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Antiviral Activity of Bacteria-Derived Human Alpha Interferons Against Encephalomyocarditis Virus Infection of Mice

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Bacteria-derived human leukocyte interferon (IFN) subtypes, IFN-αA, -αB, and -αD, and two hybrid IFNs, IFN-αAD and -αDA, were examined for both in vitro and in vivo antiviral activity. Two of these materials in highly purified form (IFN-αD and -αAD) protect mice against lethal doses of encephalomyocarditis virus infection. A single dose of 1 μg of protein of IFN-αAD 3 h before infection conferred protection in both BDF_1 and CD-1 mice against encephalomyocarditis virus infection, and multiple treatments with IFN-αD or IFN-αAD extend the mean survival time of infected mice. On a weight basis, IFN-αAD was approximately 100-fold more effective than IFN-αD. There is a direct correlation between the antiviral activity of the various human IFN species in L-929 cells and in mice for both single and multiple treatments before infection, but none of the cloned human IFN subtypes were effective when administered 24 h after infection. Mixtures of the two parental materials, IFN-αA and -αD, were not as protective as the hybrid molecule IFN-αAD, suggesting that IFNs with unique and altered species specificity can be produced by recombinant DNA methods.

The recent cloning and expression of human leukocyte interferon (Hu IFN-α) genes in bacterial systems (2, 3, 9, 10) has allowed the production of a number of distinct IFN subtypes that have unique antiviral properties (22; P. K. Weck, S. Apperson, L. May, and N. Stebbing, J. Gen. Virol., in press; P. K. Weck, S. Apperson, N. Stebbing, P. W. Gray, H. M. Shepard, and D. V. Goeddel, Nucleic Acids Res., in press). Cell culture studies indicate that these Hu IFN subtypes have distinct activities against different viruses (Weck et al., J. Gen. Virol., in press). In addition, the construction of hybrid IFN genes with recombinant DNA technology has produced IFNs that have relatively high specific activities on human, bovine, and murine cell lines (17; Weck et al., Nucleic Acids Res., in press). Natural Hu IFN preparations do not show a notable activity in mouse cells. However, in some cases, indirect mechanisms appear to be involved in the antiviral effects of IFNs in vivo (4, 13, 14). For these reasons, we examined the antiviral effects of various bacteria-derived Hu IFNs against encephalomyocarditis (EMC) virus infection of mice.

The IFNs used in these experiments were produced by direct expression in *Escherichia coli* in a plasmid construction with the *trp* system (2, 3) and consisted of 165 or 166 amino acid residues. IFN-αA and IFN-αD (2) correspond to IFN-α-2 and IFN-α-1, respectively, described by Nagata et al. (9). The presence of common restriction endonuclease sites in the gene sequences for these two IFNs has facilitated the production of four hybrid IFNs designated IFN-αAD (*Bgl*), IFN-αAD (*Pvu*), IFN-αDA (*Bgl*), and IFN-αDA (*Pvu*) (Weck et al., Nucleic Acids Res., in press). However, the present in vivo studies involved only IFN-αAD (*Bgl*) and IFN-αDA (*Bgl*), which correspond to IFN-α-2(B)α-1 and IFN-α-1(B)α-2, respectively, of Streuli et al. (17). We compared the ability of these two hybrid interferons to protect mice against EMC virus infection. These two hybrid IFNs, generated at the *Bgl*II restriction enzyme site, consist of 61 amino acids from the amino terminus of IFN-αA and 104 amino acids from the carboxy terminus of IFN-αD or 62 amino acids from the amino terminus of IFN-αD and 104 amino acids from the carboxy terminus of IFN-αA (Weck et al., Nucleic Acids Res., in press). They have different specific activities when assayed in mammalian cell cultures.

MATERIALS AND METHODS

Animals. Female white CD-1 and hybrid BDF_1 mice 4 to 6 weeks old were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. All animals were housed 10 per cage after initial injections and were provided with food and water ad libitum. All treatments with IFN and virus injections were given intraperitoneally in 0.1-ml volumes.

Cells and viruses. Mouse L-929, human WISH, and bovine MDBK cells used for determining IFN titers were obtained from the American Type Culture Collection (ATCC), Bethesda, Md., and maintained in minimum essential medium containing 2% fetal calf serum, 100 U of penicillin per ml, and 10 μg of streptomycin per ml (GIBCO Laboratories, Grand Island, N.Y.).

