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Antiviral Activity of Alpha Interferon in Sindbis Virus-Infected Cells Is Restored by Anti-E2 Monoclonal Antibody Treatment

PHILIPPE DESPRÈS,[1] JOHN W. GRIFFIN,[1] AND DIANE E. GRIFFIN[1,2,3]*

Departments of Neurology[1] and Medicine,[2] Johns Hopkins University School of Medicine, and Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Hygiene and Public Health,[3] Baltimore, Maryland 21205

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Pretreatment of AT3 rat prostatic carcinoma cells expressing the inhibitor of apoptosis *bcl-2* (AT3-*bcl-2* cells) with alpha interferon (IFN-α) affected replication of a virulent strain of Sindbis virus (SV) but did not protect against virus-induced cell death. Treatment of cells with IFN-α late during infection affected ongoing SV replication very little. Previous studies have shown that cross-linking of the viral glycoprotein E2 with antibody delays the inhibition of K^+ influx by improving the function of Na^+K^+ATPase and the Na^+-K^+-$2Cl^-$ cotransport system in SV-infected cells (P. Desprès, J. W. Griffin, and D. E. Griffin, J. Virol. 69:7006–7014, 1995). In these studies, we have shown that treatment of infected cells with anti-E2 monoclonal antibody also restored the ability of IFN-α to induce antiviral activity in infected cells late during infection. The very low rate of virus release in SV-infected cells treated simultaneously with anti-E2 monoclonal antibody and IFN-α was postulated to be linked to inhibition of virus maturation. Synergistic effects of antibody and IFN-α are likely to be important for control of SV replication in vivo.

Protection of mice from fatal encephalitis with Sindbis virus (SV) (family *Togaviridae*, genus *Alphavirus*) and clearance of infectious virus particles from the brains of mice with encephalitis can be accomplished by passive transfer of monoclonal antibodies (MAbs) to viral envelope proteins (6, 11, 12, 17, 25). Clearance occurs through a mechanism distinct from classical antibody-dependent cell-mediated cytotoxicity or complement-dependent lysis (6). Anti-E2 MAbs are able to control SV replication in neurons in vivo (11, 12, 17, 25) and in persistently infected cultures of rat dorsal root ganglion neurons in vitro (12).

Envelope glycoproteins E2 and E1 and the capsid protein C are the three major structural proteins in the mature virion (26). These structural proteins are synthesized from a subgenomic RNA as a large polyprotein in the order NH2-C-E3-E2-6K-E1-COOH (26). The capsid is autoproteolytically cleaved from the developing nascent chain and rapidly assembled to form nucleocapsid (NC). Precursor of E2 (pE2) and E1 are transported as a noncovalently associated heteroligomeric complex through the cell secretory pathway to the plasma membrane. Late in the pathway, pE2 is processed to produce E2 and a small glycopeptide, E3. At the plasma membrane, the specific association of E2 tails with NCs initiates a budding process which leads to the release of mature virions (9, 14, 15).

To begin to understand how anti-E2 MAbs inhibit SV replication in mature nondividing neurons, we have used a model system involving replication of TE, a recombinant neurovirulent strain of SV, in rat AT3 cells expressing the inhibitor of apoptosis *bcl-2* (2, 12). We have shown that treating SV-infected AT3-*bcl-2* cells with anti-E2 MAb G5 alters virus production in a dose-dependent manner (2). Cross-linking E2 with MAb G5 improved the functions of the Na^+ pump (Na^+K^+ ATPase) and the Na^+-K^+-$2Cl^-$ cotransport system in SV-infected cells, resulting in improved K^+ flux. MAb treatment was also associated with a delay in the shutoff of host cell protein synthesis. However, the infected cells were only transiently rescued from the effects of virus infection, raising the question of whether other components of the host antiviral response may contribute to the long-term control of viral replication observed in vivo.

