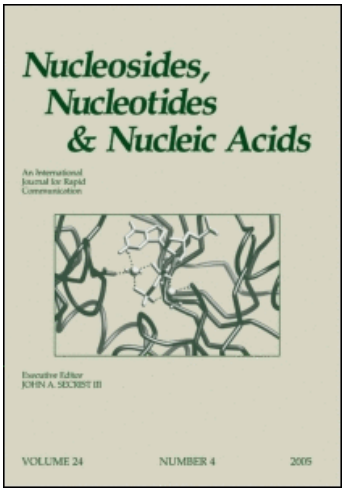

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CHARACTERIZATION OF A NOVEL 2'-5' OLIGOADENYLATE IN STIMULATED LYMPHOCYTES

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SUMMARY

We have analysed Con A-stimulated mouse lymphocytes for the presence of 2'-5'-linked oligoadenylates using a radioimmunoassay based on a monoclonal antibody raised against adenylyl (2'-5') adenosine (A2'pA). Time-course and Con A dose dependence were performed.

We found that Con A induced, in a dose-dependant manner, the accumulation of immunoreactive material, together with the incorporation of ^{3}H-thymidine in DNA. We showed that the immunoreactive material was constituted for the essential, by a novel 2'-5' oligoadenylate. It was isolated and characterized as adenylyl 2'-5'adenylic acid (2' and 3'P) according to the combination of criteria such as immunoreactivity, enzyme susceptibility, chromatographic behaviour and comparison with A2'pA3'(^{32}P)p, A2'pA3'p and A2'pA2'p that we have chemically synthetized. This is the first example of significant variations of a 2'-5' oligoadenylate level in circumstances other than the antiviral mechanism of interferon.

INTRODUCTION

Studies on the mechanism of activation of interferons lead to the discovery in interferon-treated, virus-infected cells of low molecular weight inhibitors of protein synthesis of general formula $p_x(A2'p)_nA$, $2 < x < 3$, $n > 1$ collectively referred to as 2-5A. They are synthesized from ATP by the 2-5A synthetase, an enzyme induced by interferon and activated by double-stranded RNA. They bind to and activate a latent endoribonuclease which in turn can control the level of cellular and viral RNA (reviewed in 1). With the progress of analytical procedures, combined with chromatographic analyses, an increasing number of small molecular weight 2'-5' linked oligoadenylates were detected in biological extracts. The 2-5A dimer (pppA2'pA) and 2-5A "cores" lacking the 5'-terminal triphosphate ($(A2'p)_nA$, $n > 1$), which are not protein synthesis inhibitors, have been detected in virus infected cells and tissues, in addition to functional 2-5A (2-8). These molecules have also been found under normal circumstances in numerous biological extracts from animals, yeast and bacteria (9).

In addition to these unambigously identified molecules, other compounds, displaying abnormal behaviour in high performance liquid chromatography, were detected in several instances by their ability to compete in radiobinding or radioimmune assays (10-12).

If it is now generally agreed that 2-5A acts as intracellular mediator of the antiviral effect of interferon, its wider role in the normal control of cell proliferation and differentiation and the biological relevance of the other 2'-5' oligoadenylates remain to be established.

A possible involvement of the 2-5A system in the control of T cell functions has been inferred from several observations : 1. the level of 2-5A synthetase increases with the degree of maturation of T cells (13), 2. when T cells are treated with Con A, the catabolism of the 2-5A is increased (14, 15), 3. when micromolar amount of (A2'p)2A are present in the culture medium, the Con A-induced synthesis of protein, RNA and DNA are reduced (14). In the present work the question about the implication of 2'-5' oligoadenylates in T cell proliferation-differentiation process was adressed directly by analysing the oligomers present in T cell during polyclonal stimulation.

For this purpose, we coupled to specific procedures of HPLC a radioimmunoassay based on a monoclonal antibody specific for the 5'OH-A2'pA epitope (3). Such a specificity renders possible to evaluate from the same sample 5'OH oligomers $(A2'p)_nA$, as such and 5'-phosphorylated oligomers, $P_x(A2'p)_nA$, after dephosphorylation by bacterial alkaline phosphatase. This RIA operates in the femtomole range.

