# Brefeldin A inhibits the antiviral action of interferon against encephalomyocarditis virus

G.S. Sidhu[a,*], A.K. Singh[b], P.N. Raghunath[a], S. Sivaram[b], R.K. Maheshwari[a]

[a]Department of Pathology, Uniformed Services University of Health Sciences, Bethesda, MD 20814-4799, USA
[b]Birla Institute of Technology and Science, Pilani (Rajasthan), India

## 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 $L_B$ cells; Interferon; Encephalomyocarditis

## 1. Introduction

Brefeldin A (BFA), a fungal metabolite antibiotic containing a 13-member macrocytic 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) (Fujiwara 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 secre-

* Corresponding author.

tion of various proteins in various cell types (Fujiwara et al., 1988; Misumi et al., 1986; Magner and Papagiannes, 1988). BFA inhibits the glycoprotein 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 formation (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, transport 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 immunoregulatory (Pestka et al., 1987; Adams and Hamilton, 1988). Several studies (Staeheli, 1990; Samuel, 1988; Gupta et al., 1982; Rice et al., 1985; Hovanessian et al., 1987; Galabru and Hovanessian, 1987) have shown that IFN treatment is associated with the induction of two double stranded RNA-dependent enzymes, 2'-5' oligoadenylate (2-5A) synthetase and protein kinase. These biochemical 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 synthetase, 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 antiviral 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. Buckley, NIAID, NIH, Bethesda, MD, USA) was plaque purified and passaged at a multiplicity of 0.01 TCID$_{50}$/cell. The virus stock was assayed by determining its cytopathological effects (cpe) in L$_B$ cells and the titer was 2 × 10$^9$ TCID$_{50}$/cell.

### 2.3. Reagents

Recombinant murine IFN-$\beta$ was obtained from Toray Industries, Japan. BFA was purchased from Epicenter, Madison, WI. Polyionosinic-polycytidylic acid (poly I:C), a double stranded RNA, was purchased from Pharmacia LBK Biotechnology, USA. [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol), [$\alpha$-$^{35}$S]dCPT (1000 Ci/mmol) and [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) were supplied by DuPont/NEN, Boston, MA.
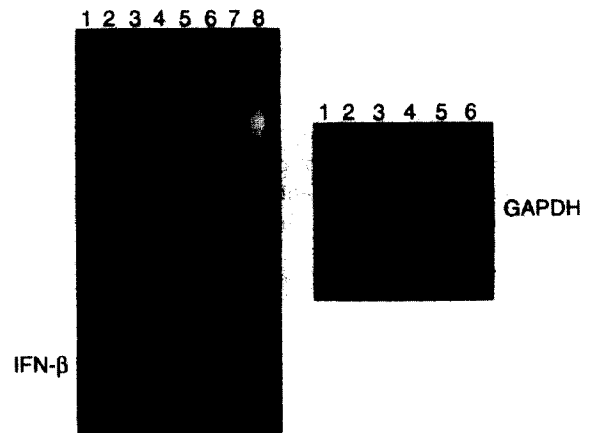


