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# OLIGO(2'-5')ADENYLATE SYNTHETASE IN HUMAN LYMPHOBLASTOID CELLS

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## SUMMARY

The enzyme oligo(2'-5')adenylate synthetase, when activated by double-stranded RNA, polymerizes ATP into the novel oligonucleotide (2'-5')ppp(Ap)$_n$A. We describe conditions for assay of this enzyme in crude extracts of a human lymphoblastoid cell line, Namalwa. The production of (2'-5')ppp(Ap)$_n$A by Namalwa extracts was 3-5 times greater than the production by extracts of interferon pretreated mouse L cells, and 700 fold higher than the production by extracts of untreated mouse L cells. The relatively high level of oligo-(2'-5')adenylate synthetase in Namalwa cells was not attributable solely to their constitutive secretion of low levels of interferon. Analysis of the size distribution of the oligomers formed at different times suggested that the enzyme can add ATP to a free pppApA. Infection by Newcastle disease virus or treatment with interferon raised the apparent synthetase levels only marginally. Experiments that employed antibody to interferon suggested that the interferon must be externalized from the NDV-infected cell to induce maximal synthetase levels.

## INTRODUCTION

One current hypothesis of the mechanism of interferon action suggests that interferon induces the synthesis of an enzyme, oligo(2'-5')adenylate synthetase which, when activated by dsRNA's arising from viral replication, elaborates from ATP the unique 2',5'-linked oligoribonucleotide, 2-5(A) (1 and references therein). This oligomer, a potent protein synthesis inhibitor, activates a latent cellular endonuclease which degrades mRNA and thereby inhibits protein synthesis.(2,3) While the relevance of this metabolic pathway in the mode of interferon action remains to be established, it seems likely that the role of the dsRNA-dependent oligo (2'-5') adenylate synthetase is not confined to interferon-treated cells. Extracts of rabbit reticulocytes (4) as well as estrogen-treated and withdrawn chick oviduct (5) have been reported to have high levels of this 2-5(A) synthetase.

We report here that two lines of human lymphoblastoid cells, namely RPMI 1788 and Namalwa, have oligo(2'-5')adenylate synthetase levels equal to

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or exceeding those found in interferon-treated mouse L cells. The Namalwa cell line is a constitutive interferon producer (6), but when the cells are grown in the presence of antibody to interferon, oligo(2'-5')adenylate synthetase levels remain elevated.

## MATERIALS AND METHODS

Mouse L cells were maintained and pretreated overnight with 20 N.I.H. reference units/ml (1 effective unit/ml, $2 \times 10^7$ units/mg protein) as described previously (7).

The maintenance and induction of lymphoblastoid cells with Newcastle Disease Virus and purification of lymphoblastoid interferon have been reported (8,9). Interferon treated Namalwa cells were exposed to $1\text{-}2 \times 10^3$ N.I.H. reference units/ml of lymphoblastoid interferon ($3\text{-}5 \times 10^5$ units/mg protein) (9). Cells were harvested about 18 hr after NDV induction or exposure to interferon. Cells grown in the presence of antibody or normal gamma globulin were exposed for five to six passages to $3 \times 10^3$ to $5 \times 10^4$ neutralizing units (NU) per ml of sheep anti-lymphoblastoid and rabbit or goat anti-fibroblast antibodies, or the equivalent amount of normal rabbit gamma globulin.

The production and assay of sheep anti-lymphoblastoid antibody and goat anti-fibroblast antibody has been described (10,11, I. A. Braude, Ph.D. dissertation 1978, Leuven, Belgium). Rabbit anti-fibroblast antibody was the kind gift of Dr. J. Vilcek, (New York University School of Medicine, New York, N.Y.).

The RPMI 1788 cells were the gift of Dr. B. Papermaster (Cancer Research Center, Columbia, MO.).