Mouse thymocytes and splenocytes were studied. Time-course after Con A stimulation and Con A dose-response were performed. ^3H thymidine incorporation in DNA and intracellular levels of 2'-5' oligoadenylates were measured. We found that Con A treatment provoked an accumulation of immunoreactive material in lymphocytes and that the same dose of Con A induced maximal DNA synthesis and maximal immunoreactivity. Fractionation of the immunoreactive material showed that a unique component accounted for the immunoreactivity. Its characterization as an adenylyl (2'-5') adenylic 2'(3') acid is described here. It is the first natural 2-5A related molecule which is identified other than the oligomeric series $p_x(A2'p)_nA$, $0 < x < 3$, $n > 1$.

MATERIAL AND METHODS

Animals

The experiments were performed with 3 weeks old Swiss mice, purchased from two independent sources : Iffa Credo (L'Abresle, France), the animal breeding facilities of the Faculty of Pharmacy (Montpellier, France). The animals were sacrificed in most cases between 10 and 11 a.m. and in some cases at 2 p.m.

Lymphocyte cultures

The animals were sacrificed by cervical dislocation. Thymuses and spleens were removed immediately under sterile conditions and immersed in Hank's balanced salt solution (HBSS). The thymuses were teased through a sterile nylon mesh to obtain single cell suspensions ; the spleens were disrupted with a glass tissue homogenizer. The cells were pelleted at 200 x g for 10 mn and either processed for analysis or gently resuspended in RPMI 1640 (Gibco Bio-Cult) for use in cell cultures. For mitogenic stimulation, 15 ml cell cultures were incubated at 37°C in a 5 % CO_2/air humidified atmosphere in RPMI 1640

supplemented with 10 % heat-inactivated fetal calf serum, penicillin (100 U/ml) and gentallin (40µg/ml). The cells were cultured either alone (controls) or in the presence of lectins for 3 to 5 days at a density of 2.5 to 4 x 10^6 cells ml^{-1}. Concavalin (Con A, Pharmacia) was used at 5 µg ml^{-1} for thymocytes and 2.5 µg ml^{-1} for splenocytes in time course experiments and at variable concentrations in the range from 1 to 20 µg ml^{-1} in dose-response studies. In this latter case, the extent of stimulation was also modulated by treatment with peanut agglutinin (PNA, reactifs IBF) : freshly prepared thymocytes (2 x 10^7 cells ml^{-1}, in RPMI 1640) were incubated at room temperature for 30 mn with 0.04 units neuraminidase (type VI, Sigma) and 3 µg PNA. The cells were then extensively washed with RPMI 1640 and cultured as above (16). Cell countings were performed in the presence of Trypan blue to assess viability. Spleen cells were also counted after staining with Gentian violet (0.01 % w/v in 3 % acetic acid). For DNA synthesis evaluation, aliquots of 5 x 10^5 cells were withdrawn in triplicate from 15 ml culture medium for 4 h with 1 µCi of (^{3}H) thymidine (5 Ci $mmole^{-1}$, the Radiochemical Center, Amersham). The cells were then collected onto glass fiber paper using a cell harvester and the filters processed for liquid scintillation counting.

Preparation of samples for RIA

At the time of sampling, the cells were collected by centrifugation and the cell pellet disrupted by sonication in chilled 1N $HClO_4$. Insoluble material was removed by centrifugation and the acidic extracts were brought to neutrality with 9N KOH. The insoluble potassium perchlorate was eliminated by centrifugation. All these operations were performed in the cold. The neutralized extracts were stored at -20°C until use. (17)

Analytical procedures

The RIA of 2'-5' oligoadenylates has been described elsewhere (3, 17). It was performed either on crude neutral extracts or on the fractions collected after HPLC fractionation. Each sample was assayed in duplicate for the presence of immunoreactive material, either directly or after enzymatic treatments.

Enzymatic treatments : Bacterial alkaline phosphatase (BAP, type IIIR from E. Coli, Sigma) and snake venom phosphodiesterase (SVPDE from Crotalus durissus terrificus (Boehringer Mannheim) were employed in

0.1 M Tris HCl pH 9.3, 1mM $MgCl_2$, T2 RNase (grade VI, Sigma) in 0.1M sodium citrate pH 5.8 using A3'pA as a substrate to control the enzymatic activity in parallel experiments. All the incubations were performed at 37 °C.

