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The Mechanism of Poly I:C-Induced Antiviral Activity in Peritoneal Macrophage

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Macrophages play an important role in defense against virus infection by intrinsic resistance and by extrinsic resistance. Since interferon-induced enzymes which are 2'-5' oligoadenylate synthetase and P1/eIF-2 protein kinase have been shown to be involved in the inhibition of viral replication, I examined the mechanism by which poly I:C, an interferon inducer, exerts its antiviral effects in inflammatory macrophages infected with herpes simplex virus type 1 (HSV-1). The data presented here demonstrate that poly I:C-induced antiviral activity is partially due to the activation of 2'-5' oligoadenylate synthetase. The activation of 2'-5' oligoadenylate A synthetase by poly I:C is also at least partly mediated via the production of interferon-β. Taken together, these data indicate that interferon-β produced in response to poly I:C acts in an autocrine manner to activate the 2'-5' oligoadenylate synthetase and to induce resistance to HSV-1.

Key words: Poly I:C, Interferon, 2'-5' oligoadenylate synthetase, Protein kinase

INTRODUCTION

Macrophages, important cells of a host defense system, have been shown to be involved in antiviral activity by killing virus infected cells (extrinsic resistance) and by inhibiting intracellular replication of virus (intrinsic resistance) (Morahan, 1984; Morahan *et al.*, 1985). Indeed macrophages are generally resistant to infection with a variety of viruses, including flaviviruses, hepatitis viruses, herpes viruses, cytomegaloviruses, and influenza viruses (Allison, 1974; Mims, 1964; Mogensen *et al.*, 1989). The mechanisms by which macrophages are resistant to virus infection remains unclear but in some instances interferon has been implicated (Proietti *et al.*, 1986; Linnavuori *et al.*, 1983) while in other cases it has not (Sit *et al.*, 1988).

Macrophages can be activated by a variety of agents and some of these have also been shown to increase resistance to viruses when administered *in vivo* (Gangemi *et al.*, 1987; Glasgow *et al.*, 1977; McGeoch, 1989; Sarzotti *et al.*, 1989; Singh *et al.*, 1989). For instance, treatment of mice with *Propionibacterium acnes* has been shown to result in increased resistance to infection with herpes viruses (Glasgow *et al.*, 1977; Kirchner *et al.*, 1977; Morahan *et al.*, 1977). This increased resistance may be partially due to increased intrinsic antiviral activity of macrophage since macrophages from *P. acnes*-treated mice were shown to be more resistant than inflammatory macrophages to infection with HSV-1 *in vitro* (Sit *et al.*, 1988).

Polyriboinosinic : Polyribocytidylic acid (Poly I:C) has been shown to be a potent macrophages activator (Alexander *et al.*, 1971; Schultz *et al.*, 1977) and interferon inducer (Field *et al.*, 1967; Greene *et al.*, 1986), which is effective in the prophylactic and therapeutic treatment of viral infections (Catalano *et al.*, 1970; Heckman *et al.*, 1981; Kern *et al.*, 1975). In addition, poly I:C has been shown to be effective *in vitro* in inducing resistance to virus infection in various cell types (Van Damme *et al.*, 1987).

The mechanisms by which poly I:C exerts its antiviral effects are not fully understood. Recently, poly I:C has been shown to exert its intrinsic antiviral activity in inflammatory macrophages via the production of IFN-β (Pyo *et al.*, 1991). Interferons have been shown to induce the production of several proteins including 2'-5' oligoadenylate (2-5A) synthetase and P1/eIF-2α protein kinase (protein kinase) which are activated by double-stranded RNA. These two enzymes are involved in the inhibition of viral replication (Lengyel, 1982). The role that IFN-β produced by poly I:C activated macrophages plays in virucidal activity has not been

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fully elucidated. In this study I have examined whether poly I:C-induced antiviral activity in macrophage is mediated via 2-5A or protein kinase and poly I:C-induced IFN-β is involved in the activation of these enzymes. The results demonstrated that 2-5A is partially involved in poly I:C-induced antiviral activity and activation of 2-5 A by poly I:C is at least partly mediated by the production of IFN-β.

