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Brefeldin A inhibits the antiviral action of interferon against encephalomyocarditis virus

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Abstract

Brefeldin A (BFA), a unique fungal metabolite of a 13-membered lactone ring, exhibits various biological actions, including antitumor, antifungal and antiviral activities. In the present study, mouse L B cells were treated with various concentrations of interferon (IFN) and/or BFA overnight and infected with encephalomyocarditis virus (EMCV) after removal of IFN and BFA. Doses of BFA which neither inhibit the metabolism of the cell nor the infectivity of EMCV, decreased the IFN-induced antiviral activity against EMCV as demonstrated by virus titer from supernatants. Since 2-5A synthetase and double-stranded RNA (dsRNA)-dependent protein kinase (PKR) have been suggested to be involved in the antiviral action of IFN against EMCV, their activities were investigated in L B cells after BFA treatment. Northern blot analysis and in situ hybridization showed a decrease (2-3-fold) in the mRNA of 2'-5' oligoadenylate (2-5A) synthetase after BFA treatment. BFA also inhibited the activity of 2-5A synthetase, 2-5A dependent RNase and phosphorylation of PKR in cellular extracts, indicating that BFA may be exerting its inhibitory effect both at the transcriptional and post-transcriptional levels. This study reports a new biological action of BFA, demonstrating that BFA antagonized the antiviral action of IFN by inhibiting IFN-induced enzymatic pathways. These studies also suggest that both 2-5A and PKR are important in the antiviral activity of IFN against EMCV.

Keywords: Brefeldin A; Mouse LB cells; Interferon; Encephalomyocarditis

1. Introduction

Brefeldin A (BFA), a fungal metabolite antibi-otic containing a 13-member macrocyclic lactone ring has antiviral, antibacterial, and antifungal activities (Harri et al., 1963; Tamura et al., 1968). Morphological studies have shown that BFA causes disassembly of the Golgi-apparatus and
dilation of the endoplasmic reticulum (ER) (Fujii- wara et al., 1988; Lippincott-Schwartz et al., 1989). BFA causes rapid dissociation of proteins associated with the cytosolic face of the Golgi and subsequent retrograde movement of components of the cis, medial and trans-Golgi network (TGN) to reassemble into extensive tubular processes without redistributing in the ER (Wood et al., 1991; Lippincott-Schwartz et al., 1989; Reaves and Banting, 1992). BFA effectively blocks secr-
tion of various proteins in various cell types (Fuji-
wara et al., 1988; Misumi et al., 1986; Magner
and Papagiannes, 1988). BFA inhibits the glyco-
protein secretion in VSV-infected BHK cells
(Takatsuki and Tamura, 1985) and processing
and secretion of the envelope glycoproteins of
human immunodeficiency virus (HIV-1) in
MOLT-3 cells infected with HIV-III_B strains,
leading to the inhibition of viral particle forma-
tion (Pal et al., 1991). BFA reversibly arrests
assembly and release of various viral particles
including Herpes Simplex virus (Cheung et al.,
1991), pseudorabies virus (Whealy et al., 1991)
and Punta Toro virus (Chen et al., 1991). BFA
has been used to study folding, assembly, trans-
port and intramolecular interactions of HIV-1
envelope proteins (Earl et al., 1991).

Interferons (IFNs) are proteins or glycoproteins
and have potent biological functions including
antiviral, growth inhibitory and immunoregula-
tory (Pestka et al., 1987; Adams and Hamilton,
1988). Several studies (Staeheli, 1990; Samuel,
1988; Gupta et al., 1982; Rice et al., 1985; Hov-
anessian et al., 1987; Galabru and Hovanessian,
1987) have shown that IFN treatment is associ-
ated with the induction of two double stranded
RNA-dependent enzymes, 2’-5’ oligoadenylate (2-
5A) synthetase and protein kinase. These bio-
chemical pathways have been implicated in the
IFN-mediated inhibition of a number of viruses.