The replication of alphaviruses is sensitive to the effect of alpha/beta (type 1) interferon (IFN) (19). IFNs have been reported to act synergistically with antibody to protect mice from fatal infection with Semliki Forest virus (1, 24) and to decrease replication of a number of viruses in vitro (10). IFN is present in the brains of mice with SV-induced encephalitis, but it has not been recognized to play an important role in recovery (7, 20, 23, 28). We have studied the effects of combined IFN and anti-E2 MAb treatment on SV-infected cells in vitro and show that the antiviral activities are synergistic.

SV replication is inhibited by IFN-α treatment. We first determined whether alpha interferon (IFN-α) inhibits SV replication in rat AT3-*bcl-2* cells. Prior to virus infection, AT3-*bcl-2* cells were treated with IFN-α (recombinant human IFN-α; Biosource International, Camarillo, Calif.) at a final concentration of 10^3 IU/ml. This dose of IFN-α had been shown to inhibit Mayaro virus replication in primate cells without significant effects on cellular growth (19). After incubation for 8 h at 37°C, IFN-α-treated cells were infected with SV (TE strain, 10 PFU per cell) in the continued presence of IFN-α (10^3 IU/ml). Previous studies have shown that IFN-α treatment does not modify the absorption and penetration of alphavirions in cells (19). The rate of production of infective particles was examined at the time of maximum production in untreated SV-infected cells (15 h postinfection). Preincubation of AT3-*bcl-2* cells with IFN-α before SV infection reduced the yield of infectious virus particles more than 99% ($P < 0.01$) (Table 1). Therefore, the AT3-*bcl-2* cell line is sensitive to IFN-α stimulation.

Labeled viral proteins in IFN-treated and untreated, infected cells were immunoprecipitated by anti-E2 MAb or monospecific anti-nonstructural protein (nsP) sera (Fig. 1). Expression of E2-precursor polyproteins or mature E2 in IFN-

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Public Health, Baltimore, MD 21205. Phone: (410) 955-3459. Fax: (410) 955-0105.

TABLE 1. Effects of IFN-α treatment in SV-infected AT3-*bcl-2* cells

Treatment	Time of treatment relative to infection	Virus production (PFU/cell/h)	Total protein synthesis[a] (cpm/cell/h)
No virus			208 ± 1
No virus + IFN-α[b]			227 ± 3
Virus + no treatment[c]		27.50 ± 2.50[d]	29 ± 4 ([14 ± 2]%)[e]
Virus + IFN-α[f]	−8 h	0.08 ± 0.05[g] ([0.3 ± 0.2]%)	141 ± 1 ([68 ± 1]%)
Virus + IFN-α[h]	+11 h	20.50 ± 0.50 ([74.5 ± 2.0]%)	35 ± 2 ([17 ± 1]%)
Virus + anti-E2 MAb[i]	+11 h	9.40 ± 2.00 ([34.2 ± 7.2]%)	71 ± 2 ([34 ± 1]%)
Virus + anti-E2 MAb + IFN-α[j]	+11 h	0.62 ± 0.24 ([2.7 ± 1.0]%)	95 ± 4 ([46 ± 2]%)

[a] Measurement of total protein synthesis for 1 h as previously described (2).
[b] Mock-infected AT3-*bcl-2* cells treated with IFN-α (10^3 IU/ml) for 24 h.
[c] SV-infected AT3-*bcl-2* cells (TE strain; multiplicity of infection of 10).
[d] The virus yield at 15 to 16 h postinfection (p.i.).
[e] Total protein synthesis at 16 to 17 h p.i.; the level of labeled protein compared with untreated mock-infected cells.
[f] IFN-α-pretreated cells infected with SV; the virus yield at 15 to 16 h p.i.; total protein synthesis at 16 to 17 h p.i.
[g] The level of virus release compared with untreated infected cells.
[h] SV-infected cells treated with IFN-α (10^4 IU/ml) for 2 h at 11 h p.i.; the virus yield at 2 to 3 h posttreatment; total protein synthesis at 3 to 4 h posttreatment.
[i] SV-infected cells treated with anti-E2 MAb G5 (10^{-2} $PRNT_{50}$ per cell) for 2 h at 11 h p.i.
[j] SV-infected cells treated simultaneously with anti-E2 MAb G5 (10^{-2} $PRNT_{50}$ per cell) and IFN-α (10^4 IU/ml) for 2 h at 11 h p.i.