Routinely, 10-500 ml of cells at saturation density were harvested and washed three times with 35 mM Tris-HCl, pH 7.5, 140 mM NaCl. Lysis was achieved either by suspension of the cells in 1.4 vol. lysis buffer, 10 mM Tris.HCl, pH 7.5, 1.5 mM $Mg(OAc)_2$, 10 mM KCl and 7 mM β-mercaptoethanol followed by Dounce homogenization, or by suspension of the cells in the same buffer containing 0.5% NP40. Control experiments showed that NP40 did not effect the synthesis or assay of 2-5(A) at the concentrations of NP40 employed. Lysed cells were first centrifuged at 10,000 x g for 10 min and the S10 supernatants were centrifuged further at 100,000 x g for 2 hr.

The synthesis of 2-5(A) in solution was essentially as described by Minks et al., (12) except that 0.1 mM fructose diphosphate was not regularly employed since its inclusion did not effect the titers or the time course of the reaction. Standard conditions for 2-5(A) synthesis by Namalwa S100 extracts were 20 mM Hepes, pH 6.5-7.0, 10 mM $Mg(OAc)_2$, 100 mM KCl, 1 mM dithiothreitol, 5 mM ATP, $10^{-4}$ M poly (I)·poly (C), and 10% S100 (0.03-0.30 $A_{260}$ units). Synthesis of 2-5(A) by the synthetase pre-bound to 2',5'ADP-Sepharose or to poly(I)·poly(C) Sepharose was performed as described previously (13).

The yield of 2-5(A) was estimated in most cases by titration of the boiled reaction mixture into an EMC-viral RNA programmed, micrococcal nuclease pretreated cell free system from mouse L cells, as described previously (13). The titer of the reaction mixture is defined as the inverse of the dilution required to inhibit protein synthesis by 50%. We have determined that this inhibition is attained with 0.5-1 nM 2-5(A) (M.I.J., R.M.F., P.F.T., submitted for publication). In some experiments

the amount of 2-5(A) produced was determined by the DEAE cellulose binding assay described by Baglioni et al. (14).

RESULTS

The levels of 2-5(A) produced by extracts of NDV-induced Namalwa cells or interferon-treated mouse L cells first were compared using the solution assay and the 2',5'-ADP-Sepharose assay. As reported earlier (M.I.J., R.M.F., P.F.T., submitted for publication), under solution assay conditions (2 hr), the yield of 2-5(A) from interferon-treated mouse L cell extracts was poor (titer = 2000), probably because of high levels of 2-5(A) inactivating enzymes. As determined by the 2',5'-ADP-Sepharose assay (6 hr), the amount of 2-5(A) made by the mouse cell extracts (titer = 20,000) was about five times lower than the corresponding Namalwa sample (titer = $10^5$) although the mouse cell extract used in this experiment contained twice the protein of the Namalwa sample. When bound to poly(I)·poly(C)-Sepharose, the Namalwa extract produced the same titer of 2-5(A) as did the extract bound to 2',5'-ADP-Sepharose (titer = 2 x $10^5$).

The product of the Namalwa reaction was identified as 2-5(A) by the following criteria i) heat stability (ii) analysis of $^3$H-labelled, bacterial alkaline phosphatase (BAP) digested products on PEI cellulose (iii) analysis of undigested and BAP digested products by DEAE-cellulose chromatography in the presence of 7 M urea, and iv) comigration of the DEAE-cellulose isolated trimer species with chemically synthesized (2'-5')ApApA on DEAE cellulose chromatography and HPLC (M.I.J., P.J. Bridgen, J. Imai, P.F.T., unpublished data).

When [$^3$H]ATP was included in a solution reaction mixture and 2-5(A) production was measured by binding to DEAE cellulose (14), the yield of 2-5(A) increased linearly with time (Figure a). The protein synthesis inhibition titers and the binding data plotted on a semi-log scale displayed a similar time dependence (Figure b).