Studies reported in this paper show that low
and non-toxic doses of BFA which do not have
any effect on the EMCV, abolished the antiviral
activity of IFN against EMCV in L_B cells by
inhibiting the activities of IFN-induced 2-5A syn-
thesase, PKR and 2-5A dependent RNase. This
study reports that a new biological action of BFA
is the inhibition of the IFN-induced biochemical
enzymatic pathways which are involved in antivi-
ral activity of IFN against EMCV.

2. Materials and methods

2.1. Cells

A subclone, 11 (eleven), was derived from the
mouse L_B cell line (originally obtained by D.C.
Burke, University of Warwick, Coventry, UK) by
a single cell cloning method (Singh et al., 1988).
Cells were maintained in Eagle’s MEM (EMEM)
supplemented with 10% fetal bovine serum.

2.2. Virus

The EMCV (originally obtained from C. Buck-
ley, NIAID, NIH, Bethesda, MD, USA) was
plaque purified and passaged at a multiplicity of
0.01 TCID so/cell. The virus stock was assayed by
determining its cytopathological effects (cpe) in L_B
cells and the titer was 2 × 10^9 TCID so/cell.

2.3. Reagents

Recombinant murine IFN-β was obtained from
Toray Industries, Japan. BFA was purchased
from Epicenter, Madison, WI. Polyionosinic-poly-
cytidylic acid (poly I:C), a double stranded RNA,
was purchased from Pharmacia LBK Biotechnol-
yogy, USA. [α-32P]dCTP (3000 Ci/mm mol), [α-
35S]dCPT (1000 Ci/mm mol) and [γ-32P]ATP (6000
Ci/mm mol) were supplied by DuPont/NEN,
Boston, MA.

![GAPDH](image)

Fig. 1 PCR amplification for IFN-β in poly I:C and with or
without BFA treated L_B cells: RNA was prepared from
normal, poly I:C-treated, and poly I:C and BFA-treated cells,
reverse transcribed and amplified for IFN-β gene. Amplifica-
tion for GAPDH from the same reverse transcription reaction
was used as an internal control. Lanes: (1) normal cells; (2)
BFA; (3) poly I:C; (4) poly I:C + BFA (0.1 μg/ml); (5) poly
I:C + BFA (0.2 μg/ml); (6) poly I:C + BFA (0.3 μg/ml); (7)
amplification without template; (8) 123 bp ladder. The concen-
tration of poly I:C used was 100 μg/ml for each treatment.
2.4. Assay for antiviral activity

The antiviral activity of IFN was assayed as described previously (Maheshwari and Friedman, 1979). Briefly, the cells were grown in 96-well microtiter plates until 70% confluent and were treated in quadruplicate with IFN in the presence or absence of various doses of BFA (0.01, 0.1, 0.2, 0.3 μg/ml) for 16 h. IFN and BFA were then removed, and cells were washed and infected with EMCV for 1 h at a multiplicity of 0.1 TCID<sub>50</sub>/cell. The unabsorbed virus was removed, cells were washed, and fresh medium was added. Cells were incubated further for 24 h and examined under a light microscope for cytopathic effects. Supernatant from replicate wells was pooled and used for virus yield estimations. Data were plotted as inhibition of virus yield (log<sub>10</sub>) compared to control.

2.5. Polymerase chain reaction (PCR) for IFN-β

L<sub>929</sub> cells were treated with BFA (0.1, 0.2 and 0.3 μg/ml) and after 10 h poly I:C (100 μg/ml) was added for an additional 6 h. Total cellular RNA was isolated with an RNAzol B kit (Tel-Test Inc., Friendswood, TX). The RNA was further purified by lithium chloride precipitation and analyzed by electrophoresis in a denaturing 1.5% formaldehyde gel to ascertain that the RNA was undegraded. The reverse transcription was performed using reverse transcriptase (RT) (Life Technologies, Gaithersburg, MD) and random primers. To 3 μl of RT mix, the following components were added; 6 μl of dNTP mix (100 mM); 10 μl of 10 × PCR buffer and 0.5 μl of Taq polymerase (5 U/μl); 0.4 μl of sense primer (1 μg/μl) and 0.4 μl of antisense primer (1 μg/μl) and 79.7 μl of distilled water. The samples were incubated in a DNA thermocycler (Perkin Elmer Cetus, Gene Amp PCR system 9600) for 25 cycles. The temperature
cycling was as follows: 95°C for 1 min 30 s (denaturing), 50°C for 1 min (annealing of primers) and 72°C for 1 min 30 s (primer extension). To confirm that equal amounts of RNA were taken in each PCR reaction in the experiment, primers for the ‘housekeeping gene’, glyceraldehyde phosphate dehydrogenase (GAPDH) were used for comparison.

