dent methods such as X-ray crystallography are needed for site identification. Since there is some doubt that affinity reagents react exclusively at or relatively near the binding site, their most useful function may be to identify proteins binding ligands in complex systems such as membranes.

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Antibodies to the Codons ApApA, ApApC, and ApUpG†

Rose M. D'Alisa‡ and Bernard F. Erlanger*

ABSTRACT: Antibodies to three triplet codons (ApApA, ApApC, and ApUpG) were elicited in rabbits by immunization with trinucleoside diphosphate-bovine serum albumin conjugates. Both cold and tritiated ApUpG used in these studies were synthesized by a modification of the method of P. Leder *et al.* ((1965), *Biochemistry 4,* 1561). The specificities of the antibodies were determined by gel diffusion, microquantitative precipitation, and radioimmunossay. Precipitation analysis using rabbit serum albumin conjugates showed that antibodies to ApApC and ApUpG recognized the entire trinucleotide sequence but also cross-reacted with the components of the hapten closest to the carrier protein. However, radioimmunossay using radiolabeled ApUpG and unabsorbed anti-ApUpG globulin showed that there was a population of antibody highly specific for the ApUpG sequence. Precipitation analysis showed that the anti-ApApA immunoglobulin reacted only with its parent antigen and not at all with conjugates of A or ApA, even without prior absorption. This unusually high specificity may reflect the unique three-dimensional conformation of the ApApA molecule. Many immunizations were required to obtain antibodies specific for ApUpG and, even then, the antibody was of low titer. The possibility that synthesis of antibody specific for ApUpG may inhibit further protein synthesis by reaction with the initiator codon is discussed.

In 1964, this laboratory reported a new method for the preparation of immunogenic protein conjugates of ribonucleosides and ribonucleotides which were used to elicit nucleic acid reactive antibodies highly specific for the purine or pyrimidine determinant groups (Erlanger and Beiser, 1964). Subsequently, this method was used, in our laboratory as well as in others, to prepare antibodies to inosine (Bonavida *et al.,* 1971; Inouye *et al.,* 1971), to methylated bases (Levine *et al.,* 1971; Sawicki *et al.,* 1971), and to various dinucleotides (E. Nahon in Beiser and Erlanger, 1966; Wallace *et al.,* 1971; Erlanger *et al.,* 1972). The unusually high specificity of the antibodies and their ability to bind to single-stranded regions of nucleic acids made them useful tools for probing biological and biochemical systems (Klein *et al.,* 1967; Seegal *et al.,* 1969; Wallace *et al.,* 1969; Freeman *et al.,* 1971; Liebeskind *et al.,* 1971, 1974; Erlanger *et al.,* 1972) and for the examination of the architecture of metaphase chromosomes (Dev *et al.,* 1972; Schreck *et al.,* 1973).

The purpose of this paper is to report the elicitation and properties of antibodies specific for the codons ApApA,

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ApApC, and ApUpG.[1] The antibodies were shown by precipitation and radiohapten competition studies to be capable of recognizing the trinucleotide sequence present in the determinant group of the antigen. It should be noted that while this work was in progress, a report appeared (Bonavida *et al.*, 1972) describing the raising of antibodies to the trinucleotides ApApU and ApApC by immunization with conjugates prepared by the same method used in this paper.

Materials and Methods

Trinucleotides. ApApA was purchased from Miles Laboratories and ApApC from Sigma Chemical Co.

ApUpG was synthesized by a modification of the method of Leder *et al.* (1965). The incubation mixture (30 ml total volume) was made up in 0.5 M Tris-HCl buffer (pH 9.0) containing 0.5 mM EDTA and 0.1 M $MgCl_2$. Included in this solution were 180 μmol of ApU (Sigma), 250 μmol of GDP (Sigma), 100,000 units of T_1 ribonuclease (P-L Biochemicals), and 50 units of polynucleotide phosphorylase (prepared by E. Kisailus by a modification of the method of Singer, 1966; Klee and Singer, 1968). Incubation was at 37° for 18 hr after which the reaction mixture was heated in a boiling water bath for 3 min. Bacterial alkaline phosphatase (150 units, code BAPF, Worthington Biochemical Corp.) was added and incubation at 37° for 90 min followed. After being heated in a boiling water bath for 5 min to terminate the reaction, the mixture was passed through a 65 × 1.5 cm column of Bio-Gel P-10 (Bio-Rad Laboratories), which had been equilibrated in distilled water, and elution with distilled water was begun; 5-ml fractions were collected and read at 260 nm in a Beckman DU spectrophotometer; fractions with an OD greater than 1.0 were spotted on Whatman 3MM paper. The paper was developed by descending chromatography in 1-propanol–ammonia–water, 55:10:35 v/v/v, for 18 hr and dried, and the spots were located by scanning under a uv light source (see Results). ApUpG began to appear in fraction 10 and was present in all fractions up to fraction 21. The fractions containing ApUpG were pooled, concentrated by evaporation *in vacuo,* and rechromatographed on paper (Schleicher and Schuell Co., No. 2040B) as described above to remove all traces of contaminants. ApUpG was eluted from the paper with distilled water and lyophilized. Total yield from this batch was 12 mg of ApUpG.

