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Interferon-α inhibits the emergence of cellular stress response-dependent morbillivirus large plaque variants

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Abstract

Cellular levels of heat shock proteins (HSPs) are elevated in response to physiologic states accompanying acute virus infection (e.g. fever). The objective of the present work was to define the antiviral effect of purified human lymphoblastoid IFN in the presence of HSP over-expression. For this purpose, canine distemper virus (CDV) was used since the response of CDV transcription and persistent infection phenotype to elevated HSP is characterized. First, the effect of elevated HSP on CDV lytic infection phenotype in Vero and CV1 cells was defined, and results extended to the closely related measles virus (MV). Cells expressing elevated levels of the major inducible 70-kDa HSP (hsp72) supported the emergence of large plaque variants of both CDV and MV from small plaque purified inocula. IFN treatment concurrent with infection caused a dosage-dependent reduction in the expression of large plaque variants without affecting hsp72 levels or total plaque number. In contrast to the stress response-induced large plaque variant, small plaques were resistant to the antiviral effects of IFN. These data demonstrate the ability of IFN to selectively abrogate the pro-viral effects of HSP over-expression, inhibiting the formation of a plaque phenotype that is correlated to enhanced virulence in animal models of morbillivirus encephalitis.

Keywords: Interferon α; Distemper; Measles; Plaque variants; Heat shock protein

1. Introduction

The cellular stress response is induced by loss of cellular homeostasis following the unfolding of newly synthesized protein. Denaturation of protein results in transcriptional activation of heat shock protein (HSP) genes and suppressed synthesis of non-heat shock proteins, hallmarks of the stress response (Westwood et al., 1991; Beckmann et al., 1992). The induced HSPs, in their role as chaperonins, facilitate the refolding of denatured

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protein and target terminally denatured protein for degradation, thereby mediating cell recovery (Riabowol et al., 1988; Gaitanaris et al., 1990; Wagner et al., 1994). Elevations in HSP persist into the post-stress interval, protecting cells from subsequent insults that are lethal to non-stressed cells. This protection is the basis for HSP-mediated induced thermotolerance (Mizzen and Welch, 1988). Thus, heat shock proteins are viewed as protective to the cell by mediating either recovery or protection from injury. This role has been particularly well illustrated for the major inducible 70-kDa HSP (hsp72) (Riabowol et al., 1988), a highly conserved HSP family member that is expressed at only very low constitutive levels but which can accumulate to high levels following cellular stress.

Among the many effects of viruses upon cells, induction of the stress response has only recently been identified. Induction may be a direct consequence of viral replication, reflecting virus-mediated cellular damage or the increased production of newly synthesized viral proteins that require a cellular chaperonin function to mediate proper folding. Direct virus induction of HSP has been shown for several virus-host systems in vitro (Khandjian and Turler, 1983; Sheshberadaran and Norrby, 1984; DiCesare et al., 1992; MacKowiak et al., 1993; Donofrio et al., 1995), with the biological relevance of those findings substantiated by in vivo observations: viral antigen positive astrocytes within the brains of dogs suffering from canine distemper virus (CDV) encephalitis express elevated hsp72 (Oglesbee and Krakowka, 1993). However, CDV antigen negative astrocytes also express elevated hsp72 within inflamed foci, suggesting that stress response induction may be both direct (i.e. direct viral induction) or indirect and mediated by inflammatory cytokines. Further and more general elevations in HSP levels can be caused by other physiologic conditions that accompany acute viral infections, such as the hyperthermia associated with the febrile response (Li et al., 1992).

The potential exists for the stress response to modify the antiviral activity of IFN by either altering the virus infection phenotype or by altering the cellular response to IFN. Stress-induced increases in viral gene expression are observed in multiple viral families, including Retroviridae (Stanley et al., 1990; Andrews et al., 1995), Herpesviridae (Williams et al., 1989), and Paramyxoviridae (Oglesbee et al., 1993). For the paramyxovirus CDV, viral transcription is enhanced when HSPs are induced prior to lytic virus infection or during persistent virus infection, resulting in increased virus-induced cytopathic effect in the latter (Oglesbee et al., 1993). There is also evidence to suggest that HSPs and IFN act synergistically. Evidence for the direct modulation of IFN antiviral activity by the stress response includes the observation that the antiproliferative and antiviral activities of IFN-α are potentiated at elevated temperatures in vitro (Hirai et al., 1984). However, the experimental design used in that study did not distinguish between the antiviral effects of elevated temperature per se (Nover, 1991) and the effects of induced HSPs on IFN activity. The cellular stress response may modulate IFN antiviral activity by increasing cellular levels of enzymes mediating the IFN-induced antiviral state. MDCK and WISH cells removed from heat or ethanol exhibit increased levels of 2′,5′ oligoadenylate synthetase activity commensurate with stress response induction (Chelbi-Alix and Chousterman, 1992). Synergism between induced HSP and the antiviral activities of interferon is further suggested by the finding that IFN can potentiate HSP induction by other stressors (Morange et al., 1986; Dubois et al., 1988).

The objective of the present study was to define the antiviral activity of purified IFN-α following induction of the cellular stress response, using a virus-cell system of defined stress-responsiveness. For this purpose, African green monkey kidney epithelial cells (Vero and CV1) were infected with CDV and, for comparison, the closely related measles virus (MV). Both CDV and MV belong to the morbillivirus genus of Paramyxoviridae. The antiviral activity of IFN-α was defined in both the presence and absence of prior HSP induction.