2.6. PKR assay

Two sets of Lβ cells were treated with IFN-β (0–300 I.U./ml) and/or BFA (0.20 μg/ml) for 16 h. One set of cells were infected with EMCV at a multiplicity of 1 TCID₅₀/cell for 8 h. Cellular extracts from infected or uninfected cells were prepared using NP40 lysis buffer (10 mM Hepes pH 7.6, 1.5 mM magnesium acetate, 90 mM KC1, 0.5% (v/v) NP40, 2 mM mercaptoethanol), containing 2 μg/ml leupeptin and phenylmethylsulfonfly fluoride (PMSF). Protein kinase assay was done as described (Dubois and Hovanessian, 1990) in these extracts. Briefly, cellular extracts (100 μg protein) were incubated with poly I:C at 1 × 10⁻⁶ g/ml, ATP at 0.1 mM, MgCl₂ at 10 mM and 1 μl [γ⁻³²P]ATP (6000 Ci/mmol) for 30 min at 30°C. The phosphorylation reaction was stopped by addition of 2 × SDS-gel sample buffer, and heated at 90°C for 5 min. Phospho-proteins were separated by 10% SDS-PAGE and analyzed by autoradiography.

2.7. Assay of 2-5A dependent RNase

2-5A dependent RNase was analyzed as described by Nolan-Sorden et al., 1990. A bromide substituted and radioactive derivative of 2-5A, the 5'-monophosphate, P(A₂'-br₂'p₂')₃⁻[³²P]Cp, was used as a probe for the nuclease; this probe was a generous gift from Dr. Robert Silverman, Cleveland Clinic Foundation, Cleveland, OH. Cells were lysed with NP40 lysis buffer containing 2 μg/ml protease inhibitor leupeptin and PMSF. The lysates were centrifuged at 12000 × g and the cellular extracts (100 μg protein) were incubated with the probe (50000 counts/min) for 60 min in ice and then under UV light for an additional 1 h. After cross linking, reaction mixtures were heated at 95°C for 5 min in SDS-gel sample buffer and the proteins were separated by 10% SDS-PAGE. Gels were dried and autoradiograms were prepared using XAR-5 X-ray film (Kodak).

2.8. Assay of 2-5A synthetase

The synthetase present in 12000 × g supernatant fractions was bound to poly (I)poly (C)-cellulose by the method of Stark et al., 1982. The 2-5A synthesis was determined using the radiobinding method of Knight et al., 1980. Briefly, the NP40 lysates obtained from cells were centrifuged at 12000 × g and the aliquot of the supernatant containing 170–200 μg protein was made up to 15 μl with DBG (10 mM Hepes pH 7.5, 50 mM KC1, 1.5 mM magnesium acetate, 7 mM mercaptoethanol, 20% v/v glycerol) buffer and 15 μl of washed poly I:C cellulose with DBG was added to the aliquot. The mixture was incubated for 1 h at room temperature, the supernatant was removed without disturbing the cellulose and the cellulose was washed twice. Assay mix (15 μl) (DBG, 8 mM ATP pH 7.0, 15 mM MgCl₂) was added to the cellulose and incubated for 2 h at 37°C, centrifuged and the supernatant was assayed for 2-5A using radiobinding assay.

2.9. DNA probes

Purified murine cDNA (2.2 kb) insert of 2-5A synthetase (kindly provided by Dr. Bryan R.G. Williams, Cleveland Clinic Foundation, Cleveland, OH) was labelled with [α⁻³⁵S]dCTP (1000 Ci/mmol) with random primers for in situ hybridization or [α⁻³²P]dCTP (3000 Ci/mmol) with Klenow enzyme for Northern hybridization.