Vesicular stomatitis virus (VSV), Indiana strain, was also purchased from ATCC and maintained by passage in BHK-21 cells. The mean titer of the virus preparation used in this study was 2.2×10^7 PFU/ml, as determined in L-929 cells. EMC virus used to infect mice was grown and stored as described previously (15) and was diluted in sterile phosphate-buffered saline (PBS) just before infection.

IFN preparation and assays. The construction and expression of bacterial plasmids containing Hu IFN genes has been described previously (2, 3). The purification of the bacteria-derived IFNs was achieved by standard column chromatography (21) or antibody affinity columns (T. Stachlin, D. S. Hobbs, H. S. Kung, C. Y. Lai, and S. Pestka, Proc. Natl. Acad. Sci. U.S.A., in press) or both. The specific activities of the highly purified preparations of IFN-αA, -αD, -αAD, and -αDA ranged from 0.6 to 2.2×10^8 U of protein per mg when assayed on MDBK cells challenged with VSV (see Table 3). These IFNs were electrophoretically homogeneous and >95% pure. The biochemical properties of one of these purified IFNs has been described previously in detail (20, 21). A comparative study was also carried out with partially purified preparations of IFN-αA, -αB, -αD, and -αAD, and these materials had specific activities of 1×10^7 U of protein per mg, as determined on MDBK cells. The mouse IFN used in these studies was a serum preparation from mice injected intraperitoneally with the double-stranded polynucleotide polyinosinic acid · polycytidylic acid, from P-L Biochemicals, Inc., Milwaukee, Wis. Serum was obtained 3 h after intraperitoneal administration of 100 μg of polyinosinic · polycytidylic acid per mouse. This IFN preparation had a titer of 6,400 U/ml, as determined on mouse L-929 cells challenged with VSV.

IFN titers were determined in a cytopathic effect inhibition assay performed in 96-well microtiter plates as described previously (1). The NIH standard for Hu IFN (G-023-901-527) or mouse IFN (G002-904-511) was used in these assays, and all titers are in international units.

Statistical methods. The survival time of mice was obtained from records prepared twice daily for at least 21 days after infection. For convenience of presentation, deaths occurring on any 1 day are shown at one time only on the mortality curves (Fig. 1). The average survival time for a group of mice was determined by calculating the mean of the reciprocals of the survival times (in hours) as described previously (14) and taking the reciprocal of this value. These harmonic mean survival (HMS) times account for surviving mice and indicate the relative efficacy of different treatments. Significant differences in the survival times of different groups of mice were determined by calculating logrank χ^2 values and accounting for surviving mice by the method of Peto and Pike (12). The significance levels of these χ^2 values, which have one degree of freedom, are indicated where applicable, and values of $P > 0.05$ are not considered significant. In all experiments, the number of mice per group was 20.

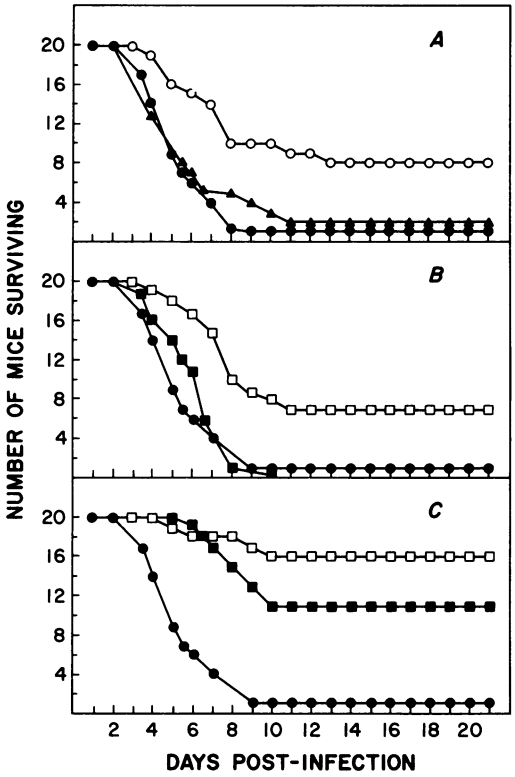

FIG. 1. The relative protective effects of partially purified IFN-αA and -αD (A), a mixture of equal units of IFN-αA and -αD (B), and the hybrid IFN-αAD (C) against EMC virus infection of CD-1 mice. All treatments were given intraperitoneally at the doses indicated 3 h before infection with $100 \times LD_{50}$ of EMC virus, as described in the text. IFNs used in this experiment had a specific activity of 1×10^7 U of protein per mg. Symbols: (A) ▲, 10^5 U of IFN-αA per animal; ○, 10^5 U of IFN-αD per animal; (B) □, 10^5 U of IFN-αA plus 10^5 U of IFN-αD; ■, 10^4 U of both materials; (C) □, 10^5 U of IFN-αAD per animal; ■, 10^4 U of IFN-αAD per animal. The survival curve for animals treated with bacterial lysate as controls (●) is shown in each panel.