treated cells is below the level of detection. Ultrastructural analysis of IFN-treated cells confirmed the very low level of expression of viral NCs and mature viral particles. The failure to detect the viral structural proteins in IFN-α-treated cells suggested that the expression of subgenomic 26S mRNA coding for structural proteins or translation of these proteins had been inhibited.

Incubation of AT3-*bcl-2* cells with IFN-α before SV infection may confer a high degree of resistance to virus replication through one of at least two major IFN-inducible enzymatic pathways that act in cooperation with double-stranded RNA. The endogenous 2,5A synthetase-nuclease system degrades viral and cellular single-stranded RNAs and the protein kinase PKR phosphorylates the protein synthesis initiation factor α subunit of eukaryotic initiation factor 2 and inhibits protein synthesis at initiation of translation (8, 22). The mechanism by which IFN inhibits alphavirus replication has not been determined.

IFN-α pretreatment resulted in a decrease in the rate of nsP synthesis by 20-fold (Fig. 1). nsPs are translated from genomic RNA to produce two large polyproteins (P123 and P1234) which are processed to several intermediate proteins and then to mature nsP1, nsP2, nsP3, and nsP4 (26). These nsPs compose the RNA transcriptase-replicase complex for the synthesis of viral RNAs.

The rate of intracellular protein synthesis in SV-infected

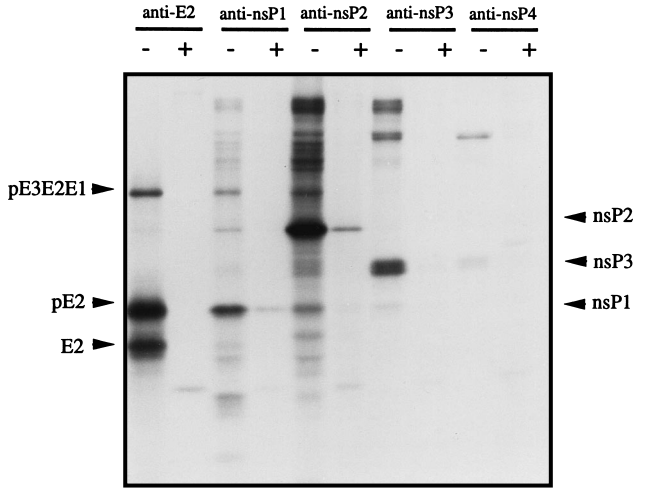

FIG. 1. Analysis of viral protein synthesis in IFN-α-treated cells. AT3-*bcl-2* cells were treated with IFN-α (10^3 IU/ml) for 8 h and then infected with SV (TE strain; multiplicity of infection of 10) in the presence of IFN-α. At 16 h postinfection, IFN-α-treated (+) or untreated (−) infected cells were labeled with 100 μCi of ^{35}S-Translabel (ICN) per ml for 1 h, and cell lysates were obtained as previously described (2). Labeled proteins were immunoprecipitated with anti-E2 MAb 106 or monospecific anti-nsP sera. Too little E1 (52 kDa) and nsP4 (70 kDa) were present for visualization on this gel. Proteins were separated by SDS–8% PAGE, autoradiographed, and quantitated by Image Quant (Molecular Dynamics).