2.10. In-situ hybridization

Lβ cells were treated with BFA (0.2 μg/ml) and/or IFN-β (100 and 300 I.U./ml) overnight. The cells were then trypsinized, adjusted to a concentration of 1 × 10⁶ cells/ml, and 200 μl of the cell suspension was collected on silanated and activated slides by cytocentrifuging at 350 × g for 5 min. In situ hybridizations were performed as described by Peltonen et
with slight modifications. Briefly, the slides were fixed in 4% paraformaldehyde and acetylated for 10 min, then dehydrated and hybridized for 18 h at 42°C. Hybridization solution contained 0.1 μg/ml 35S-labelled cDNA probe of 2-5A synthetase, 10 mM dithiothreitol, 500 μg/ml acetylated bovine serum albumin, 0.3 M sodium chloride, 50% deionized formamide, 10% (w/v) dextran sulfate, 500 μg/ml tRNA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 1 mM EDTA, and 50 mM Tris–HCl, pH 7.6. Post-hybridization was performed by sequential incubation of the slides in a progressively more dilute saline-citrate solution with 5 mM dithiothreitol and 0.1% Triton-X, dehydrated, and dipped in NTB-3 emulsion for autoradiography. After exposure for 7–10 days at 4°C, the slides were developed with D-19 developer, stained with cresyl violet acetate and mounted with permount. The cells were examined and photographed with a Zeiss microscope equipped with camera attachments.

2.11. RNA isolation and Northern hybridization

RNA was isolated by phenol/chloroform extraction (Maniatis et al., 1982). Briefly, 2 × 10⁷ cells were washed with cold PBS, trypsinized using trypsin/EDTA and centrifuged at 1500 rev./min for 10 min. Cells were lysed using cold NP40 lysis buffer (1% NP40, 1.5 mM MgCl₂, 10 mM NaCl, 10 mM Tris–HCl, pH 7.4), centrifuged, and supernatant (RNA) was transferred to SDS-buffer (0.5% SDS, 10 mM EDTA, 50 mM NaCl, 200 mM Tris–HCl, pH 9.0). Phenol/chloroform extraction was repeated 3 times and to the aqueous phase (RNA), 3 M sodium acetate (1/10 vol) and 100% ethanol were added to precipitate the RNA at −20°C overnight. RNA was recovered by centrifugation at 4°C, 3000 rev./min, for 20 min and resuspended in DEPC-treated H₂O (20–30 μl) by vortexing, and stored at −80°C.

RNA was run on 1% agarose, formaldehyde denaturing gels, and blotted onto Nytran filter overnight. The probe was denatured by heating at 95°C for 10 min and quickly cooled on ice. Probe (1 × 10⁶ counts/min/ml) was added to the hybridization solution and filters were incubated overnight at 42°C. Blots were washed (hybridization/washing conditions used were stringent), exposed to X-ray film, and autoradiograms were developed.

3. Results

3.1. Induction of IFN-β gene in poly I:C and BFA treated cells

It has been shown that BFA (1–5 μg/ml) incubated with cells after virus infection acts as an antiviral agent against several viruses. However, these doses of BFA were cytotoxic in L_B cells as demonstrated by a decrease in cell number and uptake of [3H]thymidine, therefore, the lower doses of BFA were examined for cytotoxicity. We found that the concentration of BFA above 0.3 μg/ml was cytotoxic when incubated for 16 h with cells as demonstrated by cell count and uptake of [3H]thymidine (data not shown), therefore, concentration of BFA ranging from 0.01–0.3 μg/ml were used to study its effects on virus inhibition in the presence of IFN.

It is reported that dsRNA directly induces IFN-β gene in fibroblasts (Memet et al., 1991). To demonstrate whether the inhibition of IFN-induced gene expression is specific for the IFN-induced genes, not simply the result of poor metabolism in the cell in the presence of BFA, an examination of the induction of IFN-β gene in poly I:C and BFA-treated cells was undertaken. The RT-PCR showed that IFN-β RNA can still be induced by dsRNA in the presence of BFA (Fig. 1), demonstrating that cell function was normal during BFA incubation.