Fig. 1. PCR amplification for IFN-$\beta$ in poly I:C and with or without BFA treated L$_B$ cells: RNA was prepared from normal, poly I:C-treated, and poly I:C and BFA-treated cells, reverse transcribed and amplified for IFN-$\beta$ gene. Amplification for GAPDH from the same reverse transcription reaction was used as an internal control. Lanes: (1) normal cells; (2) BFA; (3) poly I:C; (4) poly I:C + BFA (0.1 $\mu$g/ml); (5) poly I:C + BFA (0.2 $\mu$g/ml); (6) poly I:C + BFA (0.3 $\mu$g/ml); (7) amplification without template; (8) 123 bp ladder. The concentration of poly I:C used was 100 $\mu$g/ml for each treatment.
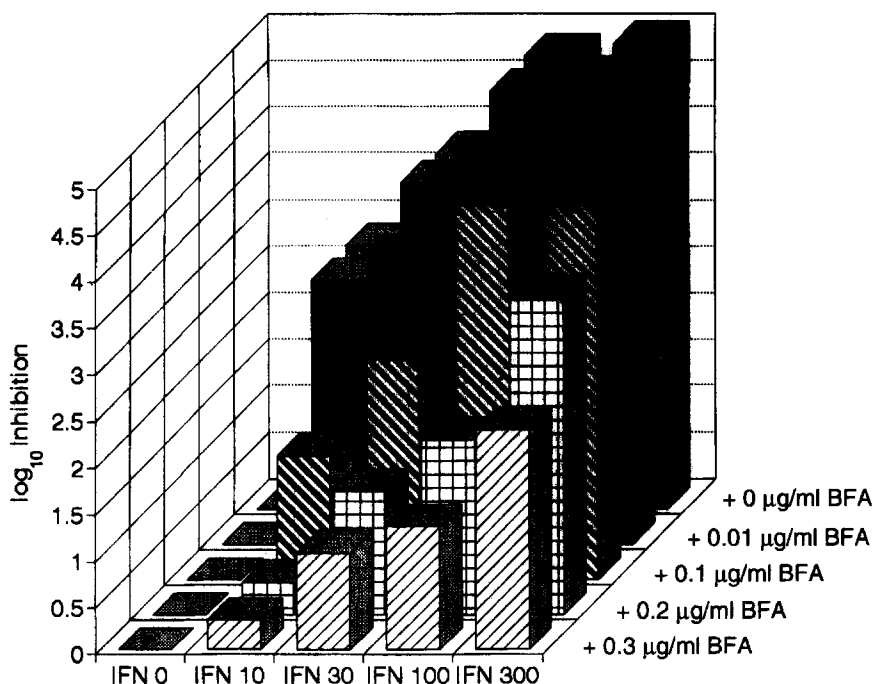
Fig. 2. Effect of BFA on the reversal of IFN-induced inhibition of EMCV. Inhibition in virus yield from cells treated with various doses of IFN (0, 10, 30, 100, 300 I.U./ml) and BFA (0, 0.01, 0.10, 0.20 and 0.30 $\mu$g/ml). These doses of BFA alone did not inhibit the virus yield.

## 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 $\mu$g/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 $TCID_{50}$/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 ($\log_{10}$) compared to control.

## 2.5. Polymerase chain reaction (PCR) for IFN-$\beta$

$L_B$ cells were treated with BFA (0.1, 0.2 and 0.3 $\mu$g/ml) and after 10 h poly I:C (100 $\mu$g/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 denaturating 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 $\mu$l of RT mix, the following components were added; 6 $\mu$l of dNTP mix (100 mM); 10 $\mu$l of 10 × PCR buffer and 0.5 $\mu$l of Taq polymerase (5 U/$\mu$l); 0.4 $\mu$l of sense primer (1 $\mu$g/$\mu$l) and 0.4 $\mu$l of anti-sense primer (1 $\mu$g/$\mu$l) and 79.7 $\mu$l 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 $L_B$ cells were treated with IFN-$\beta$ (0–300 I.U./ml) and/or BFA (0.20 $\mu$g/ml) for 16 h. One set of cells were infected with EMCV at a multiplicity of 1.0 $TCID_{50}$/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 KCl, 0.5% (v/v) NP40, 2 mM mercaptoethanol), containing 2 $\mu$g/ml leupeptin and phenylmethylsulfonyl fluoride (PMSF). Protein kinase assay was done as described (Dubois and Hovanessian, 1990) in these extracts. Briefly, cellular extracts (100 $\mu$g protein) were incubated with poly I:C at $1 \times 10^{-6}$ g/ml, ATP at 0.1 mM, $MgCl_2$ at 10 mM and 1 $\mu$l [$\gamma$-$^{32}$P]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. Phosphoproteins 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, $P(A2';)_2(br^8A2'p)_2A3'-[^{32}P]Cp$, 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 $\mu$g/ml protease inhibitor leupeptin and PMSF. The lysates were centrifuged at 12000 × g and the cellular extracts (100 $\mu$g 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 $\mu$g protein was made up to 15 $\mu$l with DBG (10 mM Hepes pH 7.5, 50 mM KCl, 1.5 mM magnesium acetate, 7 mM mercaptoethanol, 20% v/v glycerol) buffer and 15 $\mu$l 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 $\mu$l) (DBG, 8 mM ATP pH 7.0, 15 mM $MgCl_2$) 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 [$\alpha$-$^{35}$S]dCTP (1000 Ci/mmol) with random primers for in situ hybridization or [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) with Klenow enzyme for Northern hybridization.