In an analysis of the size of the oligomers synthesized at early (4 hr) and late (20 hr) times in solution we observed proportionally more tetramer and pentamer species after longer periods of incubation (Table 1). A similar but less dramatic shift to longer molecules was observed in an analysis of 2 hour and 4 hour samples (data not shown). The ratio of moles of dimer:trimer:tetramer:pentamer was 25:30:6:1 at 4 hours and 1:10:6:1 at 20 hours. The decrease in the amount of dimer present suggests that the enzyme can incorporate AMP units onto "free" dimers to form trimers. A similar conclusion has been reached recently by Minks et al. (15).

A comparison of the levels of (2'-5') oligoadenylate synthetase in extracts of NDV-infected and control (uninfected with NDV) Namalwa cells

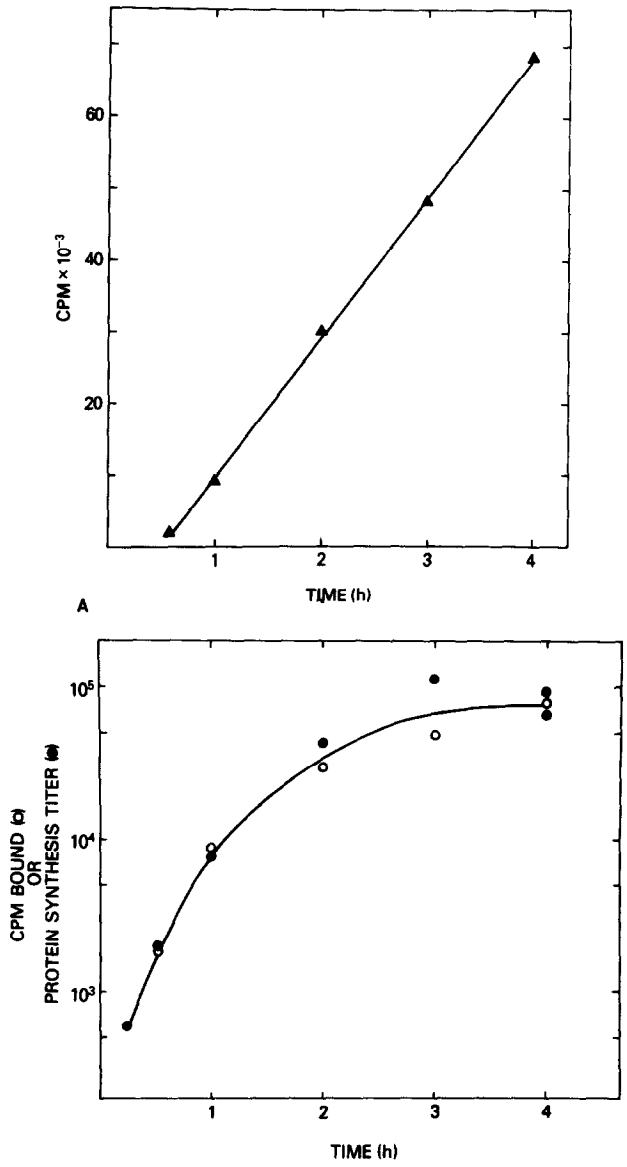


FIGURE 1. Time Course of 2-5(A) synthesis. Synthesis of 2-5(A) in a solution reaction mixture that included [$^3$H]ATP (50 Ci/mole) was terminated at the times shown. The 2-5(A) content of each sample was estimated by two methods. (A) DEAE-cellulose binding assay, performed as described by Minks et al. (12). (B) Protein synthesis titers (●) of 2-5(A) obtained from curves as in Figure 1 as described in Methods, and the corresponding data from part A replotted on the semi-log scale (o).

showed that the titers obtained from extracts of the NDV-infected cells were only two to five times higher than those from control Namalwa cells (Table 2). Maximum 2-5(A) titers were obtained from extracts prepared

