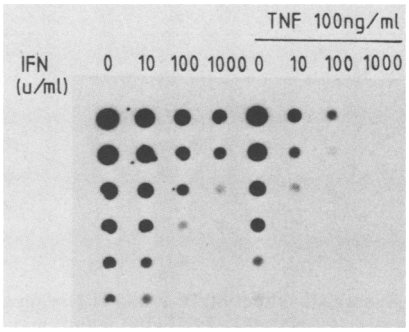

FIG. 5. Synergistic effect of IFN-γ and TNF on the replication of viral DNA. Cells were grown in 60-mm dishes, treated with TNF and IFN-γ at the indicated concentrations for 24 h, and infected with HSV-1 at an MOI of 25 PFU per cell. At 16 h p.i., total DNA was extracted and subjected to hybridization to a nick-translated *Eco*RI TK fragment.

more sensitive to IFN-γ and TNF when a low MOI was employed. However, there was still a significant inhibition in the expression of several HSV-1 proteins, even when high MOIs of HSV-1 were analyzed.

To analyze the effect of TNF and IFN-γ on viral DNA replication, total DNA from infected cells was extracted 16 h after infection. The amount of HSV-1 DNA was quantitated by hybridization with a TK probe. Figure 5 shows that 10 U of IFN-γ per ml alone did not diminish the amount of HSV-1 DNA present in infected cells. However, 100 and 1,000 U of IFN-γ per ml inhibited replication of the HSV-1 genome four- and eightfold, respectively. Although by itself TNF is not inhibitory to HSV-1 DNA replication, the combination of TNF and IFN-γ clearly suppresses viral DNA replication. These results agree well with the findings obtained on viral protein synthesis and suggest that the step blocked by TNF plus IFN-γ is prior to HSV-1 DNA replication.

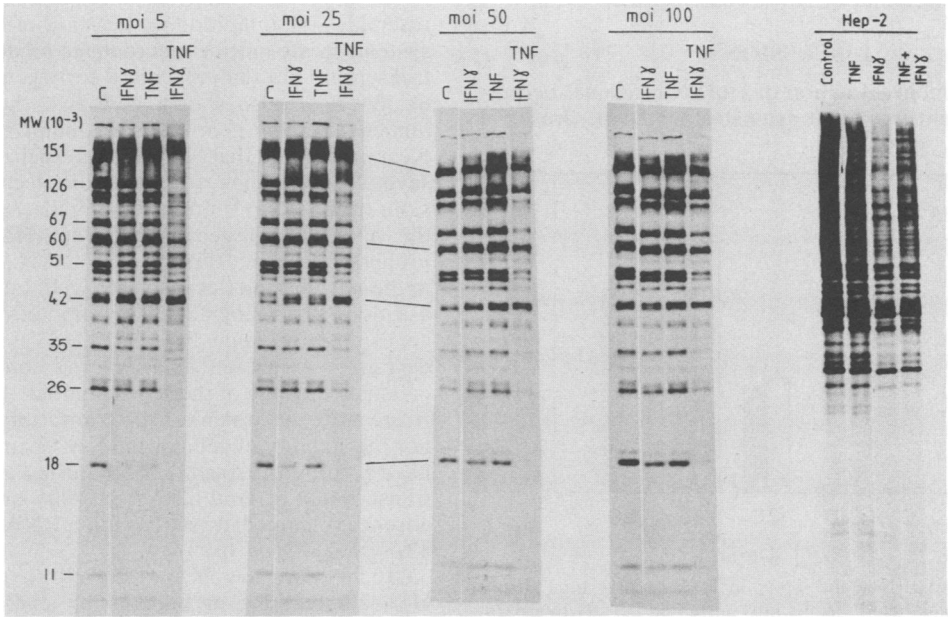

FIG. 4. Synergistic effect of IFN-γ and TNF treatment on protein synthesis in HEp-2 cells infected with HSV-1. Cells were grown in 96-well dishes and treated with TNF (100 ng/ml) and IFN-γ (10 U/ml) for 24 h before infection. Cells were labeled for 2 h with [^{35}S]methionine, and proteins were analyzed as described in Materials and Methods. C, Control. The numbers indicate molecular size in kilodaltons.

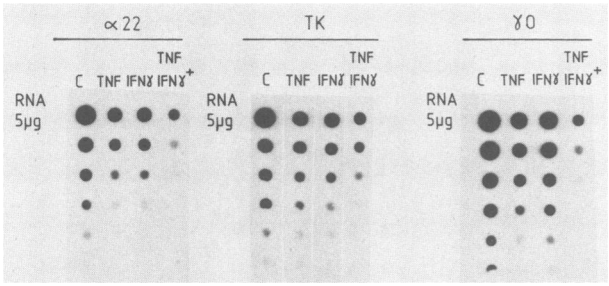

FIG. 6. Synergistic effect of TNF and IFN-γ on transcription of HSV-1 genes in HEp-2 cells. TNF (100 ng/ml) and IFN (10 U/ml) were added 24 h before infection. Conditions of infection, RNA extraction, and hybridization were as described in Fig. 2. C, Control.

The transcription of HSV-1 genes α22, TK, and γ0 was assayed as described above. TNF or IFN-γ used separately had negligible effects on the transcription of early genes α22 (α class) and TK (β class), although both of them when used alone clearly induced some inhibition (Fig. 6). However, the combination of TNF plus IFN-γ prevented the expression of early (α22 and TK) and late (γ0) genes. The conclusion from these results is that TNF plus IFN-γ blocks an early step in HSV-1 replication that comes either at or prior to the transcription of early genes.