Chromatographic procedures (18) : HPLC fractionations were performed on a Waters system registering the optical density at 260 nm. The following conditions were used :

Conditions A : A μ Bondapak C18 (30 cm x 3.9 mm id) (Waters) was run in a 4 mM potassium phosphate, pH 6.5 at 1 ml/min with a 25 min 0-30 % linear gradient of 50:50 methanol water.

Conditions B : The same column was run in 10 % (w/v) ammonium acetate, pH 5.9 at 2 ml/min with a 30 min, 0-30 % linear gradient of 50:50 methanol 10 % ammonium acetate pH 5.9. When necessary collected fraction were lyophilised and reconstituted with distilled water.

Conditions C : A Ultrasil NH_2 (25 cm x 4.6 mm) (Beckman) was run at 1 ml/min with a 20 min linear gradient starting after 10 min of isocratic conditions in 7mM potassium chloride, 7mM potassium phosphate, pH 4, from 0 to 100 % of 0.5 m potassium chloride, 0.25M potassium phosphate, pH 5.0.

Gel filtration was performed on a Tris Acryl GF05 (1 cm x 5.9 cm) (l'Industrie Biologique Française) eluted at 3 ml/hr in 0.1 M sodium citrate pH 6.2. The accurate elution volumes were determined by gravimetry.

The standard products were either of commercial origin (A2'pA and $(A2'p)_2A$ or prepared enzymatically (3) ($P_x(A2'p)_nA$, x=3, 1 <n <4, $(A2'p)_3A$ and $(A2'p)_4A$).

Obtention of A2'pA2'p and A2'pA3'p : $(A2'p)_2A$ purchased from PL Biochemicals was purified on C18 reverse phase HPLC. 3×10^{-4} M $(A2'p)_2A$ was partially hydrolysed by incubation in 0.3 N KOH for 1 h at 37°C. The medium was then adjusted to pH 1 with $HClO_4$, incubated for 15 min at 4°C and finally brought to neutrality. The products were purified on HPLC using the conditions C (see above).

Synthesis of A2'pA3'(^{32}P)p : A2'pA (Sigma) was linked to (5'^{32}P) pCp (2.3×10^6 Ci mol^{-1}, the Radiochemical Center, Amersham, U.K.) using T4 RNA ligase (PL Biochemicals) (19). A2'pA3' (^{32}P)pCp was digested to A2'pA3' (^{32}P)p by T2 RNase. The successive steps of the synthesis were controlled, and the products purified on HPLC using conditions B.

RESULTS

Levels of 2'-5' oligoadenylates in mitogen-stimulated lymphocytes

In a first set of experiments, we have measured the immunoreactivity of the cell extracts and the (^{3}H) thymidine uptake at different time-points after addition of Con A to the culture. Similar analyses were performed on cells cultured without Con A as control.

In Con A-treated lymphocytes, both thymocytes and splenocytes, the immunoreactive material accumulated until 50 h (up to 2 and 5 pmoles/10^6 cells for thymocytes and splenocytes, respectively), then decreased. In control cells, it decreased continously (down to 0.2 pmole/10^6 cells). (Fig.1 - Fig.2). This evolution is rather similar to that of DNA synthesis : particularly, the maxima were reached at the same time-period (Fig.1 - Fig. 2).

In a second series of experiments, we have studied in parallel the effect of doses of Con A on the immunoreactivity of the cell extracts and the (^{3}H) thymidine uptake in order to know if 2'-5' oligoadenylate levels depend on the dose of Con A as DNA synthesis does. Different doses of Con A or Con A plus PNA (16) were used to modulate the degree of stimulation of thymocyte cultures. Analyses were performed at 48 h, time when stimulation is at its maximum. We observed the same dose-dependence for the induction of DNA synthesis and elevation of 2'-5' oligoadenylate levels either with Con A alone or with combination of Con A and PNA (Fig. 3).

Immunological characterization and chromatographic behaviour

The results of enzymatic treatments and RIA performed on the crude extracts of stimulated lymphocytes indicated that the immunoreactive material is constituted by one or more molecules which contain phosphodiester bonds since snake venom phosphodiesterase (SVPDE) abolished the immunoreactivity. They are essentially non-phosphorylated at the 5'OH end since bacterial alkaline phosphatase (BAP) did not reveal additional material. If substantial amounts of 5'-phosphorylated oligomers had been present, they would have been detected by an increased immunoreactivity upon BAP treatment this was clearly not the case.