### 2.10. In-situ hybridization

$L_B$ cells were treated with BFA (0.2 $\mu$g/ml) and/or IFN-$\beta$ (100 and 300 I.U./ml) overnight. The cells were then trypsinized, adjusted to a concentration of $1 \times 10^6$ cells/ml, and 200 $\mu$l of the cell suspension was collected on silanated and activated slides by cytospinning in a cytocentrifuge at 350 × g for 5 min. In situ hybridizations were performed as described by Peltonen et

al., 1989, 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 $\mu$g/ml $^{35}$S-labelled cDNA probe of 2-5A synthetase, 10 mM dithiothreitol, 500 $\mu$g/ml acetylated bovine serum albumin, 0.3 M sodium chloride, 50% deionized formamide, 10% (w/v) dextran sulfate, 500 $\mu$g/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, $2 \times 10^7$ 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 MgCl$_2$, 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 H$_2$O (20–30 $\mu$l) 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 $(1 \times 10^6$ counts/min/ml) was added to the hybridization solution and filters were incubated overnight at 42°C. Blots were washed (hybridiza-

tion/washing conditions used were stringent), exposed to X-ray film, and autoradiograms were developed.

## 3. Results

### 3.1. Induction of IFN-$\beta$ gene in poly I:C and BFA treated cells

It has been shown that BFA (1–5 $\mu$g/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 [$^3$H]thymidine, therefore, the lower doses of BFA were examined for cytotoxicity. We found that the concentration of BFA above 0.3 $\mu$g/ml was cytotoxic when incubated for 16 h with cells as demonstrated by cell count and uptake of [$^3$H]thymidine (data not shown), therefore, concentration of BFA ranging from 0.01–0.3 $\mu$g/ml were used to study its effects on virus inhibition in the presence of IFN.

It is reported that dsRNA directly induces IFN-$\beta$ 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-$\beta$ gene in poly I:C and BFA-treated cells was undertaken. The RT-PCR showed that IFN-$\beta$ 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 $\mu$g/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 $\mu$g/ml or 0.2 $\mu$g/ml BFA, 0.2 $\mu$g/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).
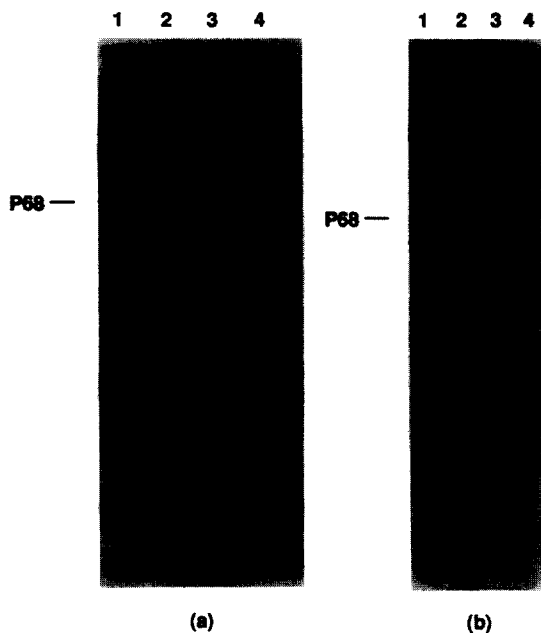


Fig. 3. Phosphorylation of PKR in extracts from uninfected (a) or EMCV infected (b) cells. The cellular extracts were prepared using NP40 lysis buffer from BFA, IFN, or BFA and IFN treated, infected or uninfected cells. PKR phosphorylation was assayed using [$\gamma$-$^{32}$P]ATP and dsRNA. Phosphoproteins were analyzed by 10% SDS-PAGE and autoradiography. Lanes: (1) untreated; (2) BFA (0.20 $\mu$g/ml)-treated; (3) IFN-$\beta$ (300 I.U./ml)-treated; (4) IFN-$\beta$ (300 I.U./ml) and BFA (0.20 $\mu$g/ml)-treated cells.