RESULTS

Comparison of the activities of IFN-αA, -αD, -αAD, and -αDA against EMC virus infection of mice. The relative antiviral activities of highly purified (>95%) preparations of IFN-αA, -αD, -αAD, and -αDA against EMC virus infection of CD-1 mice are shown in Table 1. The single doses of the Hu IFN administered in this experiment were adjusted to give comparable units of antiviral activity titrated on human WISH cells, but these doses are also shown in terms of micrograms of protein. A single intraperitoneal

TABLE 1. Effectiveness of a single dose of IFN-αA, -αD, -αAD, and -αDA and mouse serum IFN against EMC virus infection of CD-1 mice

IFN	IFN dose[a]		n^b	HMS	χ^2inf[c]	P
	U	μg				
None			1	120.2		
IFN-αA	10^4	0.1	2	141.2	1.8	NS[d]
	10^3	0.01	2	138.7	0.8	NS
	10^2	0.001	0	118.8	0.2	NS
IFN-αD	10^4	10	14	694.4	24.2	<0.001
	10^3	1	8	332.2	19.3	<0.001
	10^2	0.1	4	167.8	4.9	<0.05
IFN-αAD	10^4	0.1	14	549.5	19.2	<0.001
	10^3	0.01	3	178.6	6.5	<0.05
	10^2	0.001	4	157.5	3.5	NS
IFN-αDA	10^4	0.3	0	122.2	0.3	NS
	10^3	0.03	0	126.3	0.4	NS
	10^2	0.003	1	122.2	0.002	NS
Murine	400		10	320.5	16.9	<0.001
	40		11	393.7	18.0	<0.001
	4		3	177.0	5.7	<0.05

[a] Doses are expressed as μg per animal or U per animal, as determined on WISH cells for IFN-αA, -αD, -αAD, and -αDA or L-929 cells for murine IFN as described in the text. The IFN were given as single intraperitoneal doses 3 h before infection with a 100 times the LD_{50} dose of EMC virus.

[b] Number of mice surviving out of 20 animals per group.

[c] Logrank χ^2 comparisons with the infected control group (none).

[d] NS, Not significant.

injection of 10^4 U of IFN-αD or -αAD 3 h before infection caused highly significant protection. However, when compared on a microgram basis, IFN-αAD appeared to be much more effective than IFN-αD; a dose of 0.1 μg of IFN-αAD per animal was significantly more effective ($P < 0.01$) than a dose of IFN-αD. Treatment with various concentrations of IFN-αA or IFN-αDA did not cause significant protection against EMC virus infection (Table 1). Even at doses of 10^5 U per animal, IFN-αA and IFN-αDA (data not shown) failed to cause significant protection of EMC virus-infected mice. The mouse IFN used as a positive control in these experiments caused significant protection at doses as low as 4 U per animal, and previous experiments have demonstrated that a dose of 10^5 U of a human buffy coat IFN preparation does not confer protection against EMC virus infection.

The efficacy of multiple treatments before infection with the various IFN preparations was also examined. Repeated treatments with 10^2, 10^3, or 10^4 U (titrated on WISH cells with the human standard) of IFN-αD or IFN-αAD caused significantly greater protection than single treatments at the same doses given once 3 h before infection. In the repeated treatment regimen, treatments were given at 96, 72, 48, 24, and 3 h before EMC virus infection. IFN-αA and IFN-αDA were ineffective in the multiple treatment regimen before infection, even at doses of 10^4 U per treatment. Single or multiple treatments at or after 24 h postinfection did not cause significant protection against EMC virus infection with any of the IFN preparations described here (data not shown).