MATERIALS AND METHODS

Mouse and Herpes Simplex Virus Strains

Male CD-1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and the KOS strain of HSV-1 was kindly provided by Dr. M. Nachtigal (Department of Pathology, University of South Carolina, Columbia SC USA). Stock virus was prepared by propagation in Vero cells (African green monkey) for 24 hours in RPMI-1640 supplemented with 2% fetal calf serum at a multiplicity of infection (moi) of 0.02.

Chemicals and Antibodies

Unless otherwise indicated, all chemicals were purchased from Sigma chemical Co. (St. Louis, MO). Polyclonal anti-mouse IFN-β antibodies (10,000 IU/ml) were purchased from Lee Biomolecular Research Laboratories, Inc. (San Diego, CA 92121). Culture media were assayed for endotoxin contamination by the Limulus lysate test (E-Toxate; Sigma) and found to be less than 0.05 units/ml.

Isolation of Inflammatory Peritoneal Macrophages

Thioglycolate-elicited peritoneal exudate cells were obtained from 4 to 6 weeks old male CD-1 mice following intraperitoneal injection of 1ml Brewer thiglycolates Broth (4.05 g/100 ml) (Difco Laboratories, Detroit, MI) and lavage of the peritoneal cavity with 5 ml of medium 3~4 days later. The cells were washed twice and resuspended in RPMI-1640 (Gibco, Grand Island, New York) containing 10% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 mg/ml) (RPMI-FCS).

Macrophages were isolated from peritoneal exudate cells (Klimetzek *et al.*, 1980). Briefly, peritoneal exudate cells were seeded at densities of $5-6\times10^5$ cells/cm^2 on teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2~3 hours in a 5% CO_2 humidified atmosphere. Teflon-coated petri dishes were prepared by spraying with aerosolized teflon (Fisher Scientific, Pittsburgh, PA) and sterilizing with ultraviolet light for 3 hours. The nonadherent cells were removed by washing the dishes twice with 10 ml prewarmed RPMI-1640 and the dishes were incubated for 10 min at 4℃. The supernatants were then carefully removed and discarded and the plates were washed once with prewarmed Dulbecco's Phosphate Buffered Saline (PBS) (Gibco). Cold PBS (15 ml) containing 1.5% FCS (PBS-FCS) was added followed by 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing 10 times using a 10 ml syringe. The detached cells were washed once with PBS-FCS and resuspended in RPMI-FCS. The viability of the detached cells was assessed by trypan blue exclusion and the proportion of macrophages was determined after cytoplasmic staining with acridine orange and examination using a fluorescence microscope. Cell preparations were >95% viable and contained > 90% macrophages.

Treatment of Peritoneal Macrophages with Poly I:C

Peritoneal macrophages were seeded at a concentration of 2×10^5 cells/well in 96 well tissue culture plates (Costar Products, Cambridge, MA). Nonadherent cells were removed after 1 hour at 37℃ by washing twice with RPMI-FCS and the macrophages were incubated with 5 μg/ml of poly I:C for the times indicated in the text. This concentration of poly I:C has been shown to induce maximal antiviral activity against HSV-1 infected macrophages (Pyo *et al.*, 1991). Macrophage monolayers were washed with RPMI-FCS to remove poly I:C and then infected with virus as described below. In some experiments inhibitors of cytotoxic pathways, which are known to be involved in macrophage cytotoxicity, were included along with poly I:C.

Infection of Inflammatory Macrophages with HSV-1

Macrophages were infected with the KOS strain of HSV-1 at a moi of 7 in a volume of 25 μl. After 1 hour at 37℃, the nonadsorbed virus was removed by washing with RPMI-FCS and the cells were incubated for 48 hours at 37℃ in 5% CO2. Cytopathic effects (CPE) were determined using a neutral red dye uptake assay (Pyo *et al.*, 1991). Assessment of CPE by this method correlated well with visual observation of the cells.