2. Materials and methods

2.1. Virus-cell systems

Small plaque purified variants of the Onderstepoort strain of CDV (Ond-CDV) and the Hallé strain of MV were used to infect subconfluent Vero and CV1 cell monolayers. Cells were grown at 37°C in MEME containing 10% fetal calf serum. To induce the cellular stress response, cells were exposed to a transient 1.5 h thermal (43°C) or heavy metal (80 or 160 μM sodium arsenite) insult at 24 h post-seeding, followed by a media change and return to pre-shock culture conditions. Cells were infected at 16 h post-shock. Levels of hsp72 present within cells at the time of infection were based upon Western blot analysis of replicate cell populations as previously described (Oglesbee et al., 1996).

2.2. Viral infection parameters

To measure viral plaque number and area, cells were cultured under methylcellulose overlays for 48 h following infection, followed by formalin fixation and Giemsa staining of the monolayers. Titers were calculated as plaque forming units per ml (pfu/ml) of inoculum and plaque areas were measured using a Zeiss Interactive Digital Analysis System (Oglesbee et al., 1990). Cell-free infectious viral progeny release was measured in cells infected at a multiplicity of infection (MOI) of 0.01. Monolayers were harvested by scraping the cells into the culture media, disrupting the cells using two freeze-thaw cycles, and the lysates clarfied by centrifugation at $12000 \times g$, 4°C, 10 min. The infectivity of the lysates was calculated as pfu/ml as described above. Viral antigen expression was measured by immunocytochemistry of methanol-fixed cell suspensions, using canine CDV convalescent serum as the source of primary antibody (Oglesbee et al., 1993). Bound primary antibody was detected using a goat anti-canine IgG FITC conjugate, and specific signal detected by flow cytometry as previously described (Oglesbee et al., 1993). The same hyperimmune serum was also used as primary antibody in Western blot analysis of total viral antigen (Oglesbee et al., 1993). For these assays, cells were infected at an increased MOI (i.e. 0.3) and were harvested immediately prior to the development of syncytia (i.e. 12 h post infection) so that antigen expression would reflect individual cellular production unaffected by differences in the rate of spread of syncytia.

2.3. Interferon treatment

Interferon-α was purified from the human lymphoblastoid B cell line (BALL-1) by Hayashibara Biochemical Laboratories, Okayama, Japan. The BALL-1 cells were propagated in immunosuppressed newborn golden hamsters and then stimulated in vitro with Sendai virus (HVJ). IFN-α was purified from the supernatant of the latter by immuno-affinity chromatography using an anti-human IFN-α monoclonal antibody, followed by gel filtration chromatography (Ando et al., 1987). This commercially available preparation consists of three IFN-α subtypes (75% α-2B, $\approx$ 25% α-8, $<1\%$ α-7) based upon N-terminal sequence analysis and is 99% pure based upon SDS-PAGE followed by staining with Coomassie brilliant blue. Quality control procedures rule out the existence of contaminating cytokines, including tumor necrosis factor (Fukuda et al., 1988). Absence of Sendai virus is established using PCR and ELISA assays for viral RNA and protein, respectively. The activity of the IFN-α preparation was defined in international units (IU) based upon the inhibition of Sindbis virus cytopathic effect in FL cells, using the NIH international IFN-α reference (Ga-23-901-532) as a standard (Fukuda et al., 1988). IFN was added at the time of virus infection (i.e. concurrent treatment), remaining in the culture supernatant for the duration of the post-infection interval, or IFN was added to the culture medium for 8 h prior to infection (i.e. interferon pre-treatment), corresponding to 8 h post-shock.