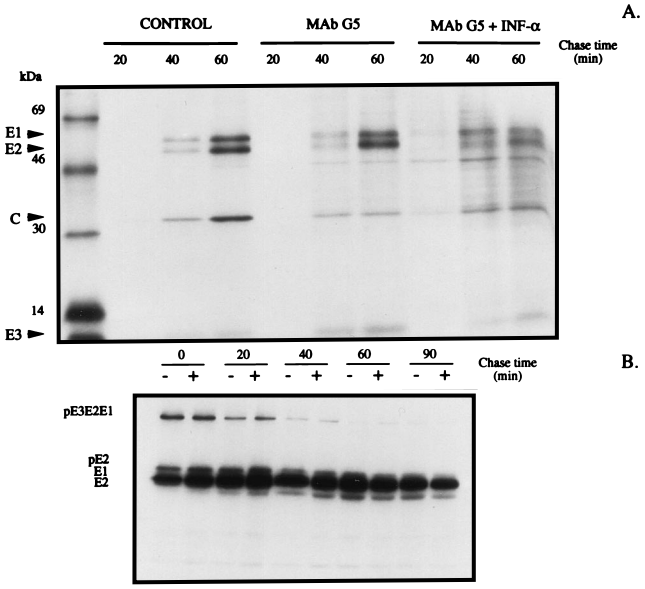

FIG. 2. Analysis of viral protein synthesis in SV-infected cells treated with anti-E2 MAb G5 and IFN-α. Viral proteins were pulse-labeled for 20 min and chased for various times as previously described (2). (A) SV-infected cells were treated with anti-E2 MAb G5 in the presence (MAb G5 + IFN-α) or in the absence (MAb G5) of IFN-α for 2 h at 11 postinfection. After 2 h, infected cells were extensively washed as previously described (2). Two hours posttreatment, infected cells were pulse-labeled with 200 μCi of ^{35}S-Translabel per ml and chased. Total proteins in supernatant fluids were ethanol precipitated and directly analyzed. (B) Intracellular pulse-chase-labeled proteins in MAb G5-treated cells with (+) or without (−) IFN-α were immunoprecipitated with anti-SV polyclonal serum. Proteins were separated by SDS–15% PAGE.