To be assured that the protective effects of cloned IFN-αAD were not peculiar to CD-1 mice, a comparative study with BDF_1 animals and two virus doses was performed (Table 2). In this experiment, animals were challenged with 30 or 300 times the median lethal dose (LD_{50}) of EMC virus 3 h after IFN treatment. Statistical analyses demonstrated that single treatments with 10^5 U of IFN-αAD which is equivalent to 1 μg, caused highly significant protection of both strains of mice infected with either dose of virus. IFN-αAD appeared to be slightly more effective in the BDF_1 mice. Because the HMS time is almost the same in the infected control groups of CD-1 and BDF_1 mice given either 30 or 300 LD_{50} of EMC virus, it is possible to compare the efficacy of IFN-αAD between the two strains of mice, and such logrank χ^2 comparisons demonstrate that the difference in the protection of the two strains is significant at $P = 0.05$. A second-

TABLE 2. Protective effects of IFN-αAD against EMC virus infection in two different strains of mice[a]

Mouse strain	Treatment	Virus dose of 30 × LD_{50}				Virus dose of 300 × LD_{50}			
		n^b	HMS	χ^2inf[c]	P	n^b	HMS	χ^2inf[c]	P
BDF_1	PBS	4	171.2			0	113.8		
	IFN-αAD, 10^5 U	19	4,348.8	25.3	<0.001	7	276.2	22.6	<0.001
	Mouse IFN, 500 U	12	404.8	10.9	<0.001	3	167.5	7.4	<0.01
CD-1	PBS	4	175.1			1	107.1		
	IFN-αAD, 10^5 U	13	543.2	10.2	<0.01	5	234.2	13.4	<0.001
	Mouse IFN, 500 U	10	312.5	3.9	<0.05	5	196.1	9.7	<0.01

[a] The IFN doses are U of IFN-αAD per animal determined on WISH with the human standard or polyinosinic · polycytidylic-induced mouse IFN assayed on L-929 cells with the mouse standard.
[b] Number of mice surviving out of 20 animals per group.
[c] Logrank χ^2 comparisons with the group receiving PBS.

ary challenge of the surviving CD-1 and BDF_1 mice with a lethal dose of EMC virus 30 days after the initial infection revealed that all of the animals were susceptible to infection.

Comparison of various partially purified IFN preparations against EMC virus infection of mice. The antiviral effects of IFN-αD and -αAD against EMC virus infection of mice are consistent with earlier studies of cell cultures that showed that of the materials examined, only these two IFNs had notable antiviral activity in mouse L-929 cells (Weck et al., J. Gen. Virol., in press; Weck et al., Nucleic Acids Res., in press). To assess the antiviral activity of other Hu IFN subtypes obtained from bacteria, we compared the efficacy of partially purified preparations of IFN-αA, -αB, -αD, and -αAD against EMC virus infection of CD-1 mice. A dose of 10^5 U (titrated on WISH cells with the human standard) of each IFN preparation per mouse was given 3 h before infection, and the results (data not shown) demonstrated that IFN-αA and -αB did not cause significant protection. IFN-αAD was highly protective and a bacterial lysate-treated group acted as a control. The amount of bacterial lysate administered per mouse was the same as the amount of protein administered with the IFN treatments (10 μg), and the survival of the bacterial lysate-treated group was not significantly different from a PBS-treated control group. In addition, the administration of a dose of 10^5 U of a preparation of naturally derived human buffy coat IFN did not confer significant protection on animals infected with EMC virus.

Because the hybrid IFN-αAD is composed of 61 amino acids from the amino-terminus of IFN-αA and 104 amino acids from the carboxy terminus of IFN-αD, it seems possible that a mixture of the two parental molecules could have activity similar to IFN-αAD. Comparisons of the protective effect of partially purified IFN-αAD, -αA, -αD, and a mixture of equal units of IFN-αA and IFN-αD are shown in Figure 1. Treatment of the mice with 10^5 U of IFN-αD resulted in significant protection ($P < 0.001$), whereas the same dose of IFN-αA failed to protect mice against EMC virus infection (Fig. 1, A). Combined treatment with IFN-αA plus IFN-αD did not seem to enhance survival (Fig. 1, B), and this group was not significantly different from that treated with IFN-αD alone. In contrast, doses of 10^4 or 10^5 U of IFN-αAD protected 11 of 20 and 16 of 20 mice, respectively, when given 3 h before infection (Fig. 1, C). The difference between the groups treated with 10^5 U of IFN-αAD and IFN-αD is significant at $P = 0.05$. Thus, it appeared that the hybrid IFN-αAD had unique activity not shared with either parental IFN subtype or mixtures thereof.