3. Results

3.1. Stress response induction increased mean plaque area

The present work established the effect of the

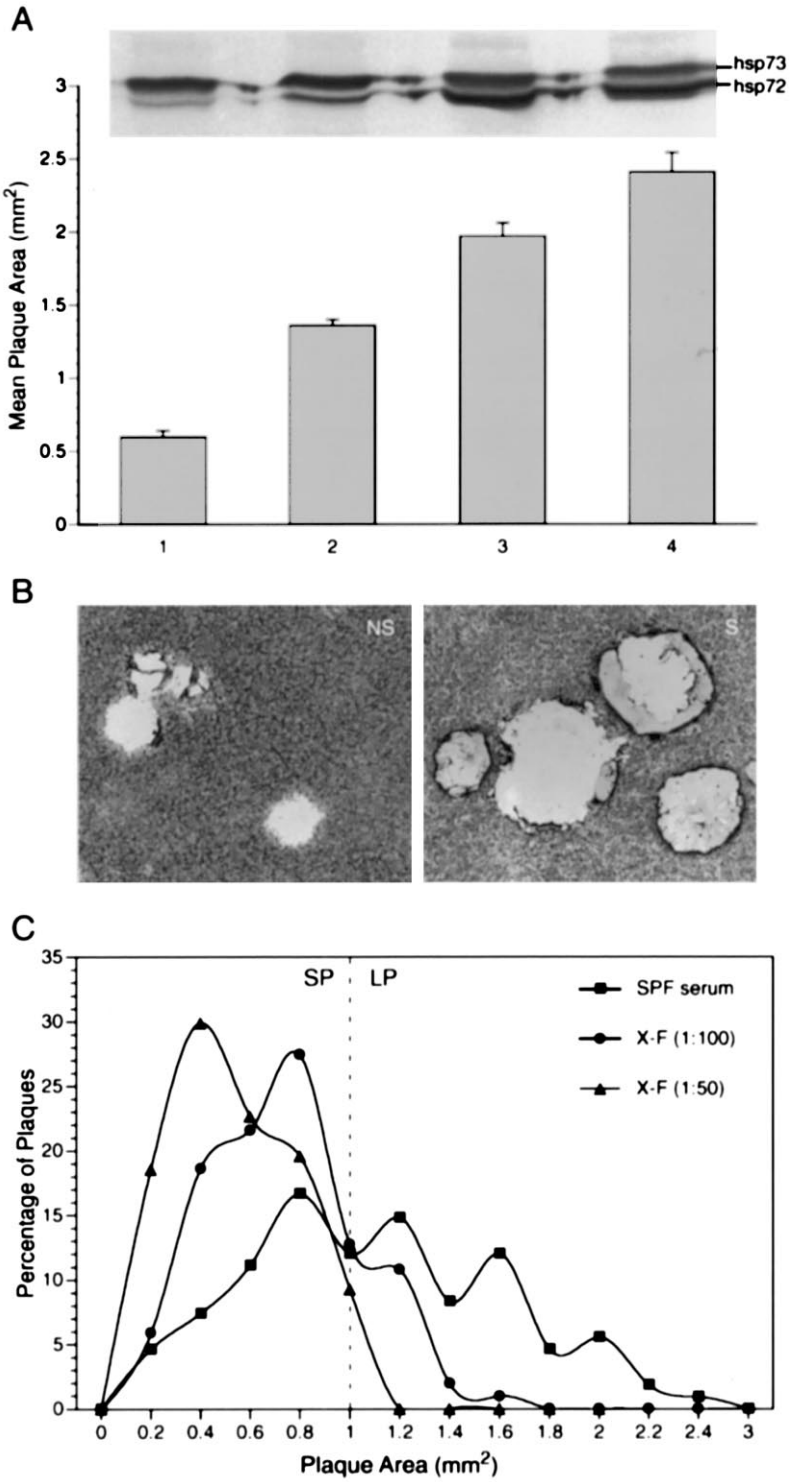
A
hsp73
hsp72
Mean Plaque Area (mm²)
0
0.5
1
1.5
2
2.5
3
1
2
3
4
B
NS
S
C
SP
LP
SPF serum
X-F (1:100)
X-F (1:50)
Percentage of Plaques
0
5
10
15
20
25
30
35
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
3
Plaque Area (mm²)

Fig. 1.

stress response upon infection parameters used to define the antiviral activity of IFN: viral plaque number and size, viral protein expression, and cell-free infectious progeny release. Both hyperthermia and sodium arsenite shock of Vero cells increased levels of the major inducible 70-kDa HSP (hsp72) at 16 h post-shock, the time of viral challenge. Increasing the severity of cell stress (i.e. increasing the concentration of sodium arsenite or prolonging the duration of the 43°C hyperthermia) resulted in greater intracellular accumulation of hsp72 based upon Western blot analysis of cell lysates (Fig. 1A).

Cellular levels of hsp72 were, in turn, proportionate to resultant mean plaque area (Fig. 1A). Measurements of plaque areas were reported at 48 h post-infection (PI), an interval supporting maximal plaque area development prior to healing of the monolayer. Mean plaque areas were based upon an average of 100 plaques and were 0.6 ± 0.04 (standard error of the mean, S.E.M.) mm^2 from non-shocked cells, whereas cells exposed to a 1.5 or 3 h 43°C shock yielded mean plaque areas of 1.36 ± 0.04 and 1.97 ± 0.09 mm^2, respectively. The largest plaques (2.41 ± 0.13 mm^2) followed a 1.5 h 160 μM sodium arsenite shock, a treatment that also yielded the greatest degree of HSP induction. All differences in mean plaque area were significant ($P < 0.01$) using a two-tailed Student's t-test. Stress-response mediated increases in mean plaque area were independent of post-infection intervals, being observed at 18, 20, 22, 24 and 36 h PI (data not shown).

3.2. Increased mean plaque area reflected emergence of large plaque variants

Analysis of the distribution of plaque areas showed that infection of shocked monolayers resulted in the emergence of distinct large plaque variants. Bimodal distributions were predominant, where shocked monolayers contained large and small plaques. The latter were comparable in size to the unimodal distribution of small plaques on non-shocked cells (Fig. 1B). Plaque numbers were not significantly different between shocked and non-shocked treatment groups; variability in the stress-induced mean plaque area reflected changes in the ratio of large to small plaques. Stress-induced changes in plaque distributions were consistent between experimental trials and were confirmed on CV1 cells. Stress- induced large plaque variants were unstable, requiring previously stressed cells to maintain the phenotype and precluding isolation of specific variants for further analysis; large plaque isolates yielded small plaques on non-shocked monolayers and a bimodal distribution of large and small plaques on shocked monolayers (data not shown). A correlation between hsp72 levels at the time of viral challenge and resultant mean plaque area was also shown for Hallé-MV infection of both Vero and CV1 cells (Vasconcelos et al., 1998).