Table 1

Size Distribution of 2-5(A)

| | Nanomoles of 2-5(A)+ (AMP equivalents) | | | |
|---|---|---|---|---|
| | 4 h | (% Total) | 20 h | (% Total) |
| Dimer | 30 | (30) | 10 | (3) |
| Trimer | 53 | (53) | 158 | (48) |
| Tetramer | 14 | (14) | 132 | (40) |
| Pentamer | 3 | (3) | 26 | (28) |

+The 2-5(A) in 4 and 20 hour reaction mixtures (0.5 ml, [$^3$H]ATP at 50 Ci/mole), was purified by chromatography on DEAE-cellulose (14). Initial input of ATP was 5 µmole. The 4 and 20 hour samples contained 100 and 330 nmoles of 2-5(A), respectively, as determined from the radioactivity eluting from the DEAE cellulose column. The relative ratios of dimer, trimer, tetramer and pentamer were estimated by chromatography of purified, bacterial alkaline phosphatase digested inhibitor on DEAE cellulose in the presence of 7 M urea.

16 to 18 hours post infection when the amount of interferon present in the medium is considerable (data not shown). Treatment of control cells for 18 hours with high levels of interferon (see Methods) produced extracts that yielded a similar increase (4-fold) of 2-5(A) titer. In contrast, extracts from cells carried through five to six passages in the presence of anti-interferon antibody in 10-100 fold excess of that required to neutralize the constitutive interferon showed a decreased (4-20-fold) ability to produce 2-5(A). This 4-20-fold decrease in 2-5(A) titer corresponds to a 2-4-fold decrease in enzyme levels as determined from experiments in which 2-5(A) titer was measured as a function of crude extract added to the assay. Normal gamma globulin had no effect on apparent 2-5(A) synthetase levels. Experiments in which 2-5(A) was synthesized by control extract mixed with various amounts of extract of antibody-treated cells showed that the latter extracts did not have increased levels of 2-5(A) degrading activities that would account for their decreased ability to synthesize 2-5(A). When Namalwa cells maintained in the presence of anti-fibroblast interferon antibody and anti-lymphoblastoid interferon antibody were infected with NDV, synthetase was induced at reduced levels when the subsequent interferon production overcame the capacity of the antibody in the medium to neutralize the externalized interferon (Table 2, experiment 1). When detectable antibody was present 18 hours after induction, apparent synthetase levels remained

Table 2. EFFECT OF INTERFERON AND ANTI-INTERFERON ANTIBODY ON APPARENT 2-5(A) SYNTHETASE LEVELS IN NAMALWA CELLS

| | Experiment 1[a] | | | Experiment 2[a] | | |
|---|---|---|---|---|---|---|
| | 2-5(A) Titer[b] | Interferon[c] (log units/ml) | Antibody[d] (NU/ml) | 2-5(A) Titer[b] | Interferon[c] (log units/ml) | Antibody[d] (NU/ml) |
| Control | 53,000 | < 1.4 | - | 71,000 | < 1.4 | |
| Normal γG | 43,000 | < 1.4 | - | 40,000 | < 1.4 | |
| Normal γG +NDV | 200,000 | 3.1 | - | 170,000 | 2.6 | |
| Interferon | - | - | - | 250,000 | 3.2 | |
| Antibody | 2,900 | < 1.4 | Le $8.8X10^3$<br>F $3.7X10^4$ | 17,000 | < 1.4 | Le $3.5X10^4$<br>F $4.7X10^4$ |
| Antibody +NDV | 59,000 | 2.6 | - | 29,000 | < 1.4 | Le $3.5X10^4$<br>F $6X10^3$ |

[a] For each experiment cultures were grown, treated, extracted and assayed simultaneously.

[b] Titers were determined as described in Methods from reaction mixtures containing 0.15 $A_{260}$ units of S100 and incubated for 1 hour.

[c] N.I.H. reference units/ml determined as described previously (8).

[d] Neutralizing units/ml determined as described by Smith (10).