### 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) L$_B$ 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) L$_B$ cells. A significant increase in the activity

Fig. 4. (a and b) Expression of 2-5A synthetase: $L_B$ cells were treated with BFA, IFN, or BFA and IFN for 16 h. RNA was isolated, blotted, and Northern hybridization was done using $^{32}$P-labelled cDNA of murine 2-5A synthetase probe. (a) Lanes: (1) untreated control cells; (2) BFA (0.20 $\mu$g/ml)-treated; (3) IFN-$\beta$ (200 I.U./ml)-treated; (4) BFA (0.20 $\mu$g/ml) and IFN-$\beta$ (200 I.U./ml)-treated cells. $\beta$-actin (housekeeping gene) served as internal control. (b) Lanes: (1) IFN-$\beta$ (300 I.U./ml)-treated; (2) BFA (0.2 $\mu$g/ml) and IFN-$\beta$ (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-$\beta$ (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 $^{32}$P-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
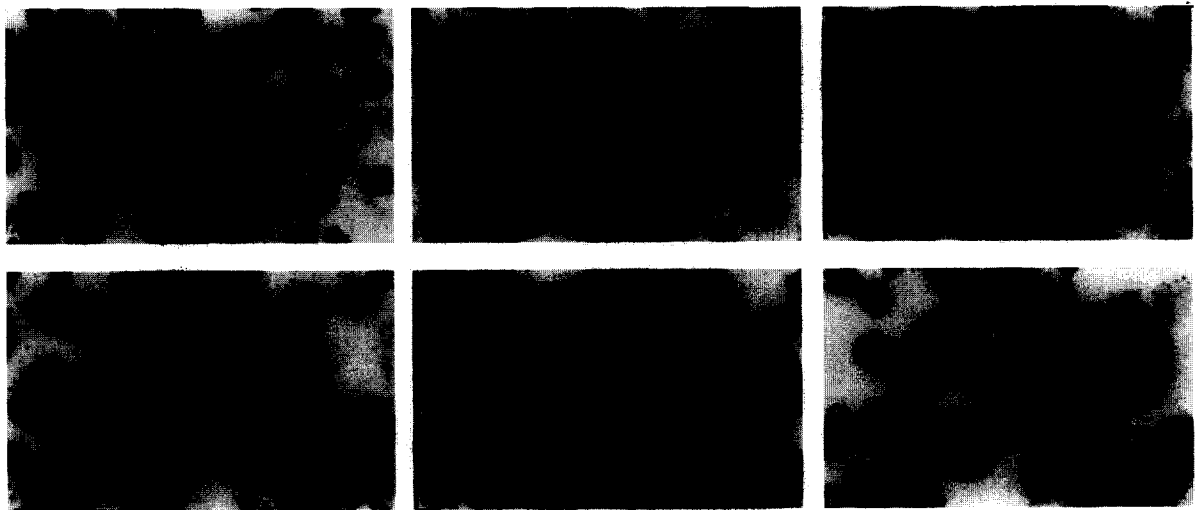
Fig. 5. Effect of 2-5A synthetase mRNA by in situ hybridization in $L_B$ cells: cells were cytospinned on activated slides and hybridized with [$^{35}$S]-labelled murine cDNA probe of 2-5A synthetase. (A) untreated control cells; (B) BFA (0.2 $\mu$g/ml)-treated cells; (C and E) cells treated with IFN-$\beta$ (100 I.U./ml) and (300 I.U./ml), respectively; (D and F) cells treated with BFA (0.2 $\mu$g/ml) plus IFN-$\beta$ (100 I.U./ml) and (300 I.U./ml), respectively.