Stress response mediated increases in CDV mean plaque area (i.e. emergence of large plaque variants) reflected increased cell-surface viral anti-

Fig. 1. Induction of the cellular stress response resulted in the emergence of large plaque variants of CDV from small plaque-purified inocula. (A) The stress response was induced in Vero cells using a 43°C 1.5 h shock (2), a 43°C 3 h shock (3), or a 160 μM sodium arsenite shock (4). Cytoplasmic levels of hsp72 at 16 h post-shock were measured by Western blot analysis (inset) and compared to non-shocked controls (1). Levels of hsp72 increased progressively as the severity of the shock increased, contrasted to levels of the constitutively expressed $\approx$70-kDa HSP (hsp73). When parallel cell populations were infected, the resultant mean plaque area ($\pm$ standard error of the mean, S.E.M.) increased in concert with progressive increases in hsp72 at the time of infection (i.e. 16 h post-shock). (B) The basis for the increase in mean plaque area was the emergence of large plaque variants on shocked cell monolayers (S), contrasted to the unimodal distribution of small plaques on non-shocked cells (NS). (C) The emergence of large plaque variants (LP) was dependent upon expression of the CDV F glycoprotein, based upon inhibition of large plaque formation by antibody against CDV F (rabbit anti-F hyperimmune serum). A multimodal distribution of large plaques formed in the presence of a 1:50 dilution of specific pathogen-free gnotobiotic dog serum (SPF) lacking antibodies against CDV, whereas a 1:100 dilution of anti-F serum reduced and a 1:50 dilution of anti-F serum eliminated expression of large plaque variants. Plaque number was not affected by treatment. Each distribution represented $\geq$100 plaques.

gen expression. Non-shocked and shocked (160 μM sodium arsenite, 1.5 h) Vero cell monolayers were infected at an MOI = 0.3 with Ond-CDV at 16 h post-shock. Cells were harvested immediately prior to the development of syncytia at 12 h PI and stained with CDV hyperimmune canine serum (Y2504). This serum contains antibodies recognizing all six structural proteins of CDV, including the membrane glycoproteins F and H (Oglesbee et al., 1993). Cells were then formalin fixed, stained with an FITC-conjugated anti-canine IgG, and the signal quantitated by flow cytometry. Uninfected cells and infected cells stained with gnotobiotic dog serum lacking antibodies against CDV were used as negative controls. The percentage of viral antigen positive cells was equivalent between treatment groups, being 45.1% for non-shocked cells and 43.8% for shocked cells in one experimental trial. However, the mean fluorescence intensity (i.e. fluorescence channel number) of antigen positive cells was 3.83 for non-shocked cells and 4.74 for shocked cells on a logarithmic scale, an almost 10-fold difference in mean fluorescence per cell.

To confirm the role of cell-surface viral membrane glycoproteins in mediating the difference in shocked versus non-shocked plaque areas, parallel plaque assays were performed using shocked (160 μM sodium arsenite, 1.5 h) and non-shocked Vero cells, where culture supernatants contained 1:100 or 1:50 dilutions of rabbit anti-CDV F hyperimmune serum (Örvell and Norrby, 1980) during the development of virus-induced syncytia. The F (fusion) glycoprotein is directly responsible for the cell-to-cell fusion that is the basis for virus-induced syncytium formation (Rima, 1983; Morrison, 1988). Gnotobiotic canine serum lacking antibodies against CDV was used as a negative control. Antibodies were added at 4 h PI. The 1:100 dilution of antibody reduced both shocked and non-shocked mean plaque areas by 37 and 49%, respectively, while the 1:50 dilution of anti-F antibody reduced plaque areas by 56 and 64%, respectively. An analysis of plaque area distribution showed that the anti-F antibody eliminated expression of large plaque phenotypes, converting a multimodal distribution of large plaque variants to the small plaque phenotype (Fig. 1C). The mean area of the small plaque phenotype was, however, 50% larger than the mean of the unimodal distribution of small plaques observed on non-shocked cells cultivated under the same concentration of anti-F antibody.

3.3. Stress response induction increased viral progeny release

Cell-free infectious viral progeny release is another infection parameter used to assess the antiviral activity of IFN. Similar to the analysis of viral antigen expression, cells used for the quantification of infectious progeny release are infected at increased MOI's relative to plaque assays, with maximal progeny release observed following 90–100% syncytial coverage of the monolayer. In the present study, cells were infected in suspension at an MOI = 0.01 at 12 h following a 1.5 h 160 μM sodium arsenite or 43°C shock and then seeded into replicate tissue culture flasks. Flasks were harvested in triplicate at different PI intervals, and each cell lysate titrated separately and used to compute an average value. Mean progeny release from shocked cells was compared to progeny release derived from non-shocked infected control cells run in parallel. Progeny release from both shocked and non-shocked monolayers peaked at 34 h PI, with small but significantly greater ($P < 0.05$) release observed from shocked monolayers (Table 1). Titers were lower at 46 h, although release from shocked monolayers remained greater than that from non-shocked cells. These data were correlated to differential progeny release observed from stress-response dependent large and small plaque variants using a standard plaque assay. Five plaques of each variant were harvested from under the methylcellulose overlays at 48 h PI and the lysates pooled and titrated in triplicate. Large plaques produced approximately twice the progeny as small plaques (5.43×10^3 vs 2.45×10^3 pfu/plaque in one experimental trial) independent of target cell population used for the titration (i.e. shocked versus non-shocked). Although these differences were shown to be statistically significant, it was not clear whether the basis for these differences was differential cellular support of viral replication or increased viral spread

Table 1
The effect of stress response induction upon CDV cell-free infectious viral progeny release in Vero cells