3.2. Effect of BFA on the antiviral activity of interferon

Data (Fig. 2) shows that IFN exhibited a significant antiviral activity (3000–5000-fold) against EMCV in a dose-dependent manner. The antiviral effect of IFN against EMCV above 300 I.U./ml of IFN was almost saturated, therefore, we restricted the use of IFN up to 300 I.U./ml in these studies. Various concentrations of BFA (0.01, 0.1, 0.2, 0.3 μg/ml) were used to study the antiviral activity...
against EMCV. BFA reversed the antiviral effect of IFN by 200–400-fold against EMCV, however, BFA alone had no antiviral activity (Fig. 2). Since there was not much difference in the inhibition of antiviral activity of IFN either by 0.3 μg/ml or 0.2 μg/ml BFA, 0.2 μg/ml BFA dosage was used for these studies.

### 3.3. Inhibition of IFN-induced PKR by BFA

PKR was strongly phosphorylated in IFN-treated, uninfected (Fig. 3a, lane 3) or EMCV-infected (Fig. 3b, lane 3) cellular extracts as compared to control (Fig. 3a and Fig. 3b, lane 1) and BFA-treated (Fig. 3a and Fig. 3b, lane 2) cells. BFA inhibited the phosphorylation of PKR in IFN and BFA treated, uninfected (Fig. 3a, lane 4) and EMCV-infected cells (Fig. 3b, lane 4).

### 3.4. Inhibition of IFN-induced mRNA of 2-5A synthetase by BFA

Northern hybridization analysis for 2-5A synthetase (Fig. 4a) showed that there was no detectable expression of 2-5A synthetase in control or BFA treated cells. 2-5A synthetase was highly expressed in IFN (200 and 300 I.U./ml)-treated cells (Fig. 4a, lane 3) and (Fig. 4b, lane 1), however, its expression was reduced substantially in the cells treated with IFN and BFA together (Fig. 4a, lane 4) and (Fig. 4b, lane 2), indicating that BFA down-regulated the IFN induced enzyme at transcriptional level. Actin (Fig. 4a) and GAPDH (Fig. 4b) was used as an internal control to verify initial equal quantities of RNA and the integrity of the RNA preparations.

In situ hybridization did not show the expression of 2-5A synthetase in control untreated (Fig. 5a) or BFA-treated (Fig. 5b) Lb cells. 2-5A synthetase was highly expressed as evident from the number of grains present in IFN-treated cells (Fig. 5c and Fig. 5e). However, its expression was significantly reduced in cells treated with IFN and BFA (Fig. 5d and Fig. 5f).

### 3.5. Inhibition in the activity of 2-5A synthetase by BFA

To correlate a decrease in mRNA levels and enzyme activity, 2-5A synthetase activity was determined using various doses of IFN in the presence of BFA. BFA inhibited the 2-5A synthetase activity induced by IFN as shown in Fig. 6. There was a decrease in the inhibition of the activity with an increase in the IFN dose, demonstrating that BFA could not completely inhibit the induction of 2-5A activity. The synthesis of 2-5A synthetase mRNA inhibited to a lesser extent than the enzyme activity indicating that BFA may be exerting its inhibitory effect both at transcriptional and post-transcriptional level.

### 3.6. Effect of BFA on 2-5A dependent RNase

Data (Fig. 7) shows the levels of 2-5A dependent RNase in uninfected (a) and EMCV infected (b) Lb cells. A significant increase in the activity...
2-5A Synthetase

Actin

B

2-5A Synthetase

GAPDH

Fig. 4. (a and b) Expression of 2-5A synthetase: L9 cells were treated with BFA, IFN, or BFA and IFN for 16 h. RNA was isolated, blotted, and Northern hybridization was done using 32P-labelled cDNA of murine 2-5A synthetase probe. (a) Lanes: (1) untreated control cells; (2) BFA (0.20 μg/ml)-treated; (3) IFN-β (200 I.U./ml)-treated; (4) BFA (0.20 μg/ml) and IFN-β (200 I.U./ml)-treated cells. β-actin (housekeeping gene) served as internal control. (b) Lanes: (1) IFN-β (300 I.U./ml)-treated; (2) BFA (0.2 μg/ml) and IFN-β (300 I.U./ml)-treated cells. GAPDH, a housekeeping gene served as internal control.