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; Staeheli, 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 (eIF2$\alpha$), 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 EMCV (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 EMCV-infected $L_B$ cells. BFA treatment along with IFN inhibited either the phosphorylation or expression of PKR in IFN-treated cells, whether EMCV-infected or uninfected, thereby suggesting an overall decrease in the phosphorylation of eIF2$\alpha$. Thus, there would be no inhibition in the viral protein synthesis in IFN-treated, EMCV-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
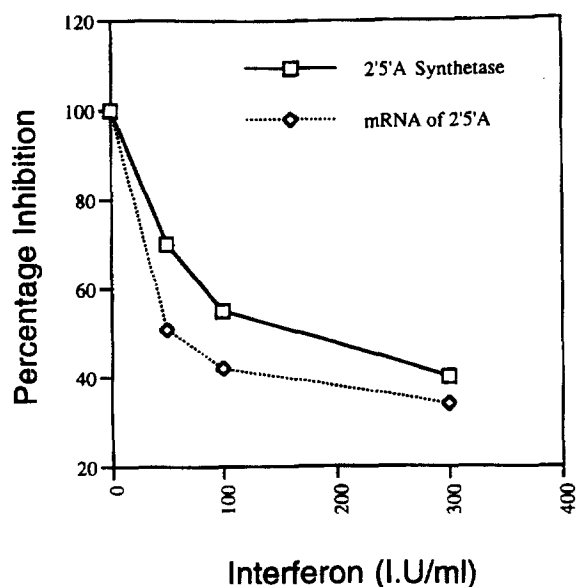


(a)                                    (b)

Fig. 7. Inhibition of IFN-induced 2-5A dependent RNase activity by BFA in uninfected (a) or EMCV infected (b) $L_B$ cells. After various treatments, cellular extracts (12000 × $g$) were prepared using NP40 lysis buffer. This cytoplasmic fraction was incubated with $P(A2'P)_2(b_2^8A_2'P)_2A_3'-[^{32}P]Cp$ 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 $\mu$g/ml)-treated cells; (3) mouse IFN-$\beta$ (100 I.U./ml)-treated cells; (4) combination of 2 and 3 treatments; (5) mIFN-$\beta$ (300 I.U./ml); (6) combination of 2 and 5 treatments.

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-



Fig. 6. Effect of correlation between the activity and mRNA of 2'5' A synthetase: $L_B$ cells (two sets) were treated with IFN using various concentrations (0, 50, 100, 200 and 300 I.U./ml) and BFA (0.2 $\mu$g/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.
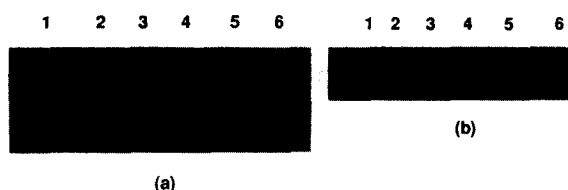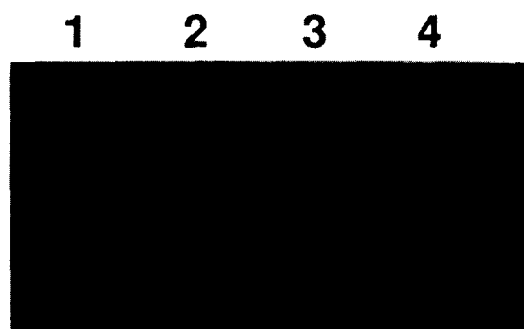


Fig. 8. Mixing experiment to ascertain the presence of soluble 2-5A in BFA-treated cells: to ascertain the presence of soluble 2-5A, equal amounts of extracts from IFN-treated, and IFN plus BFA treated cells were mixed and the activity of the nuclease was assayed using 10% SDS-PAGE and autoradiography. Lanes: (1) IFN-$\beta$ (100 I.U./ml); (2) [1/2 IFN-$\beta$ (100 I.U./ml) + 1/2 BFA and IFN-$\beta$ (100 I.U./ml)]; (3) IFN-$\beta$ (300 I.U./ml); and (4) [1/2 IFN-$\beta$ (300 I.U./ml) + 1/2 BFA and IFN-$\beta$ (300 I.U./ ml).

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.

## Acknowledgements

## References

Adams, D.O. and Hamilton, T.A. (1988) Phagocytic cells: cytotoxic activities of macrophages. In: J.I. Galkin, I.M. Goldstein and R. Synderman (Eds), Inflammation, Basic Principles and Clinical Correlates. Raven Press, New York, pp. 471–492.

Aujean, O., Sanceau, J., Falcoff, E. and Falcoff, R. (1979) Localization of enhanced ribonuclease activity and of a phosphoprotein kinase in interferon treated Mengovirus-infect cells. Virology 92, 583–586.

Cayley, P.G., Silverman, R.H., Balkwill, F.R., McMohan, M., Knight, M. and Kerr, I.M. (1982) The 2-5A system and interferon action. Biochem. Biophys. Res. Commun. 104, 376–382.