Neutral Red Dye Uptake Assay

The neutral red dye uptake assay to measure virus cytopathic effects (CPE) was performed as described below. After incubation of the HSV-1 infected macrophages the monolayers were washed twice with RPMI-FCS and stained for 2 hours with 0.006% neutral red in medium. The stained monolayers were washed with RPMI by aspiration and the dye extracted with 0.2 ml of Sorensen's citrate buffer (pH 4.1) containing 50% ethanol for 15 minutes on rotary shaker. The optical

density was read at 530 nm in an Dynatech MR 600 microplate reader (Dynatech, Alexander, VA). Virus CPE was evaluated by calculation of a viability index, which is expressed as the ratio of dye uptake by infected cells to dye uptake by uninfected cells according to the following formula:

$$\text{Viability Index (\%)} = \frac{\text{OD of virus infected cells}}{\text{OD of uninfected cells}} \times 100$$

In cultures treated with poly I:C or inhibitors, controls to assess possible toxic effects were also employed and the viability index was calculated using the values obtained from drug-treated infected and uninfected cells. However, at the drug dosages employed, no toxicity was observed.

Assay of 2'-5' Oligoadenylate Synthetase

The activity of the enzyme was estimated by measuring the extent of polymerization of [^{32}P] ATP into oligomers with modification (Minks *et al.*, 1979). Peritoneal macrophages (5×10^5 cells/ml/well) were seeded in a 24 well plastic plate. Cells were allowed to adhere to the plastic plate at 37℃ for 2 hours. The adherent macrophages were treated with poly I:C for various times (0, 6, 12, 24, and 36 hours). They were then washed three times with PBS and lysed in a 100 μl of lysis buffer (buffer A) containing 10 mM N-2-hydroxyethyl- piperazine-N'-ethane-sulfonic acid buffer, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 7 mM dithiothreitol, 20% glycerol, and 0.5% Nonidet-P40 (NP-40). After centrifugation for 6 min at 8000×g, supernatants were removed and stored at −70℃ before assay. In the assays, 20 μl of cell extracts was mixed with 50 μl of poly I:C-coated agarose beads (Pharmacia, Uppsala, Sweden) which had been prewashed for 15 minutes at 30℃. The beads which had absorbed the 2-5A synthetase were washed with 1 ml of buffer A and incubated for 20~22 hours at 30℃ with a 10 μl reaction mixture containing buffer A, 3 mM ATP, 1.6 μCi of [α-^{32}P] ATP (Amersham Co.: 400 Ci/mmol), 10mM creatin phosphate, 3 mg of creatin kinase per ml and 40 μg of poly I:C per ml. One unit of bacterial alkaline phosphatase in 40 ml of 0.1 ml Tris base was added to the tubes, which were then incubated for 2 hours at 37 ℃. After centrifugation, 20 μl aliquots of the supernatants were added to 300 μl of acid alumina columns which were previously washed with 1 ml of 1 M glycine, 0.9 M HCl, pH 2 (buffer B). The columns were then washed with 3 ml of buffer B. The labeled free phosphate, released by the phosphatase treatment, was efficiently adsorbed by the alumina, while the phosphatase-resistant cores of the oligoisoadenylates are eluted. The elute was collected and counted directly in liquid scintillation counter.