For further identification, the cell extracts were analysed in four different chromatographic system (18). Two C18 reverse phase HPLC procedures were used : in conditions A (potassium phosphate buffer,

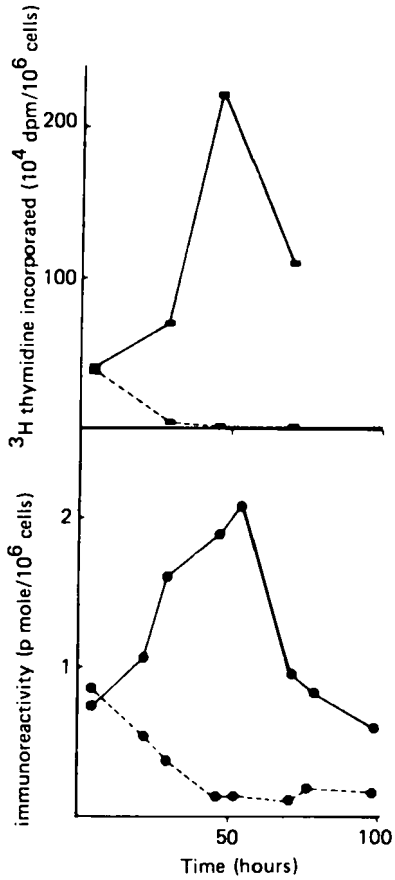


FIG. 1.. Con A-stimulation of thymocytes : time course;
- upper part : ^{3}H thymidine uptake
  + Con A ■——■ Control ■- - -■
- lower part : 2'-5' oligoadenylate levels expressed as pmole of adenylyl adenylic acid per 10^6 living cells.
  + Con A ●——● Control ●- - -●

gradient of methanol) the phosphorylated oligomers are first eluted, followed by the non-phosphorylated oligomers eluted in decreasing length order. In conditions B (ammonium acetate buffer, gradient of methanol) the phosphorylated then the core oligomers are eluted in increasing length order. Ion exchange HPLC, using combined gradients of pH and ionic strength allows to separate only the less charged molecules. In these three systems, the immunoreactive material was quantitatively recovered and eluted for more than 95 % as a unique compound displaying retention times different from those of known

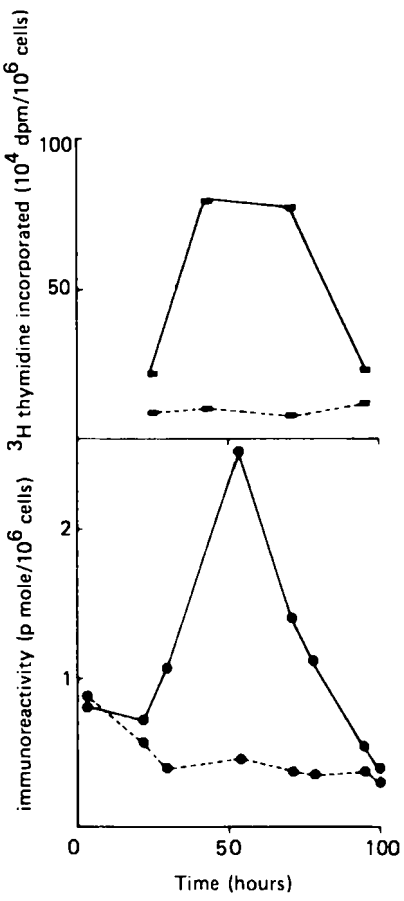


FIG. 2. Con A-stimulation of splenocytes : time course
- upper part : ^{3}H thymidine uptake
  + Con A ■——■ Control ■- - - -■
- lower part : 2'-5' oligoadenylate levels expressed as pmole of adenylyl adenylic acid per 10^{6} living cells.
  + Con A ●——● Control ●· · · ·●