Comparison of the activity of IFN-αA, -αD, -αAD, and -αDA in bovine, human, and murine cell cultures. Partially purified preparations of the various Hu IFN subtypes and the hybrid molecules had comparable activities in bovine MDBK cells, but quite different activities on human WISH and murine L-929 cells (Weck et al., J. Gen. Virol., in press; Weck et al., Nucleic Acid Res., in press). A comparison of the potency of IFN-αD and -αAD against EMC virus infection of mice was based on IFN units in human cells. To compare the antiviral activity of the various IFNs and relate this to the effects of the IFNs on mice, we assayed the highly purified materials used in the present studies in three cell lines. The data in Table 3 show that neither IFN-αA nor IFN-αDA had any detectable activity against VSV infection of L-929 cells. Only IFN-αAD was consistently effective against this virus in all three cell lines, and the titers of IFN-αD and IFN-αDA were approximately two and three logs higher, respectively, on bovine MDBK cells compared with WISH cells. The data in Table 3 show that equal amounts of IFN-αD and -αAD, titrated on human WISH cells, cause a 68-fold difference in activity, titrated on mouse L-929 cells. Thus the difference in potency of IFN-αD and -αAD in vivo is very similar to the difference in activity in vitro.

TABLE 3. Titers of bacterial-derived Hu IFN in different cell cultures

IFN	IFN titers[a]		
	WISH	MDBK	L-929
IFN-αA	7.5×10^7	6.2×10^7	NA[b]
IFN-αD	3.2×10^6	2.2×10^8	4.8×10^4
IFN-αAD	1.2×10^8	1.2×10^8	1.2×10^8
IFN-αDA	1.8×10^5	1.1×10^8	NA

[a] Titers are expressed as the number of IFN units of protein per milligram and are standardized against the NIH human leukocyte standard when assayed on WISH and MDBK cells and against a mouse IFN standard when assayed on mouse L-929 cells challenged with VSV virus. The materials used in these assays had been purified to 90% purity.

[b] NA, IFN had no detectable antiviral activity on these cell lines.

DISCUSSION

The fact that subtype IFN-αD coded by a human gene protects mice against lethal EMC virus infection is perhaps surprising because the subtype appears to be a significant component of natural Hu IFN preparations that have very low activity in mice or murine cells. Of even greater interest is the observation that hybrid IFN-αAD has relatively high antiviral activity in murine cell cultures and in vivo. The antiviral activity of IFN-αAD in BDF_1 animals correlates with the antitumor effects of this IFN against L-1210 leukemia cells in the same strain of mice (N. Stebbing, P. K. Weck, J. T. Fenno, S. Apperson, and S. H. Lee, *in* E. De Maeyer et al. [ed.], *The biology of the interferon system*, in press). This suggests that unique IFNs with altered species specificity can be produced by recombining segments of different IFN genes. A comparison of the different cloned IFNs shows that the relative activity of highly purified materials in mice is comparable to the relative activity of highly purified materials in cell cultures. That is, neither IFN-αA nor IFN-αDA has significant antiviral activity in mice, either in vitro or in vivo, and 100 times more IFN-αD than IFN-αAD is required to achieve equal protection in vivo. Single treatments with IFN-αD and IFN-αAD revealed that doses of 10 μg and 0.1 μg, respectively, conferred very similar protection (Table 1).

Many factors affect the in vivo efficacy of IFNs or mediate their activity, and IFNs can have profound effects on different aspects of immune responses (4, 13, 14, 16). IFN can alter NK cell functions (8), modulate cell-mediated cytotoxicity, and affect antibody production, all of which may alter in vivo virus replication. The suppression of macrophages reduces the effectiveness of IFN in mice against EMC infection (14). Thus, a number of cell populations that respond to exogenous IFN therapy may play a part in inhibiting EMC virus replication in infected animals. Indirect effects may be of great importance in some virus infections, such as herpes simplex type 1. Certainly, the observation that IFN-αA is not very active against herpes simplex type 1 virus in cell cultures (Weck et al., Nucleic Acids Res., in press) but reduces ocular lesions in rabbits (G. Smolin, N. Stebbing, M. Friedlaender, R. Friedlaender, and M. Okumoto, Arch. Ophthalmol., in press) suggests that indirect effects are involved with this infection in vivo. The importance of indirect effects in the rabbit system is also indicated by the efficacy of late treatments commencing 2 days after infection (Smolin et al., in press). In contrast, the purified species of IFN that we studied that are effective against EMC virus infection of mice are not effective when administered after infection, and there is a direct correlation between efficacy in murine cells and efficacy in mice. The infection of mice with EMC virus results in a viremia and seeding of target organs that eventually lead to involvement of the central nervous system (18, 19), and this course of infection is known to be altered by IFN treatment (5, 6, 7, 11). The susceptibility to reinfection of mice surviving as a result of IFN treatment suggests that EMC virus was eliminated by IFN treatment before an adequate humoral immune response could be mounted to confer long-term protection and that IFN treatment does not act by stimulating antibody protection. Thus, direct antiviral effects may be of primary importance for EMC virus infections.

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