RESULTS

Activation of 2'-5' Oligoadenylate Synthetase

The double-stranded RNA-dependent 2-5A synthetase is present in extracts of various cells (Ball *et al.*, 1978; Baglioni *et al.*, 1979; Roberts *et al.*, 1976; Zbar *et al.*, 1971), and in many cases its level can be raised by exposure to interferon (Ball *et al.*, 1978; Baglioni *et al.*, 1979; Kimchi *et al.*, 1979; Minks *et al.*, 1979). This increase seems to have an important role in the establishment of an antiviral state by interferon (Kimchi *et al.*, 1979; Wolf *et al.*, 1990; Woodhour *et al.*, 1969). In order to examine whether poly I:C activates 2-5A synthetase in mouse peritoneal macrophages, the kinetics of conversion of ATP into oligoadenylate phosphatase-resistant cores was investigated. Peritoneal macrophages were treated with 5 μg/ml of poly I:C for 24 hours at 37℃. Cell extracts were prepared using NP-40 and 2-5A synthetase absorbed onto poly I:C-agarose beads. After washing, the beads were incubated for various hours (0, 6, 12, 24, and 36 hours) at 30℃ with [α-^{32}P]ATP, and the soluble reaction products were treated with alkaline phosphatase to release the free phosphate and passed through the small alumina columns to adsorb the free phosphate. The synthesis of 2-5A proceeded linearly for up to 36 hours (data not shown).

The assay described above was then used to measure 2-5A synthetase activity in cells treated with 5 μg/ml of poly I:C for increase times. Treatment with poly I:C resulted in a gradual increase in 2-5A synthetase, while enzyme activity of control medium-treated macrophages was low (Table I). These data suggest that poly I:C induced the production of 2-5A synthetase in macrophages.

Table I. Effects of time of poly I:C treatment on the 2'-5' oligo A synthetase in macrophages by poly I:C

Time of treatment	2'-5' oligo A synthetase activity (cpm×10 ^{1}/10^6 cells)	
	Exp. 1	Exp. 2
control (24 h)	0.9± 0.2	2.0± 0.3
6 hr	4.1± 0.4*	4.4± 0.1*
12 hr	7.2± 1.1*	8.0± 0.5*
24 hr	11.9± 0.7*	16.3± 2.9*

The cells were treated with poly I:C (5 μg/ml) for indicated times. Control was cultured with medium alone for 24 hours. After extraction and 22 hours assay with extracts, phosphatase-treated supernatant was applied on alumina columns, and the effluent radioactivity was counted. The means± SD of the duplicates are indicated. *Significantly different from control (medium treated), $P<0.001$

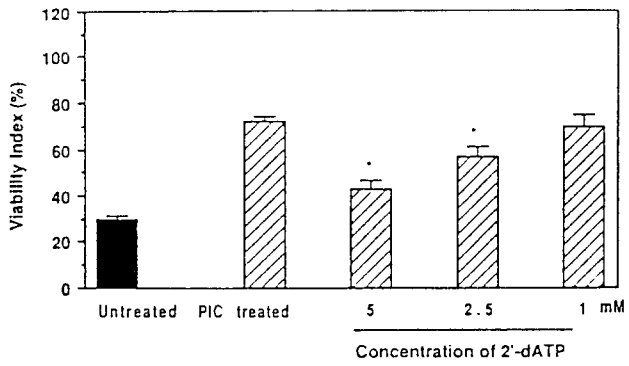

Fig. 1. Inhibition of poly I:C-induced antiviral activity by 2'-dATP.
Macrophages were treated with poly I:C (5 μg/ml) in the presence of various doses of 2'-dATP. Cells were infected with HSV-1 (KOS strain: 7 moi) 24 hours later and CPE determined after an additional 48 hours. The result were expressed as the viability index. Data presented are mean± SD (bars) of quintuplicates from a representative experiments. *Significantly different from poly I:C treatment, $p<0.005$ to $p<0.001$.