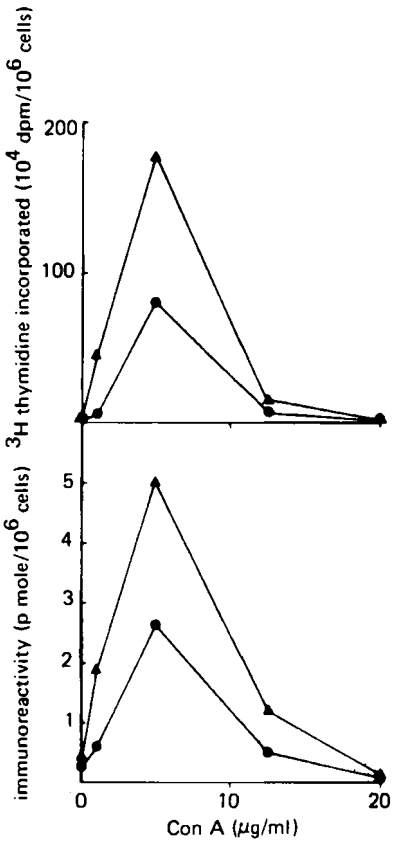


FIG. 3. Con A-stimulation of thymocytes : dose-response
- upper part : ^{3}H thymidine uptake
  + Con A + PNA ▲—▲ + Con A ●—●
- lower part : 2'-5' oligoadenylate levels expressed as pmole of adenylyl adenylic acid per 10^6 living cells
  + Con A + PNA ▲—▲ + Con A ●···●

2'-5' oligoadenylates (18) (Fig.4 A and B, Fig. 5A). Non-phosphorylated dimers and trimers were detected but always in trace amounts (Fig.4 B).

Indications about the size of the immunoreactive material were obtained in gel-filtration experiments. We found that chromatography on Tris-Acryl GF05 allows the separation of core oligomers according to their rank. The behaviour of the biological compound strongly supported the hypothesis of a small molecule, since it was eluted in a volume comparable to that of the core dimer A2'p5'A (Fig. 4D).