Chen, S.Y., Matsuoka, Y. and Compans, R.W. (1991) Assembly and polarized release of punta toro virus and effects of Brefeldin A. J. Virol. 65, 1427–1439.

Cheung, P., Banfield, B.W. and Tufaso, F. (1991) Brefeldin A arrests the maturation and egress of Herpes Simplex virus particles during infection. J. Virol. 65, 591–598.

Clemens, M.J. and Williams, B.R.G. (1978) Inhibition of cell free protein synthesis by pppA2'p5'A2'p5'A: a novel oligonucleotide synthesized by interferon treated L cell extracts. Cell 13, 565–572.

Doms, R.W., Russ, G. and Yewdell, J.W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. J. Cell Biol. 109, 61–72.

Dubois, M.F. and Hovanessian, A.G. (1990) Modified subcellular localization of interferon induced P68 kinase during encephalomyocarditis virus infection. Virol. 179, 591–598.

Earl, P.L., Moss, B. and Doms, R.W. (1991) Folding, interaction with GRP78-BiP, assembly and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. 65, 2047–2055

Farrell, P.J., Balkow, K., Hunt, T., Jackson, J. and Trachsel, H. (1977) Phosphorylation of initiation factor eIF2 and the control of reticulocyte protein synthesis. Cell 11, 187–200.

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A. and Ikenhara, Y. (1988) Brefeldin A causes disassembly of the Golgi-complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. 263, 18545–18552.

Galabru, G. and Hovanessian, A. (1987) Autophosphorylation of the protein kinase dependent on double stranded RNA. J. Biol. Chem. 262, 15538–15544.

Gupta, S.L., Holmes, S.L. and Mehra, L.L. (1982) Interferon action against Reovirus: activation of interferon induced protein kinase in mouse L929 cells upon Reovirus infection. Virology 120, 495–499.

Harri, E., LoeMer, W., Singh, H.P., Stahlin, H. and Tamm, C. (1963) Die constitution von Brefeldin A. Helv. Chem. Acta. 46, 1235–1243.

Hovanessian, A.G. (1989) The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. J. Interferon Res. 9, 641–647.

Hovanessian, A.G., Galabru, J., Meurs, E., Buffet-Janverse, C., Svab, J. and Robert, N. (1987) Rapid decrease in the levels of the double stranded RNA-dependent protein kinase during virus infection. Virology 159, 126–136.

Hovanessian, A.G., Wood, J.N., Meurs, E. and Montagnies, L. (1979) Increased nuclease activity in cells treated with pppA2'p5'A2'p5'A. Proc. Natl. Acad. Sci. USA 76, 3261–3265.

Kerr, I.M., Brown, R.E. and Ball, L.A. (1974) Increased sensitivity of cell free protein synthesis to double stranded RNA after interferon treatment. Nature 250, 57–59.

Knight, M., Caley, P.J., Silverman, R.H., Wreschner, D.H., Gilbert, C.S., Brown, R.E. and Kerr, I.M. (1980) Radioimmune, radiobinding and HPLC analysis of 2-5A and related oligonucleotides from intact cells. Nature 288, 189–192.

Levintow, L. (1974) The reproduction of picornavirus, In: Fraenkel-Conrat, H. and R.R. Wagner (Eds), Comprehensive Virology, Vol 2, pp. 109–169. Plenum Press, New York.

Lippincott-Schwartz, J., Yuan, L.C., Banifacino, J.S. and Klausner, R.G. (1989) Rapid distribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from the Golgi to ER. Cell 56, 912–920.

Magner, J.A. and Papagiannes, E. (1988) Blockade by brefeldin A of intracellular transport of secretory proteins in mouse pituitary cells; effects on the biosynthesis of thyrotropin and free cY-subunits. Endocrinology 122, 912–920.

Maheshwari, R.K. and Friedman, R.M. (1979) Production of stomatitis virus with low infectivity by interferon-treated cells. J. Gen. Virol. 44, 261–264.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning, A Laboratory Manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

Maynell, L.A., Kirkegaard, K. and Klymkowski, M.W. (1992). Inhibition of poliovirus RNA synthesis by Brefeldin A. J. Virol. 66, 1985–1994.