of 2-5A dependent RNase was found in cells treated with 100–300 I.U./ml of IFN-β (lanes 3 and 5, respectively) compared to control (lane 1) as well as BFA-treated (lane 2) cells. BFA treatment of uninfected cells along with IFN significantly reduced the level of 2-5A dependent RNase as compared to IFN alone (lanes 4, 5 and 6); however, the activity of 2-5A dependent RNase was completely inhibited by BFA in IFN-treated and EMCV-infected cells (Fig. 7b, lanes 4 and 6), raising the question whether endogenous 2-5A may compete with the 32P-labelled probe.

To rule out the presence of endogenous 2-5A, the cellular extracts [(1/2 of IFN-treated (Fig. 7b, lane 3) + 1/2 IFN and BFA-treated (Fig. 7b, lane 4)], or [(1/2 of IFN-treated (Fig. 7b, lane 5) + 1/2 IFN and BFA-treated (Fig. 7b, lane 6)] were mixed and examined for the nuclease. If 2-5A were present, an inhibition of 2-5A dependent RNase in the mixed cellular extract could be expected to be several fold higher than in IFN-treated cells. However, the level of the nuclease was not decreased more than 50–60% in the mixed cellular extract (Fig. 8, lanes 2 and 4), suggesting that soluble 2-5A or its inhibitor was not present in the IFN and BFA-treated, EMCV-infected cells.

4. Discussion

BFA inhibits the glycosylation and export of proteins. This property of BFA led to the exploration of its antiviral activity against viruses such as corona virus, retrovirus, pseudorabies viruses and alphaviruses that require newly synthesized glycosylated, secreted or membrane bound proteins during its infectious cycle (Tamura et al., 1968; Doms et al., 1989; Takatsuki and Tamura, 1985; Chen et al., 1991; Cheung et al., 1991; Whealy et al., 1991; Maynell et al., 1992). BFA did not have any effect on the infectious cycle of adenovirus because adenovirus did not involve the glycoprotein secretion for its infectivity (Maynell et al., 1992). BFA inhibits the replication of poliovirus, a non-enveloped virus, possibly by preventing the formation of vesicles which are required for RNA replication. In the present study, BFA did not inhibit the EMCV, a non-enveloped virus which is similar to poliovirus. While
studying the effect of BFA on poliovirus replication, BFA was present in the medium during the period of replication of virus, however, in the present study, 20-fold lower concentration of BFA was used and removed before EMCV adsorption and replication. The discrepancy in the inhibition of poliovirus and EMCV by BFA may be due to the BFA dosage and the incubation of the drug during the virus replication cycle.

The mechanism by which IFN inhibit the replication of viruses is not completely understood. Both IFN-inducible protein kinase and 2-5A synthetase enzymatic pathways have been described (Pestka et al., 1987; Hovanessian et al., 1979; Kerr et al., 1974; Stacheli, 1990; Williams et al., 1979; Clemens and Williams, 1978; Samuel, 1988) which appear to be involved in the inhibition of replication of viruses by interfering with the translation of viral RNA.

PKR is highly induced in many IFN-treated cells (Hovanessian, 1989). Upon activation by dsRNA, PKR binds to ATP, is phosphorylated, and catalyses the phosphorylation of the eukaryotic protein synthesis initiation factor (eIF2α), thus mediating the inhibition of protein synthesis (Farrell et al., 1977; Safer, 1983). During EMCV infection, enhanced phosphorylation of PKR has been reported, probably due to its binding to replication complexes of EMVC (Aujean et al., 1979; Levintow, 1974). Data reported in this paper show a significant increase in the level of phosphorylated PKR in IFN-treated uninfected or EMVC-infected L9 cells. BFA treatment along with IFN inhibited either the phosphorylation or expression of PKR in IFN-treated cells, whether EMVC-infected or uninfected, thereby suggesting an overall decrease in the phosphorylation of eIF2α. Thus, there would be no inhibition in the viral protein synthesis in IFN-treated, EMVC-infected cells in the presence of BFA as compared to cells treated with IFN alone.