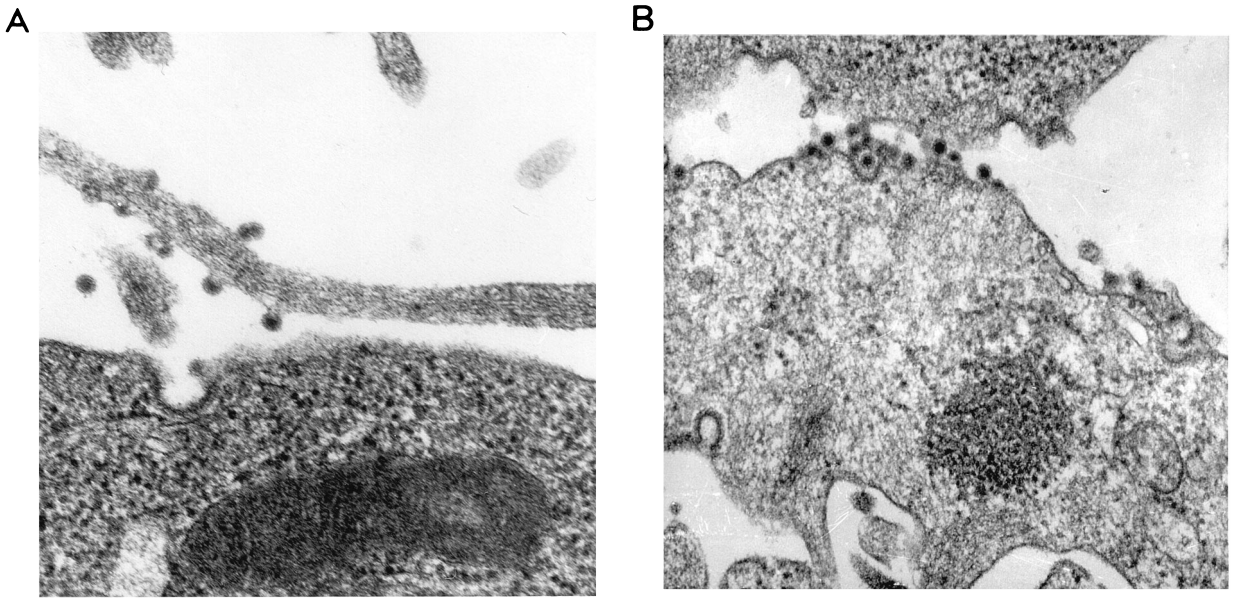

FIG. 3. Electron microscopy analysis of SV-infected cells. Ultrastructure analysis of thin sections prepared from SV-infected AT3-*bcl-2* cells. (A and B) Virus budding from the plasma membrane of untreated, infected cells (A) and accumulation of aberrant forms of viral NCs in cytoplasm and cell membrane-associated immature virus particles in infected cells treated with anti-E2 MAb G5 (10^2 $PRNT_{50}$ per cell; 1 μg/ml) and IFN-α (10^4 IU/ml) at 2 h posttreatment (B). Magnification, ×50,000.

cells was 14% of that in mock-infected cells with or without IFN-α treatment 11 h after infection ($P = 0.0005$). In cells treated with IFN-α before infection, protein synthesis was 68% of normal ($P = 0.035$) (Table 1). Therefore, a low rate of nsP expression was sufficient to induce a limited shutoff of host protein synthesis. This is consistent with previous reports that expression of genomic RNA alone affects host cell protein synthesis (3). Nuclear localization of nsP2 may contribute to the inhibition of host DNA and mRNA synthesis in alphavirus-infected cells (21).

IFN-α pretreatment did not prevent cell death induced by SV infection although the rate of survival of IFN-α-treated cells, as determined by trypan blue exclusion ([22.5 ± 2.5]%), was twice that of untreated infected cells ([11 ± 0.5]%) at 40 h postinfection ($P < 0.01$). Cell death has also been observed in IFN-α-pretreated cells infected with Mayaro virus, another alphavirus (19). These results suggest that expression of nsPs may be sufficient to induce cell death although the mechanism may be distinct from that of productively infected cells which die by induction of apoptosis (13). The nucleoside triphosphatase activity of nsP2 may be involved in these processes (21).

Anti-E2 MAb treatment restores the antiviral activities of IFN-α in SV-infected cells late during the infection. SV-infected AT3-*bcl-2* cells were incubated with IFN-α (10^4 IU/ml) 11 h after infection when the rate of virus release reached its maximum (2). After 2 h, IFN-treated cells were extensively washed, and the rate of production of infective particles was determined 3 to 4 h later (Table 1). IFN-α exhibited no significant antiviral activity when added at this stage of virus replication. There are several possible explanations for this insensitivity to IFN-α treatment late during SV infection. Shutoff of host cell protein synthesis may make the relevant transcription factors unavailable, or translation of their target mRNAs may not occur. Alternatively, modulation of Na^+K^+ ATPase activity may inhibit the specific signal transduction pathways used by IFN-α since the integrity of the Na^+ pump has been postulated to be critical for the transcriptional activation of IFN-stimulated regulatory elements (18).

To investigate the latter possibility, we determined whether cross-linking of E2 with anti-E2 MAb could restore the ability of IFN-α to induce antiviral activity. We have previously reported that the Na^+K^+ATPase and the Na^+-K^+-$2Cl^-$ cotransport system activities are maintained in SV-infected cells treated with a dose of 10^{-2} units of the highest dilution which reduced 50 virus plaques by 50% ($PRNT_{50}$) (1 μg of immunoglobulin G1 per ml) of anti-E2 MAb G5 (2). IFN-α and MAb G5 were added simultaneously to SV-infected cells for 2 h at 11 h postinfection. At 3 h posttreatment, the rate of production of infectious particles was significantly reduced compared with SV-infected cells treated with anti-E2 MAb alone ($P < 0.02$) (Table 1). There was also a synergistic effect of IFN-α and anti-E2 MAb to increase the rate of protein synthesis in SV-infected cells ($P < 0.001$) (Table 1). These results suggest that the signal transduction pathways used by IFN-α were stimulated in SV-infected cells treated with anti-E2 MAb G5 and suggest a link between the maintenance of host cell protein synthesis and the lower rate of virus particles released. At later times posttreatment, the antiviral effects of IFN-α were not sustained and the survival of IFN-α-treated cells was similar to that of anti-E2 MAb-treated cells 40 h postinfection.