To determine whether very early steps in HSV-1 replication, such as HSV-1 binding and entry into HeLa cells, were affected by TNF and IFN-γ, HSV-1 virions were labeled with [^{35}S]methionine, and the entry of these virions into cells was monitored at different times. HSV-1 entered control cells and HEp-2 cells treated with TNF and IFN with similar kinetics and to a similar extent (Fig. 7). The amounts of viral particles adsorbed to uninfected cells at 0°C with and without treatment with TNF and IFN-γ were also similar (results not shown). These results lead us to suggest that HSV-1 is internalized in cells treated with the two cytokines and that the step blocked by them occurs after entry and before or at the level of transcription of HSV-1 early genes.

DISCUSSION

The antiviral mode of action of HuIFN-α against herpesviruses is different from that against other DNA viruses in several respects. First, the production of infectious particles is significantly blocked by HuIFN-α, but viral protein synthesis is not affected (4, 17). The inhibition of virus release (4) and the production of noninfectious viral particles (17) have been documented as the mode of action of HuIFN-α against HSV-1 in human fibroblasts. Our present results point out the differences that exist between the different human IFN species with regard to the inhibitory effects on HSV-1 in HEp-2 cells. Thus, IFN-γ by itself shows some inhibition of viral translation and transcription, particularly when a low MOI is used. Higher doses of HSV-1 are able to overcome the inhibition by IFN. This experiment is of interest because it might help to explain conflicting reports from different groups (4, 6, 15, 17). Our experiments are also consistent with the finding that high IFN-β doses prevent protein synthesis in HSV-1-infected Chang cells (3), because in the HEp-2 system here described, the action of IFN-β is similar to that of IFN-γ rather than to that of IFN-α. Our findings lend additional support to the view that human and mouse IFNs show different effects against HSV-1. Therefore, it is not surprising to us that MuIFN-α-β blocks immediate-early gene transcription in mouse macrophages (5). The conclusion that can be made about the action of IFN against HSV-1 is that it varies according to the system under study in each laboratory (3, 4, 6, 17, 20). Therefore, HSV-1 is rather unique in this regard and differs from picornaviruses and rhabdoviruses, which are more consistently inhibited by high doses of IFN in the different systems analyzed (11, 19). HSV-1 is also at variance with other DNA viruses, such as vaccinia, because it is blocked by the combined action of TNF and low doses of IFN-γ, whereas vaccinia virus translation continues to control levels in cells treated with both compounds (results not shown). Another interesting aspect of the present work is that TNF by itself has no inhibitory effects on HSV-1. This result does not support the idea that TNF stimulates the production of IFN-β_2 in our HEp-2 cells, unless we assume that IFN-β_2 plus TNF has no effect on HSV-1. Experiments are now in progress in our laboratory to clarify this point.

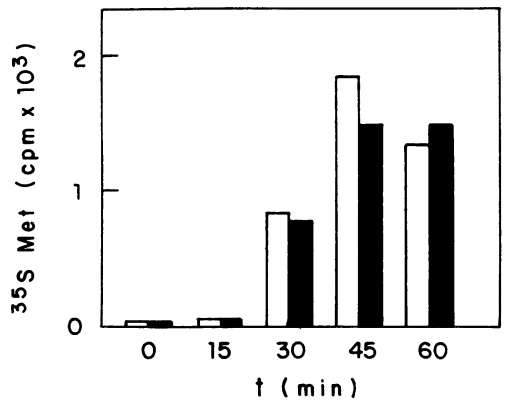

FIG. 7. Effect of IFN-γ and TNF on HSV-1 entry into HEp-2 cells. Entry of radiolabeled virions into untreated cells (white bars) or cells treated with 10 U of IFN-γ per ml and 100 ng of TNF per ml (black bars) was measured as described in Materials and Methods, at the time indicated.

The mode of action of TNF plus IFN-γ against HSV-1 resembles the inhibition by IFN-α-β of HSV-1 in the mouse system. In the murine macrophage model, a blockade in the transcription of the immediate-early genes was observed (5, 6). We have also shown here that the expression of the immediate-early gene α22 is diminished in our system. Keeping in mind that HSV-1 is internalized in cells, there are several possibilities to explain the decrease in α22 expression: (i) the HSV-1 virions enter cells, but they do not reach the nucleus; (ii) decapsidation of HSV-1 virions is not properly achieved; (iii) the HSV-1 DNA is properly located in the nucleus and decapsidates well, but the RNA polymerase does not recognize the promoters of immediate-early genes; and (iv) immediate-early HSV-1 mRNAs are rapidly degraded. At present, we have no convincing evidence to conclude any one of these possibilities. If possibilities iii and iv are true, since neither the transcription of cellular genes nor the stability of cellular mRNAs seems to be affected, we have to propose specific mechanisms acting on viral DNA transcription or viral mRNA stability or both to explain the absence of immediate-early viral mRNA in cells treated with TNF plus IFN-γ.

Much effort has been directed to control HSV-1 and HSV-2 infections in humans by chemotherapy. In fact, either IFN alone or in combination with antiherpes drugs has been described as a potential inhibitor of HSV infections (16, 24). The finding that TNF acts in synergy with HuIFN-γ

opens the possibility of using these two agents in combination against certain HSV-1 diseases.

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