IFN-induced 2-5A synthetase system includes 2-5A synthetase and latent endonuclease. In the presence of dsRNA, 2-5A synthetase polymerizes ATP into 2-5A. 2-5A activates endonuclease which then degrades mRNA (Silverman et al., 1982), and as a result viral protein synthesis is inhibited. In the present study, Northern and in situ hybridization analysis show that the 2-5A synthetase gene was highly expressed in IFN-treated cells but was significantly decreased in these cells by BFA. Since 2-5A synthetase is an
index of 2-5A, it would appear that there was a concomitant reduction in the level of 2-5A, leading to inhibition in the activation of 2-5A dependent RNase. The level of 2-5A dependent RNase was reduced in IFN plus BFA-treated cells, whether infected or uninfected, as compared to infected or uninfected cells treated with IFN alone. The loss of 2-5A dependent nuclease activity has been monitored both by loss of the 2-5A-binding protein (presumptive nuclease) and directly by loss of activatable nuclease (Silverman et al., 1982; Cayley et al., 1982). Mixing experiments failed to demonstrate the presence of any soluble inhibitor of the nuclease and 2-5A which could compete for the binding with nuclease in the inhibited extracts. Therefore, the decrease in the 2-5A dependent RNase may be attributed to the loss of the nuclease or its inactivation by BFA. The present studies suggest that BFA antagonizes the antiviral activity of IFN against EMCV probably by inhibiting the IFN-induced enzymes at both the transcriptional and post-transcriptional level. IFN induces several genes including the IFN genes themselves. Secretion of newly synthesized IFN may then contribute to the effective dose of IFN received by the cells. BFA treatment could, by inhibiting protein secretion, eliminate this supplemental dose. This explana-

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![Fig. 6. Effect of correlation between the activity and mRNA of 2'-5' A synthetase: L<sub>a</sub> cells (two sets) were treated with IFN using various concentrations (0, 50, 100, 200 and 300 I.U./ml) and BFA (0.2 μg/ml) for overnight. Using first set, RNA was prepared, blotted and hybridized with 2-5A synthetase probe. The autoradiogram was scanned and the inhibition in the transcripts was obtained. Cellular lysate was obtained from the second set and the activity of the enzyme was obtained using radiobinding assay at each dose of IFN in the presence of BFA. The data was plotted to find whether the inhibition of the enzyme by BFA is at transcriptional or post-transcriptional level.](image)

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![Fig. 7. Inhibition of IFN-induced 2-5A dependent RNase activity by BFA in uninfected (a) or EMCV infected (b) L<sub>a</sub> cells. After various treatments, cellular extracts (12000 × g) were prepared using NP40 lysis buffer. This cytoplasmic fraction was incubated with P(A2′P)2(b5′A3′P)2A′[32P]P and linked under U.V. light. After cross linking, proteins were separated on 10% SDS-PAGE. Lanes: (1) untreated control cells; (2) BFA (0.20 μg/ml)-treated cells; (3) mouse IFN-β (100 I.U./ml)-treated cells; (4) combination of 2 and 3 treatments; (5) mIFN-β (300 I.U./ml); (6) combination of 2 and 5 treatments.](image)

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![Fig. 8. Mixing experiment to ascertain the presence of soluble 2-5A in BFA-treated cells: to ascertain the presence of soluble 2-5A, equal amounts of extracts from IFN-treated, and IFN plus BFA treated cells were mixed and the activity of the nuclease was assayed using 10% SDS-PAGE and autoradiography. Lanes: (1) IFN-β (100 I.U./ml); (2) [1/2 IFN-β (100 I.U./ml)] + 1/2 BFA and IFN-β (100 I.U./ml); (3) IFN-β (300 I.U./ml); and (4) [1/2 IFN-β (300 I.U./ml)] + 1/2 BFA and IFN-β (300 I.U./ml).](image)
tion is supported with the data which showed a decreased effect of BFA on the reversal of IFN induced inhibition of EMCV, and 2-5A and 2-5A synthetase with higher (initial) doses of IFN.

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