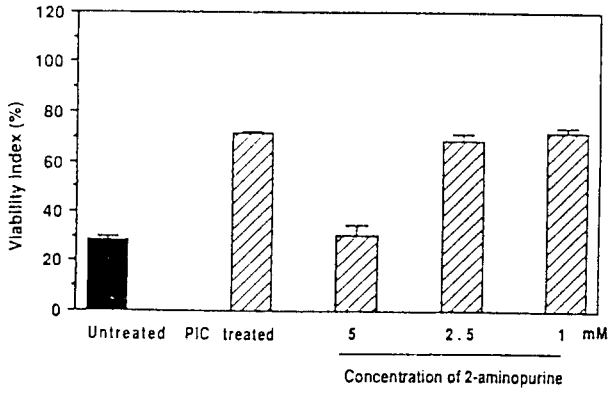

Fig. 2. Inhibition of poly I:C-inducedantiviral activity by 2-aminopurine.
Macrophages were treated with poly I:C (5 μg/ml) in the presence of various doses of 2-aminopurine. Cells were infected with HSV-1 (KOS strain: 7 moi) 24 hours later and CPE determined after an additional 48 hours. The result were expressed as the viability index. Data presented are mean± SD (bars) of quintuplicates from a representative of 3 experiments.

Table II. Effects of increasing concentrations of 2'-dATP on activation of 2'-5' oligo A synthetase in macrophages by poly I:C.

Treatment		2'-5' oligo A synthetase activity (cpm×10 ^{3}/10^{6} cells)	
Poly I:C	2'-dATP	Exp. 1	Exp. 2
−	−	1.1± 0.2	0.8± 0.1
+	−	33.6± 3.4	36.3± 1.9
+	10 mM	1.1± 0.3	1.0± 0.1
+	5 mM	5.2± 0.8	3.2± 0.5
+	1 mM	20.7± 2.7	20.0± 1.6

The cells were treated for 24 hours with poly I:C (5 μg/ml). After extraction and 22 hours assay with extracts in the presence of 2'-dATP, phosphatase-treated supernatant was applied on alumina columns, and the effluent radioactivity was counted. The means± SD of the duplicates are indicated.

Determination of the Involvement of 2-5A Synthetase Induction in the Poly I:C-induced Antiviral Activity

The following experiment was conducted to determine whether 2-5A synthetase was involved in the poly I:C-induced antiviral activity. Macrophages were treated with poly I:C for 24 hours in the presence of 2'-ATP, an inhibitor of 2-5A synthetase (Muller *et al.*, 1984). Following treatment, the cells were infected with HSV-1, cultured for an additional 48 hours in the presence of 2'-dATP and CPE was determined. The high concentration (5 mM) of inhibitor used in this experiment did not result in cytotoxicity, but concentrations greater than 5 mM were cytotoxic to cells. The results presented in Figure 1 show that the poly I:C-induced antiviral activity was partially inhibited in a dose-dependent manner. Moreover, 2'-dATP inhibited the activity of 2-5A synthetase in a dose-dependent manner (Table 2). These results suggest that in macrophages, the induction of antiviral activity may depend at least in part on a pathway involving 2-5A synthetase.

Examination of the Involvement of Protein Kinase in the Poly I:C-induced Antiviral Activity

It has been known that IFN activates ds-RNA dependent protein kinases which is also involved in the antiviral effects of IFN (Petska *et al.*, 1987). In order to examine whether a ds-RNA dependent protein kinases are involved in poly I:C-induced antiviral activity, cells were treated for 24 hours with poly I:C in the presence of 2-aminopurine, an inhibitor of both protein kinases and 2-5A synthetase (P. Tso *et al.*, 1976). The cells were infected with HSV-1, cultured for an additional 48 hours in the presence of 2-aminopurine and CPE was determined. Figure 2 shows that 2-aminopurine completely inhibited the antiviral activity of poly I:C at a high concentration (5 mM), but not at lower concentrations. This high concentration is in complete agreement with the optimum concentration of 2-aminopurine needed in other systems (Kaufman *et al.*, 1987; Zinn *et al.*, 1988). At the highest concentration used, 2-aminopurine did not exert any gross adverse effects on the cells. The data obtained from experiments using 2'-ATP showed that 2'-dATP, the inhibitor of 2-5A synthetase, resulted in a 72% reversal of the antiviral activity of poly I:C at a high concentration (5 mM). Collectively, these results suggest that

Table III. Effects of anti-IFN antibody on the 2'-5' oligo A synthetase induced by poly I:C.