Hours post infection	Progeny titer (pfu/ml)					
	Heat shock	Non-shocked control	*P*-value	Arsenite shock	Non-shocked control	*P*-value
6	24.0 ± 6.9	40.5 ± 4.5	<0.05	35.0 ± 23.5	41.7 ± 27.1	n.s.
10	4.5 ± 7.8	6.0 ± 6.9	n.s.	5.0 ± 12.2	2.0 ± 0.4	n.s.
14	6.0 ± 5.2	4.5 ± 0.0	n.s.	21.7 ± 7.5	16.7 ± 10.3	n.s.
18	39.0 ± 6.9	7.5 ± 5.2	<0.005	$(4.1 \pm 3.1)10^3$	$(2.8 \pm 0.9)10^2$	<0.005
22	$(7.8 \pm 4.3)10^3$	$(1.4 \pm 0.8)10^3$	<0.0001	$(7.3 \pm 1.5)10^4$	$(1.5 \pm 0.6)10^4$	<0.0001
26	$(3.6 \pm 1.1)10^4$	$(2.2 \pm 0.6)10^4$	<0.005	$(1.8 \pm 0.7)10^5$	$(7.4 \pm 2.9)10^4$	<0.0001
34	$(17.5 \pm 1.5)10^5$	$(15.0 \pm 2.2)10^5$	<0.05	$(12.5 \pm 1.1)10^5$	$(9.7 \pm 3.5)10^5$	<0.05
46	$(7.4 \pm 1.2)10^5$	$(4.7 \pm 0.1)10^5$	<0.05	$(3.9 \pm 0.9)10^5$	$(2.3 \pm 0.6)10^5$	<0.05

Cells were shocked for 1.5 h with either 80 μM sodium arsenite or 43°C and infected at 16 h post-shock. Cells were harvested for titration of cell-free infectious viral progeny release at varying post-infection intervals. Titers are expressed as pfu/ml and represent an average of three flasks per time point, each flask titrated in triplicate, $\pm$ the standard deviation (S.D.). Differences in titer between shocked and non-shocked cells were compared using a two-tailed Student's *t*-test. Non-significant differences ($P > 0.05$) are indicated by n.s.

in the monolayer (i.e. increased number of virus infected cells). Use of higher multiplicities of infection could not be used to address this issue due to inhibitory effects mediated by defective interfering particles, a characteristic feature of the morbillivirus system.

3.4. The activity of IFN-α against stress-enhanced virus infection parameters

To determine if IFN-α was capable of inducing the stress response in non-shocked monolayers, Vero and CV1 cells were treated with either 0 or 1000 U/ml IFN for 8 h, and cell lysates analyzed for hsp72 content by Western blot analysis at the end of the treatment interval. The addition of IFN failed to increase the signal intensity of hsp72 between samples or to alter the ratio of hsp72 to the constitutively expressed 70-kDa HSP family member (hsp73) within a sample (data not shown). Lack of stress response induction by IFN-α is supported by reports in the literature (Dubois et al., 1988). IFN treatment also failed to modify stress response mediated increases in cellular hsp72 levels, based upon Western blot analysis of shocked cell lysates 8 h following treatment with either 0 or 1000 U/ml IFN-α, representing 16 h post shock.

Cells were treated with 0, 1, 10, 100, or 1000 U/ml of IFN-α for 8 h prior to infection (IFN pre-treatment) or the IFN was added to the cells at the time of viral challenge, remaining in the methylcellulose overlays for the following 48 h (IFN concurrent treatment). IFN-α pre-treatment reduced plaque number in a dosage-dependent manner, and the dose-response curves were the same for shocked versus non-shocked cells. Results were independent of target cell line (i.e. Vero vs CV1), virus (i.e. CDV vs MV), or stressor (i.e. heat vs arsenite induction of the stress response). Results for Hallé-MV infection of heat shocked Vero cells are presented (Fig. 2). Total plaque number was reduced approximately 4-fold at 1000 U/ml for infection of both shocked and non-shocked cells. The difference was statistically significant ($P < 0.05$), although consistent with the relative resistance of the small plaque phenotype to IFN treatment (see below). The introduction of IFN-α at the time of viral challenge did not affect resultant plaque number regardless of dosage (data not shown).

Stress response-induced large plaque variants of CDV exhibited a high sensitivity to IFN-α. The multimodal distribution of CDV large plaque variants induced by a 1.5 h 160 μM sodium arsenite or 43°C shock 16 h prior to infection

(mean plaque area = 3.01 ± 0.20 mm^2) was resolved into a bimodal distribution of large and small plaques (mean plaque area = 2.12 ± 0.15 mm^2) when 1 U/ml IFN-α was administered at the time of viral challenge (concurrent IFN treatment) (Fig. 3). Increasing the concentration of IFN-α to 10 U/ml diminished the relative proportion of large plaque variants while plaque number remained constant; the resultant mean plaque area was 1.84 ± 0.13 mm^2 (S.E.M.). Formation of large plaques was totally inhibited at 100 and 1000 U/ml IFN-α. Increasing IFN-α concentrations also decreased the mean size of the small plaques, although the dose-response was relatively small. The mean small plaque area on untreated non-shocked cells was 0.89 ± 0.06 mm^2, compared to 0.71 ± 0.07 mm^2 on non-shocked cells treated with 100 U/ml IFN-α. These results were reproduced using IFN-α pre-treatment, where loss of the large plaque phenotype was accompanied by reductions in total plaque number (data not shown). The ability of IFN-α to reduce plaque area was not attributed to inhibition of cell proliferation; cell densities were equivalent between treatment groups at the time of viral challenge and at the end of the plaque assays.