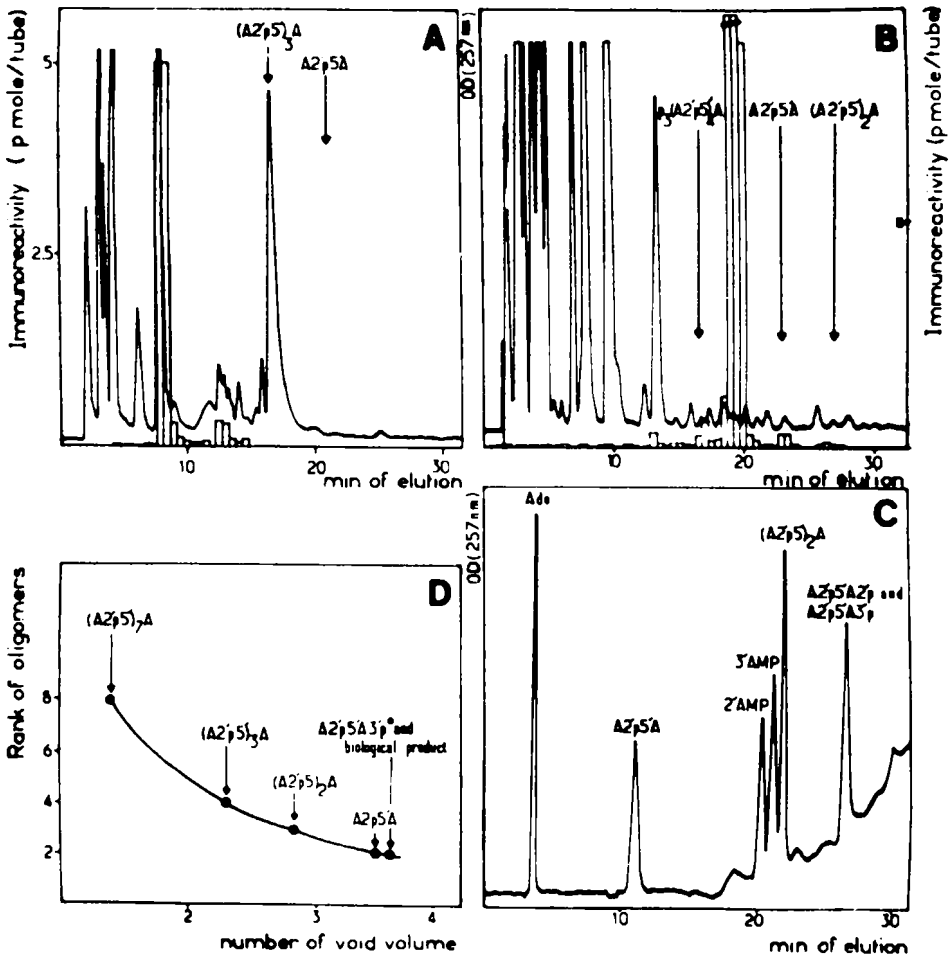


FIG. 4. Chromatographic characterization of the immunoreactive material extracted from Con A-stimulated mouse thymocytes.
A) HPLC analysis of an extract for Con A-stimulated cells (44 hr, 5 μg ml^{-1} Con A, 3 x 10^6 cells ml^{-1}) on a μ Bondapak C18 column eluted in conditions A (see Materials and Methods). 0.5 ml fractions were collected and assayed for the presence of immunoreactive products (bars).
B) HPLC analysis of another extract (48 hr stimulation) on the same column eluted in conditions B (see Materials and Methods). 0.5 ml fractions were collected and assayed for the presence of immunoreactive products (bars).
C) HPLC fractionation of a partial alkaline digest of $(A2'p)_2A$ on ultrasil NH_2 eluted in conditions C (see Materials and Methods). OD was registered at 260 nm.
D) Gel filtration behaviour of the biological product and chemically or enzymatically synthesized adenylyl 2'-5' adenylic acids on a Tris Acryl GF05 column eluted as described in Materials and Methods. 0.75 ml fractions were collected and the products were detected by their absorbance, radioactivity or immunoreactivity.

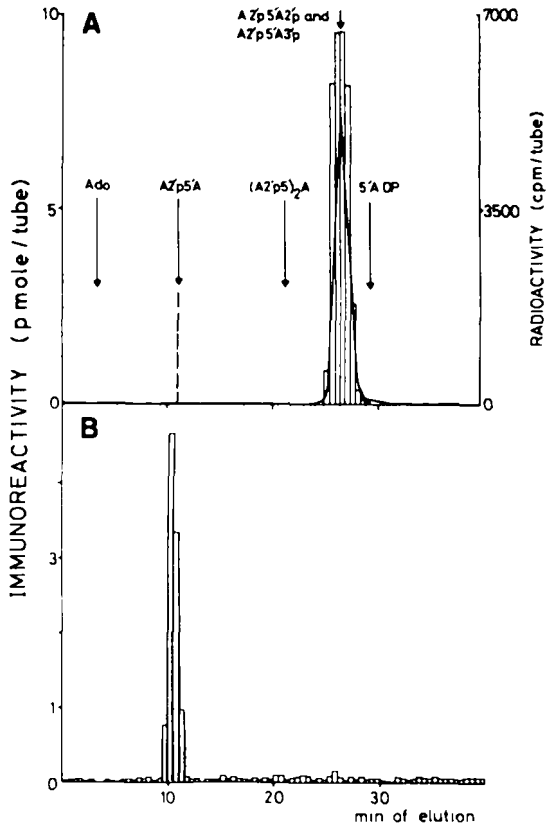


FIG. 5. HPLC analysis on ultrasil NH_2 of the biological component recovered from μ Bondapak C18 column run in conditions b. Conditions are those described in C (see Materials and Methods). Fractions were collected and assayed for their immunoreactivity (bars).
A : Sample mixed with A2'pA3' (^{32}P)p (in amount too low to mask the immunoreactivity of the biological product). In addition to immunoreactivity, radioactivity was measured in all the fractions (——)
B : Sample treated with bacterial alkaline phosphatase.

Similar elution patterns were obtained with thymocyte and splenocyte extracts. Samples taken at different time-points throughout Con A stimulation only differed by the concentrations of the unknown product. We therefore concluded that changes observed in the immunoreactivity of the crude extracts were due not to a qualitative change of the 2-5A oligomers but rather to a quantitative change of the amount of this unknown component.

Isolation

Using C18 reverse phase HPLC in conditions B, the biological unknown compound was recovered in a medium containing only volatile components. After lyophylisation, the product was submitted to ion-exchange chromatography as in figure 4C for further purification and to yet another C18 HPLC run in conditions B for desalting. Recoveries were quantitative at each step and the product was finally obtained in a salt-free solution which did not display any measurable U.V. absorbancy.