Memet, S., Besancon, F., Bourgeade, M.-F. and Thang, M.N. (1991) Direct induction of IFN-$\gamma$ and interferon $\alpha/\beta$-inducible genes by double stranded RNA. J. Interferon Res. 11, 131–141.

Misumi, Y., Miki, K., Takatsuki, A., Tamma, G. and Ikenhara, Y. (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. 261, 11398–11403.

Nolan-Sorden, N.L., Lesiak, N.L., Lesiak, K., Bayard, B., Torrence, P.F. and Silverman, R.H. (1990) Photochemical cross-linking in oligonucleotide-protein complexes between a bromine-substituted 2-5A analog and 2-5A dependent RNase by ultraviolet lamp or laser. Anal. Biochem. 184, 298–304.

Pal, R., Mumbauer, S., Hoke, G.M., Takats-Iki, A. and Sarnagadharan, M.G. (1991) Brefeldin A inhibits the processing and secretion of envelope glycoproteins of human immunodeficiency virus type I. AIDS Res. Human Retrovir. 7, 707–712.

Peltonen, J., Jaakkola, S., Gay, K., Olsen, D.R., Chu, M.L. and Uitto, J. (1989) Expression of extracellular matrix genes by cultured human cells: localization of messenger RNAs and antigenic epitopes. Anal. Biochem. 178, 184–193.

Pestka, S., Langer, J.A., Zoon, K.C. and Samuel, C.E. (1987) Interferons and their actions. Annu. Rev. Biochem. 56, 727–777.

Reaves, B. and Banting, G. (1992) Perturbations of the morphology of the trans-Golgi network following BFA treatment: redistribution of a TGN specific integral membrane protein TGN38. J. Cell Biol. 116, 85–94.

Rice, A.P., Duncan, R., Hershey, J.W.B. and Kerr, I.M. (1985) Double-stranded RNA-dependent protein kinase and 2-5A system are both activated in interferon treated, encephalomyocarditis virus-infected HeLa cells. J. Virol. 54, 894–898.

Safer, B. (1983) 2B or not 2B: regulation of the catalytic utilization of eIF2. Cell 33, 7–8.

Samuel, C.E. (1988) Mechanisms of the antiviral action of interferons. Prog. Nucl. Acid Res. Mol. Biol. 35, 27–72.

Silverman, R.H., Cayley, P.J., Knight, M., Gilbert, C.S. and Kerr, I.M. (1982) Control of the ppp(A2'p)nA system in HeLa cells. Effects of interferon and virus infection. Eur. J. Biochem. 124, 131–138.

Singh, V.K., Maheshwari, R.K., Damewood, G.P., IV, Stephenson, C.B., Oliver, C. and Friedman, R.M. (1988) Interferons alter intracellular transport of vesicular stomatitis virus glycoprotein. J. Biol. Regul. Homeostat. Agents 2, 53–62.

Staeheli, P. (1990) Interferon induced proteins and antiviral state. Adv. Virus Res. 38, 147–200.

Stark, G.R., Brown, R.E. and Kerr, I.M. (1982) Assay of (2'-5')-oligoadenylic acid synthetase levels in cells and tissues: a convenient poly(I).poly (C) paper bound enzyme assay. Methods Enzymol. 79, 194–199.

Takatsuki, A. and Tamura, G. (1985) Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitis virus G protein: intracellular accumulation of high mannose type G protein and inhibition of its cell surface expression. Agric. Biol. Chem. 49, 889–902.

Tamura, G., Ando, K., Suzuki, S., Takatsuki and Arita, K. (1968) Antiviral activity of Brefeldin A and Verrucarin A. J. Antibiotic 26, 160–161.

Whealy, M.E., Card, J.P., Meade, R.P., Robbins, A.K. and Enquist, L.W. (1991) Effect of Brefeldin A on Alphaherpesvirus membrane protein glycosylation and virus egress. J. Virol. 65, 1066–1081.

Williams, B.R.G., Golgher, R.R., Brown, R.E., Gilbert, C.S. and Kerr, I.M. (1979) Natural occurence of 2-5A in interferon-treated EMC virus infected L cells. Nature 282, 582–586.

Wood, S.A., Park, J.E. and Brown, W.J. (1991) Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosome. Cell 67, 591–600.