Time of treatment	2'-5' oligo A synthetase activity (cpm×10 ^{3}/10^{6} cells)	
	Exp. 1	Exp. 2
−	0.8± 0.1	1.7± 0.2
poly I:C	13.2± 1.2	12.3± 2.7
poly I:C ± anti IFN β	5.2± 0.3	4.6± 0.5
	(65%)[a]	(73%)[a]

[a] Inhibition of enzyme activity.
The cells were treated for 24 hours with poly I:C (5 μg/ml) in the presence or absence of anti-IFN β antibody 500 U/ml). After extraction and 22 hours assay with extracts, phosphatase-treated supernatant was applied on alumina columns, and the effluent radioactivity was counted. The Means± SD of the duplicates are indicated.

the protective effects of poly I:C may be mediated mainly by the activation of 2-5A synthetase and the ds-RNA dependent protein kinase may be much less involved in poly I:C-induced antiviral activity.

Inasmuch as poly I:C-induced antiviral activity was mediated via the production of IFN-β (Pyo *et al.*, 1991), I next determined whether the activation of 2-5A synthetase was due to the production of IFN-β. Macrophages were treated with poly I:C for 24 hours in the presence of high concentrations of anti-IFN-β antibodies (500 U/ml). Following treatment, 2-5A synthetase assay was performed. As shown in table 3, anti-IFN-β antibodies reduced the activatiion of enzyme by approximately 65~73%, suggesting that IFN-β was partially involved in the activation of enzyme. However, there may be some direct activation of the enzyme by poly I:C.

DISCUSSION

Interferon induces the synthesis of many proteins. Among these proteins are two enzymes which are activated by double-stranded RNA: 2-5A synthetase and protein kinases. These enzymes are known to play an important role in the protection of host against viruses (Lengyl, 1982). This activated enzyme, 2-5A synthetase, synthesizes oligomers of adenylate which can then activate endonuclease which cleave viral RNA (Tayler *et al.*, 1990). In addition, previous work by Benedetti *et al.* indicates that poly I:C activates protein kinase which in the presence of double-stranded RNA phosphorylates the α subunit of protein synthesis initiation factor eIF-2, thereby inactivating it. In the present studies, 2-aminopurine known as an inhibitor of protein kinase and 2-5A synthetase totally blocked poly I:C-induced antiviral activity and the inhibition of 2-5A synthetase by 2'-ATP resulted in a 72% reversal of poly I:C-induced antiviral activity. This data, therefore, suggest that 2-5A synthetase appears to be mainly involved in poly I:C-induced antiviral activity and the pathway involving a ds-RNA dependent protein kinase may be less important in the protective effects of poly I:C. In contrast with present work, there are several examples of lack of correlation between the level of 2-5A synthetase and the antiviral state (Meurs *et al.*, 1981; Sen, 1982). However, it has been suggested that the 2-5A synthetase was responsible for preferential cleavage of reovirus mRNA (Baglioni *et al.*, 1984) and a good inverse correlation was observed between the intracellular levels of 2-5A synthetase activity in peritoneal macrophages and the sensitivity of these cells to vesicular stomitis virus (Gresser *et al.*, 1985). This discrepancy may be explained on the basis of difference in both virus and cell type used in the experiment.

Since 2-5A synthetase was involved in poly I:C-induced antiviral activity and poly I:C induced the production of IFN-β, it was of interest to elucidate whether activation of 2-5A synthetase by poly I:C is mediated by the production of IFN-β. The result suggests that activation of 2-5A synthetase by poly I:C was at least partly mediated via IFN-β, but the possibility does exist that poly I:C directly activates 2-5A synthetase. Also, further studies are necessary to determine whether or not activation of protein kinase by poly I:C is mediated by the production of IFN-β for the induction of antiviral activity. In conclusion, the present study demonstrated that poly I:C-induced antiviral effect in peritoneal macrophage is mediated by the production of IFN-β which acts in an autocrine manner to render these macrophages resistant to HSV-1 infection and this process appears to involve the activation of 2-5A synthetase.

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