Sensitivity to nucleolytic enzymes

Neither the immunoreactivity nor chromatographic behaviour were modified upon incubation with T2 RNase therefore excluding the presence of 3'-5' phosphodiester bonds. The immunoreactivity was abolished by treatment with SVPDE, but the compound was in too small amounts for its degradation products to be analysed. The major breakthrough occured when we found that the unknown compound was entirely converted by BAP into a substance displaying the chromatographic properties of A2'p5'A in all systems used so far (as illustrated in Fig. 5). Moreover, this dephosphorylation did not induce the more than thousandfold increase in antigenicity demonstrated for a 5' phosphorylated dimer but provoked a change compatible with the elimination of a phosphate on the 2' or 3' end (3, 20). We therefore hypothesized that the exposed phosphoryl group was either on a 2' or 3' carbon and if so, that, adenyl 2'-5' adenylic acids would be the structure of the unknown molecule since it fits all the criteria. To check this hypothesis, we have prepared radiolabelled and OD-registering adenylyl 2'-5' adenylic acids and compared their properties to those of the biological product.

Comparison with synthetic products

High specific radioactivity A2'p5'A(^{32}P)3'p was mixed with the biological product in amounts contributing only marginally to the

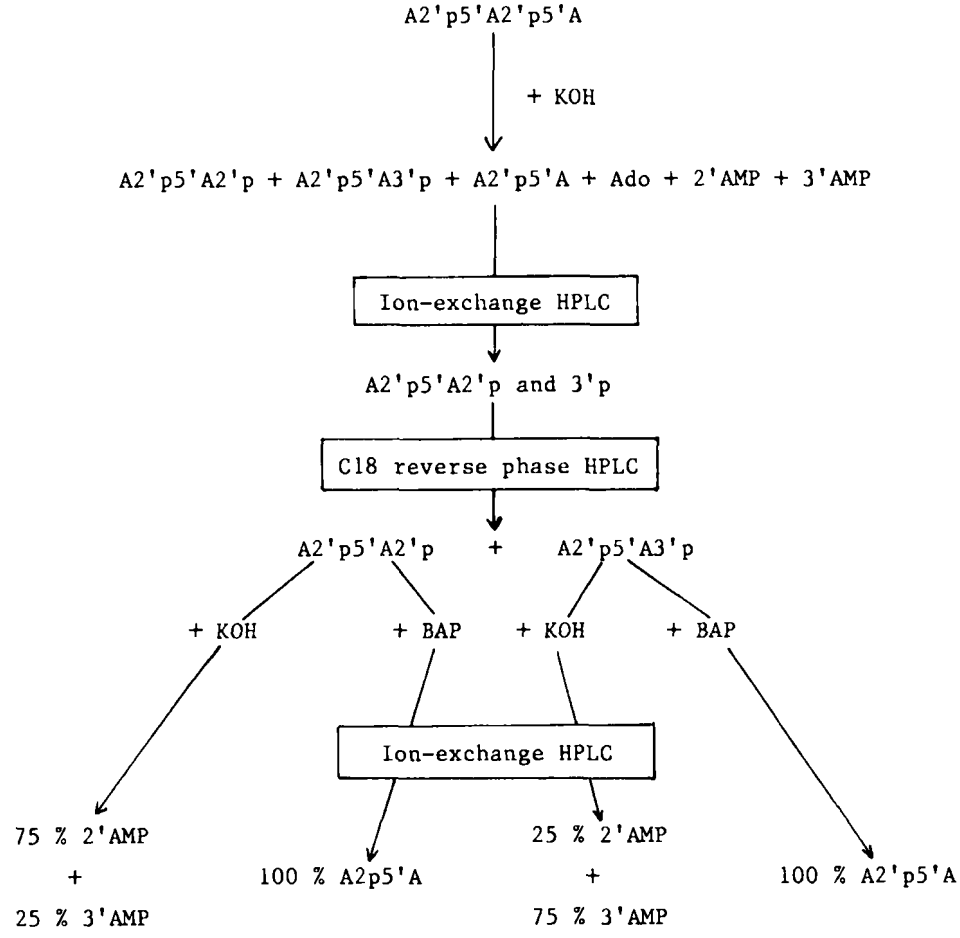
overall immunoreactivity. In all the chromatographic systems, the radioactivity was eluted exactly in the same fractions as the immunoreactivity (as illustrated in Fig. 4D and Fig. 5A).