Table 3. TITERS OF 2-5(A) SYNTHESIZED In Vitro BY VARIOUS CELL EXTRACTS

| | Solution assay | (I)·(C)-Column assay | |
|---|---|---|---|
| | Experiment 1[a] | Experiment 2[b] | Experiment 3[c] |
| Mouse L cells | < 30 | 210 | |
| Interferon-treated Mouse L cells | 3,300 | 50,000 | |
| Namalwa cells | 24,000 | 160,000 | 10,000 |
| RPMI 1788 cells | 5,300 | 67,000 | 10,000 |

[a]Titers obtained from 1 hour incubation mixture per 0.15 $A_{260}$ units of S100.

[b]Titers obtained from an overnight incubation mixture per 3 $A_{260}$ units of S100 employed in the binding step.

[c]Titers obtained from an overnight incubation mixture per 1.6 $A_{260}$ units of S100.

depressed (Table 2, experiment 2). These results suggested that some but perhaps not all of the 2-5(A) synthetase activity in control Namalwa cells resulted from the low level of constitutive interferon production, and that the interferon produced by viral infection must be externalized for maximal induction of 2-5(A) synthetase.

Data consistent with this interpretation was obtained by measurement of 2-5(A) production by extracts of a lymphoblastoid cell line, designated RPMI 1788, which does not produce detectable interferon constitutively (< 1 unit/ml, K.C.Z., unpublished results). As shown in Table 3, the 2-5(A) titers obtained with RPMI 1788 extracts were somewhat lower (15-30%) than those measured in control Namalwa cell extracts. However, the 1788 cell extracts produced 2-5(A) levels comparable to those obtained with extracts from interferon-treated mouse L cells. The higher titers obtained with the poly(I).poly(C) Sepharose method probably resulted from the longer incubation time, the greater amount of extract employed, as well as the probable removal of 2-5(A) degrading enzymes. Extracts from Daudi cells, another lymphoblastoid cell line that does not produce interferon constitutively, also yield 2-5(A) levels that were comparable to those obtained with Namalwa cell extracts (data not shown).

## DISCUSSION

The data presented here demonstrate that Namalwa cell extracts yield at least three times more 2-5(A) than extracts of interferon-treated mouse L cells when the extracts are assayed under identical conditions. An earlier

report by Stark et al (4) maintained that Namalwa cell extracts produced at least ten times less 2-5(A) than did interferon-treated mouse L cells. The source of this discrepancy is not clear. The Namalwa cells used in this study were grown in spinner culture, produced interferon constitutively, and responded to NDV induction with rapid production and high yields of interferon (8).

The occurrence of oligo(2',5')adenylate synthetase in extracts from a constitutive interferon producer, a mutant mouse 3T6 cell line, has been reported recently by Jarvis et al (16). The titer of 2-5(A) produced by these extracts was about 60-fold higher than the 2-5(A) titer of the parent cell extracts. Experiments carried out with the constitutive interferon producing Namalwa cells in the presence of antibody to interferon suggest that the 2-5(A) synthetase of Namalwa cells does not result entirely from the interaction of externalized constitutively-produced interferon with the cell. The high antibody titers observed in the medium of the antibody-treated cells at the time of extraction, as well as the previous report that the addition of antibody to the medium of induced cells can prevent the establishment of the antiviral state (17), argues against the possibility that interferon induced the synthesis of oligo(2',5')adenylate synthetase even in the presence of antibody. However, since the relationship between the antiviral state and synthetase induction has not been unequivocally established, this latter possibility cannot be dismissed.

It appears that the levels of 2-5(A) synthetase in control Namalwa cells are near maximal. Pre-treatment with high doses of interferon raises the apparent synthetase levels only five-fold or less. We are presently investigating whether peripheral resting lymphocytes like splenic lymphocytes (18) and like the lymphoblastoid cells reported here have high levels of 2-5(A) synthetase.

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