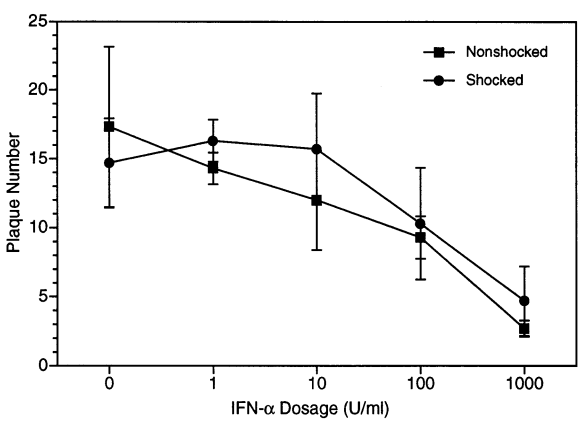

Fig. 2. An 8 h IFN-α pre-treatment (8 h post-shock) reduced MV plaque numbers on Vero cells in a dosage-dependent manner. Differences in the dose response between heat shocked (1.5 h, 43°C) (●) and non-shocked (■) cells were not present. Plaque number for each treatment represents an average of three titrations $\pm$ S.E.M. In contrast, IFN-α treatment concurrent with infection did not reduce plaque number.

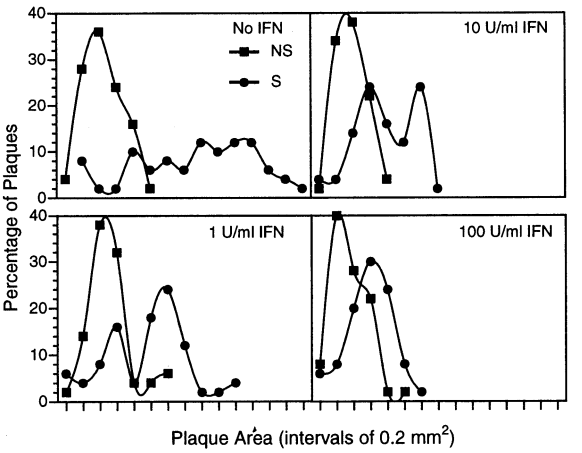

Fig. 3. Interferon-α treatment of Vero cells concurrent with infection eliminated stress response-induced emergence of CDV large plaque variants. The stress response was induced by a 1.5 h, 43°C shock. A multimodal distribution of large plaques was present on shocked cells (●) versus a unimodal distribution of small plaques on non-shocked control cells (■) in the absence of IFN-α. The loss of large plaque formation in response to IFN-α treatment was dose responsive, where elimination of large plaques was observed at 100 U/ml IFN-α. Small plaque mean area was also reduced by increasing IFN-α concentration (non-shocked > shocked). Each distribution represents ≥ 100 plaques.

The effect of concurrent IFN-α treatment on diminishing the proportion of large to small plaques and on decreasing the small plaque mean area was confirmed using CDV infection of CV1 cells. This effect was illustrated by comparing the mean area of all plaques over the range of 0–1000 U/ml IFN-α (Fig. 4). At 1000 U/ml, the difference between the mean small plaque area in shocked versus non-shocked cells was small but significant, based upon a two-tailed Student's t-test ($P < 0.05$). The effects of IFN-α treatment on total viral antigen expression and cell-free infectious viral progeny release were consistent with changes observed in total mean plaque areas. IFN-α treatment resulted in a reduction of total viral antigen within infected cell lysates for both shocked and non-shocked Vero cells, based upon Western blot analysis. Here, differences in the antigen profile and signal intensity were not discernable between shocked and non-shocked infected Vero cells at 1000 U/ml IFN-α (data not shown). In addition, differences in cell-free infectious viral progeny

release between shocked and non-shocked virus infected cells were eliminated at 1000 U/ml IFN-α (Fig. 5). For these latter assays, progeny were harvested at 24 h PI.

Stress response induced large plaque variants of MV were also sensitive to IFN-α treatment. Vero cells exposed to a transient heat shock were infected with Hallé MV at 16 h post-shock. At 48 h PI, shocked cells exhibited a bimodal distribution of large and small plaque variants (mean area of all plaques $= 0.60 \pm 0.06$ mm^2), contrasted to a unimodal distribution of small plaques on non-shocked cells (mean plaque area $= 0.36 \pm 0.03$ mm^2). Large plaque formation was inhibited at 1–1000 U/ml IFN-α (Fig. 6). The mean area of small plaques was also reduced by IFN-α in a dosage- dependent manner, although the magnitude of the response was substantially less than observed for the large plaque variant. The mean area of small plaques on shocked cells was consistently larger than the mean observed on non-shocked cells. At 1 U/ml IFN-α, the mean small plaque area was 0.51 ± 0.05 mm^2 on shocked cells compared to 0.28 ± 0.02 mm^2 on non-shocked controls. At 1000 U/ml IFN-α, the mean small plaque area on shocked cells was 0.33 ± 0.02 mm^2 compared to 0.19 ± 0.02 mm^2. These difference were small but statistically significant ($P < 0.05$).