Adenylyl (2'-5') adenylic acids were also generated by limited alkaline degradation of $(A2'p5')_2A$. Upon ion-exchange HPLC, the undigested trimer and its hydrolytic products (A2'p5'A, 2' and 3' AMP, adenosine) were identified by comparison with standards (Fig. 4C). The last eluted fraction displayed the same retention time as the biological product and co-eluted with the ^{32}P-derivative (Fig. 5A). It was expected to contain a mixture of adenylyl (2'-5') adenylic (2' and 3') acids. In our numerous attempts to separate the positional isomers, we found that an extensively used C18 column run in conditions B resolved the adenylyl 2'-5' adenylic acids in two peaks. A complete analysis of both of the two peaks was performed after an additional purification step of each one in the same system as schematically described in Table 1. To assign the position of the terminal phosphate groups, we measured the respective amounts of 2' and 3' AMP liberated by alkaline hydrolysis of each of the two molecules (Table 1). We controlled that each of them was converted into A2'pA by BAP treatment (Table 1).

The molar extinction coefficients were calculated by measuring the increase in optical density consecutive to the hydrolysis of the phosphodiester bond. These values allowed to calculate the exact concentrations and subsequently to compare the immunoreactivities of the two isomers. Ten-point displacement curves were performed with each of them in exactly the same conditions and the concentration giving half-inhibition of the binding of the tracer were determined (IC50). These data are summarized in Table 2. The small differences observed in hypochromicity increments and immunoreactivities suggest different conformations for the two isomers.

The biological product was submitted to the same type of C18 HPLC which separates the positional isomers. It gave two compounds eluted respectively at the same positions as the two adenylyl 2'-5'adenylic acids. Both of them generated A2'pA after BAP treatment, the first one displayed a 1.7 fold decrease in immunoreactivity just as the A2'pA2'p molecule eluted at the same position. In the same way, the antigenicity of the second peak was left unaffected, in agreement with

Table 1. Summary of purification and characterization of A2'pA2'p and A2'pA3'p



it being A2'pA3'p. The relative proportions were of 1/3 for the 2'p isomer and 2/3 for the 3'p isomer.

DISCUSSION

Using a highly specific and sensitive monoclonal antibody as a probe to survey the 2'-5' oligoadenylates present in Con A-stimulated lymphocytes, we did not clearly detect 2-5A and found only minute amounts of 2-5A core dimers and trimers. We showed that a unique component accounted for the immunoreactivity. We demonstrated that Con A modulates its level in the lymphocytes in the same dose-dependant manner as it does for DNA synthesis.

TABLE 2. Characteristics of A2'pA2'p and A2'pA3'p as compared to A2'pA

	A2'pA2'p	A2'pA	A2'pA3'p
M at 260 nm	26 550	25 300	25 300
% hypochromicity	11.5	15.6	15.6
Immunoreactivity (IC 50)	2.5×10^{-11} M	4.0×10^{-11} M	3.3×10^{-11} M
Sensitivity to alkaline phosphatase	A2'pA (100 %)	None	A2'pA (100 %)
Sensitivity to KOH 0.3N	2'AMP (75 %) 3'AMP (25 %)	2'AMP (25 %) 3'AMP (25 %) Ado (50 %)	2'AMP (25 %) 3'AMP (75 %)

That this molecule only detected by its antigenic properties is adenylyl 2'-5' adenylic acid lies on the combination of several criteria and all the analysis performed showed no distinction between authentic adenylyl 2'-5' adenylic acid and the biological product. Its origin and its function remained unknown and can only be matter for speculation.

Indeed, in the present status of knowledge on the 2-5A system, nothing indicates that adenylyl (2'-5') adenylic acids can be generated from functional 2-5A. The only known enzyme which degrades 2-5A liberates 5' AMP and ATP. Similarly, the 2-5A core oligomers which are known to occur naturally (2, 9) have never been reported to be the precursors of such dimers. Other possibilities need then to be considered. Among them, the hypothesis that they are breakdown products of higher molecular weight nucleic acids must deserve attention : 2'-5' linkages occur during splicing of mRNA, however, they appear to involve essentially A2'pG (21).

Until now, out of the well-characterized 2'-5' oligoadenylates produced by the 2-5A synthetase and their core-counterparts, an increasing number of 2'-5' oligoadenylates was detected either by immunoassays, radiobinding assays or functional assays. They were found in different cell lines infected with different viruses, but

neither their structure nor their function have yet been determined (10-12). However, their characterization might reveal products able to regulate the 2-5A system or "products indicative of an alternative function for this system" as pointed out by Rice et al. (12).

Here we have succeeded in characterizing such a molecule and finding a way to modulate its levels in biological conditions other than interferon treatment or deliberate viral infection.

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