To determine how IFN-α and anti-E2 MAb G5 treatment interfered with SV replication, release of viral proteins was examined 2 h after treatment. Infected cells were pulse-labeled for 20 min and chased for various times, and labeled proteins were recovered from supernatant fluids and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). Labeled structural proteins were detected in supernatant fluids of SV-infected cells after 40 min of chase. Extracellular capsid protein was used to compare the rates of virus particles released from infected cells. The C protein in supernatant fluids from SV-infected cells increased linearly during the chase period, but this increase was not seen in supernatant fluids from MAb G5-treated or IFN-α–MAb G5-treated cells. Following IFN-α treatment, labeled proteins consistent with degraded forms of viral proteins were detected early during chase (Fig. 2A). This process was significantly

accentuated in IFN-α–MAb G5-treated cells compared with MAb G5-treated cells. Therefore, the low rate of infectious virus production resulted in part from a potentiation of the antiviral effects of anti-E2 MAb by IFN-α.

IFN-α treatment did not alter the processing of newly synthesized envelope proteins in SV-infected cells treated with anti-E2 MAb (Fig. 2B). Comparable amounts of short pulse-labeled viral NCs were found in SV-infected AT3-*bcl-2* cells with and without IFN-α–anti-E2 MAb treatment (data not shown). Thus, the reduction in formation of virus particles is independent of envelope protein processing or formation of NC. Ultrastructural analysis of IFN-α- and MAb-treated cells showed that immature virus particles and NCs accumulated at the cell membranes (Fig. 3). Aberrant forms of viral NCs were also observed in the cytoplasm, suggesting that some NCs aggregate in the cytoplasm. These cytoplasmic aggregates were not observed in cells treated only with MAb. Therefore, the very low rate of infectious virus yield in IFN-α-treated cells appears to be due to a defect in virus maturation.

There are at least two potential mechanisms to explain this alteration in viral morphogenesis. One is that IFN induced the synthesis of cellular proteins that interfered with the transport of NC to the plasma membrane and thus the NC-E2 interactions failed to occur. Another is that the interruption of virus maturation resulted from inhibition of budding of virions assembled at the cell surface.

Since Mx proteins are GTPases that affect protein sorting and the transport of newly synthesized viral glycoproteins to the cell surface (22), we used reverse transcription-PCR to explore the possibility that Mx proteins were induced by IFN. Cellular mRNAs coding for rat Mx proteins (16) were detected in treated and untreated infected cells at similar levels. This is consistent with previous observations that SV replication is unaffected in avian cells expressing the murine Mx-1 protein (4). Therefore, it remains to be established what antiviral mechanisms were stimulated by IFN-α treatment later during infection that interfered with virus maturation.

In conclusion, we have shown that AT3-*bcl-2* cells replicating SV were sensitive to the antiviral action of IFN-α following anti-E2 MAb treatment. This may be related to the maintenance of Na^+K^+ATPase activity by the MAb cross-linking of E2 at the plasma membrane. IFN-α and antibody to SV are both produced locally in the brains of infected mice (5, 7, 20, 23, 27, 28). In mature mice, SV infection does not induce apoptosis in neurons and antibody is crucial for virus clearance. IFNs may also play a role and act synergistically with antibody to control virus replication. A better understanding of the combined effects of antibody and IFN will be important for understanding recovery from virus infection.

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