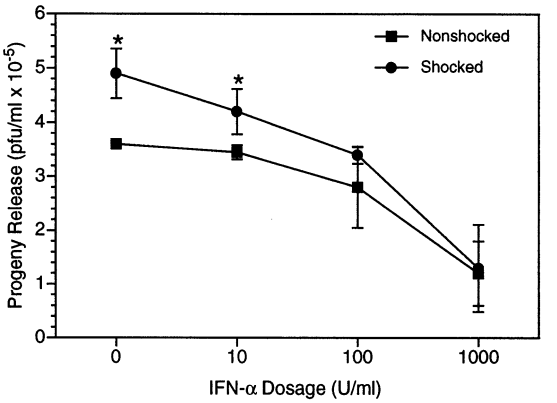

Fig. 5. The cellular stress response enhanced cell-free CDV infectious viral progeny release in Vero cells, with differences between shocked (●) and non-shocked cells (■) being eliminated by ≥ 100 U/ml IFN-α. The stress response was induced by a 1.5 h exposure to 80 μM sodium arsenite, a treatment inducing hsp72 to levels equivalent to that achieved following a 1.5 h 43°C shock. Progeny were harvested at 24 h post-infection for titration. Significant differences between progeny release on shocked and non-shocked cells (✱) were only observed at 0 and 10 U/ml IFN, conditions where large plaque variant expression was observed in parallel plaque assays. Values represent the sample mean $\pm$ standard deviation.

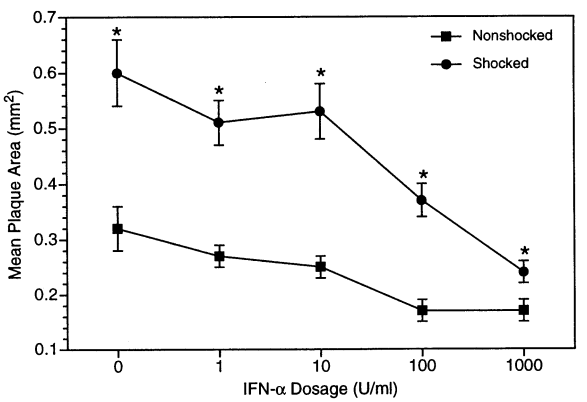

Fig. 4. The ability of IFN-α to eliminate stress response-induced CDV large plaque variants was reproduced in CV1 cells, and was reflected in the IFN-α dosage-dependent reduction in mean plaque area ($\pm$S.E.M.). The stress response was induced by a 43°C, 1.5 h shock, followed by infection and IFN-α treatment at 16 h post-shock. Mean plaque areas present on shocked (●) and non-shocked (■) cells treated with 100 and 1000 U/ml of IFN-α represented unimodal distributions of small plaques. In the latter, the mean on shocked cells was significantly greater (✱) than the mean on non-shocked cells based upon a two-tailed Student's t-test ($P < 0.05$). Each distribution represents ≥ 100 plaques. Total plaque number was unaffected by treatment.

4. Discussion

The morbillivirus-Vero system was well-suited for the characterization of the effects of stress response induction upon the antiviral activity of IFN-α. Vero cells are responsive to IFN, where treatment readily induces 2′,5′ oligoadenylate synthetase (Crespi et al., 1986), an enzyme activity correlated to the antiviral state against nonsegmented negative strand RNA viruses (Dubois et al., 1989; Chelbi-Alix and Chousterman, 1992). Secondly, morbillivirus infection does not induce endogenous IFN production in Vero cells (Crespi et al., 1986, 1988), facilitating interpretation of results where exogenous IFN is added. Finally,

the stress responsiveness of several CDV-cell systems has been established (Oglesbee et al., 1990, 1993).

Results presented in the present work indicate that elevations in cellular HSPs may mediate the emergence of large plaque variants when using small plaque purified inocula of either CDV or MV, and that purified IFN-α may inhibit the emergence of the stress response-induced large plaque variant. Although stable genotypic plaque variants of CDV and MV have been identified (Gould et al., 1976; Cosby et al., 1981), the spontaneous interconversion between large and small plaque variants is more characteristic. Until now, a basis for interconversion was not established (Carrigan, 1986). A mechanism involving a direct role for HSP in mediating the emergence of large plaque variants, versus a non-specific effect of stress on cell metabolism, is supported by the observations that stress response mediated changes in infection phenotype were independent

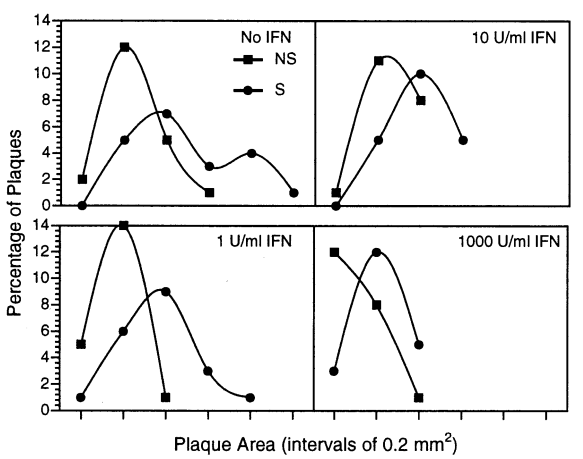

Fig. 6. Interferon-α treatment of Vero cells concurrent with MV infection eliminated stress response-induced emergence of large plaque variants similar to results with CDV. The stress response was induced by a 1.5 h, 43°C shock. A bimodal distribution of large–small plaques was present on shocked cells (●) versus a unimodal distribution of small plaques on non-shocked control cells (■) in the absence of IFN-α. The MV large plaque variant was highly sensitive to IFN-α, being eliminated at a concentration of 1 U/ml. Reductions in the mean area of small plaques on both shocked and non-shocked cells by IFN-α were small but dose-dependent. Each distribution represents ≥ 100 plaques. Total plaque number was unaffected by treatment.

of stressor (i.e. thermal or heavy metal insult) or cell lineage, and that the stressors were removed 16 h prior to infection. Cells return to their pre-shock metabolic state within 4 h following removal of non-lethal stressors while cytoplasmic levels of HSPs remain elevated, reflecting the long half life of HSPs (Duncan and Hershey, 1989). Recent work shows that hsp72 alone is responsible for supporting emergence of CDV and MV large plaques, and not other HSPs associated with stress response induction, using stably transfected human astrocytoma cells engineered to constitutively over-express hsp72 (Vasconcelos et al., unpublished observation). The interconvertibility of plaque phenotype was demonstrated by showing that plaque number was constant between treatment groups (i.e. shocked versus non-shocked cells with or without concurrent IFN-α treatment) despite changes in the proportion of large and small plaque variants; small plaques were the only phenotype observed on non-shocked cells or on shocked cells treated with ≥ 100 U/ml IFN-α or high concentrations of antibody against the viral F protein, whereas both large and small plaques were observed on shocked cells in the absence of IFN or at IFN concentrations less than 1–10 U/ml. A direct demonstration of the interconvertability of plaque phenotypes was provided by showing that progeny of stress-induced large plaques formed small plaques on non-shocked cells and a bimodal distribution of large and small plaques on shocked cells.

The biological significance of changes in plaque phenotype is that the propensity of CDV and MV to form large plaques in vitro is correlated to enhanced neurovirulence in animal models of morbillivirus encephalitis (Cosby et al., 1981; Carrigan, 1986). The effect of IFN-α on CDV and MV infection may thus be viewed as inhibitory to the emergence of virulent in vitro phenotypes while having minimal effects on low virulence (i.e. small plaque) phenotypes. Such selective IFN activity in vivo would be beneficial to the host; sustained replication of low-virulence virus would ensure the induction of immune mechanisms required for viral clearance while suppressing emergence of virulent phenotypes associated with fatal disease. The biological significance of stress re-

sponse-mediated increases in infectious progeny release is unclear. Preliminary data from this laboratory indicate that hsp72-mediated increases in viral antigen expression and cytopathic effect (i.e. large plaque formation) can occur without concurrent increases in viral progeny release. This disparity was demonstrated in CDV-infected human astrocytoma cells that constitutively over-express hsp72 (Vasconcelos et al., unpublished observation). Furthermore, although large plaque phenotypes are correlated to enhance neurovirulence, distinct profiles of infectious viral progeny release have not been described.

The mechanism by which IFN inhibited formation of stress-response enhanced large plaque phenotypes is not defined at present. Work performed in this laboratory suggests that elevations in cellular levels of hsp72 mediate stress response enhanced CDV infection parameters, where hsp72 directly stimulates transcription by the virus-encoded RNA-dependent RNA polymerase (Oglesbee et al., 1993, 1996). Based upon results of the present study, it is our hypothesis that virions within a small plaque purified inoculum of CDV or MV differ in the ability to respond to hsp72 over- expression, reflecting the high degree of genetic variability characteristic of negative strand RNA viruses (Steinhauer and Holland, 1986). When cells expressing elevated HSP (i.e. hsp72) are infected, increased transcriptional activity is manifest in hsp72-responsive viral variants resulting in increased F expression and large plaque formation, whereas non-hsp72-responsive viral variants support lower levels of transcription and F expression and thus small plaque formation. It is the hsp72-responsive viral variants that contribute most to increases in mean infection parameters (e.g. infectious progeny release, plaque area, and antigen expression) under permissive conditions, although such mean parameters necessarily under-represent the magnitude of change occurring in hsp72-responsive variants. If stress-response dependent formation of large plaques reflects elevated expression of hsp72, then IFN-α treatment must be interfering with the pro-viral effects of HSP over-expression since our data do not indicate an effect of IFN-α on hsp72 levels. This antiviral activity is observed against both CDV and MV, and for IFN pre-treatment and treatment concurrent with infection in Vero or CV1 cells. Infection of confluent cell monolayers precludes the contribution of reduced cell number as an explanation for IFN-mediated reductions in large plaque formation and mean plaque area.

Results of in vitro studies underscore the potential therapeutic significance of abrogating the pro-viral effects of the cellular stress response/HSP. Members of several viral families respond to the stress response by increasing gene expression, with infection itself being one mechanism of HSP induction. In addition, several physiologic states associated with enhanced viral virulence are characterized by increased tissue HSP expression, including psychological stress (Fukudo et al., 1997). Thus, inhibition of HSP expression (e.g. using plant-origin bioflavonoids) represents one strategy to suppress virus replication or virus-induced cytopathic effect (reviewed by Vlietinck et al., 1988; Hosokawa et al., 1992), although such treatments would necessarily be limited by the fact that the cellular protective functions of HSP would also be lost. Results from the present study indicate that IFN-α-mediated inhibition of the stimulatory effect of the stress response/HSP on virus infection phenotype represents an alternative approach.

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