Characterization of ApUpG. The material believed to be ApUpG was subjected to snake venom phosphodiesterase digestion in the following manner: the reaction mixture (0.2 ml total volume) contained 10 μmol of ammonium carbonate (pH 9.0), 2 μmol of $MgCl_2$, 10 μg of snake venom phosphodiesterase (Worthington Biochemical Corporation), and 6 A_{260} units of the trinucleoside diphosphate. Incubation was at 37° for 4 hr (Leder *et al.*, 1965). The mixture was spotted on a sheet of Whatman 3MM paper and developed by descending chromatography in 1:1:1 v/v/v isoamyl alcohol–tetrahydrofurfuryl alcohol–0.08 M potassium citrate (pH 3.0) for 18 hr (Block *et al.*, 1958).

The ApUpG was also characterized by comparison of its uv spectrum with published data (Aoyagi and Inoue, 1968).

Synthesis of Tritiated ApUpG. The reaction mixture (0.1 ml total volume) was made up in 0.5 M Tris-HCl buffer (pH 9.0) containing 0.5 mM EDTA and 0.1 M $MgCl_2$. It also contained 0.5 mCi [^{3}H]GDP (New England Nuclear, specific activity 1.29 Ci/mmol), 1.4 μmol of ApU (Sigma), 0.5 unit of polynucleotide phosphorylase (prepared by E. Kisailus; see above) and 250 units of T_1 ribonuclease (P-L Biochemicals). The procedure was identical with that used to synthesize cold ApUpG except for the ratio of starting materials which, in this experiment, was designed to obtain maximal yields while sparing [^{3}H]GDP. A fourfold excess of ApU was, therefore, used to maximize the incorporation of tritiated GDP. This reaction mixture, after dephosphorylation with 0.5 unit of alkaline phosphatase (Worthington), was spotted directly on paper and chromatographed. The ApUpG spot was located by scanning under a uv light source, cut out, eluted with distilled water, and frozen in small aliquots. The identity of the tritiated ApUpG was established by its uv spectrum and by paper chromatography using radioautography in which Kodak Medical No-Screen X-ray film was exposed for 2 weeks.

Conjugation of Trinucleotides to Protein. The trinucleotides ApApA, ApApC, and ApUpG were conjugated to bovine serum albumin or rabbit serum albumin by a modification of the method of Erlanger and Beiser (1964). Before use, BSA or RSA were passed through a column of Bio-Gel P-20 to remove any ribonuclease activity that might be present; 40 mg of BSA or RSA was dissolved in 1 ml of distilled water and passed through a 25 × 1 cm column of the gel previously equilibrated in distilled water. The column was eluted with distilled water and 2-ml fractions were collected and read at 280 mμ. The albumins usually appeared in fractions 4–7.

The procedure for the conjugation of the trinucleotides was the same as that for mononucleotides with the following exceptions: (a) incubation with sodium periodate was carried out in the dark for 30 min at room temperature, (b) after addition of sodium borohydride, the conjugate was left at 4° for only 1 hr instead of 18 hr, (c) no formic acid was added, and (d) the conjugates were dialyzed against running tap water overnight and then lyophilized. The uv spectra of the conjugates were identical with those of the corresponding trinucleoside diphosphate except for protein "end" absorption. Conjugation resulted in the substitution of 8–10 haptenic groups per protein molecule as determined by the uv absorption of the conjugates. The ApApA–BSA conjugate had been prepared earlier in this laboratory (S. S. Wallace, unpublished).

Immunization and Antisera. All antisera were prepared as described by Butler *et al.* (1962). Rabbits were immunized with 0.1 ml of an emulsion of 1 mg/ml of antigen in complete Freund's adjuvant, injected into each of the four footpads once weekly for 3 weeks, followed by monthly boosters. In the case of ApApA and ApApC, the animals began to make antibody after three immunizations. The ApUpG animals did not respond after three immunizations and they received three more immunizations at weekly intervals. The antibody response was monitored by gel diffusion and tube precipitation analysis using RSA conjugates of the trinucleotides. Animals were bled twice weekly and their sera were pooled, and globulin fractions were prepared by four successive precipitations in sodium sulfate (Strauss *et al.*, 1960). The globulin fractions were dialyzed against saline, sterile-filtered, and stored at 4°. Prior to use, the IgG fraction was isolated by passing a portion (5 ml) of each globulin through a column of DEAE-cellulose (Fahey, 1967) contained in the barrel of a 50-ml hypodermic syringe. The IgG was dialyzed against saline, concentrated to the initial volume with a B15 minicon concentrator (Amicon Corp.) and sterile-filtered.

Immunochemical Procedures. Quantitative precipitin tests were done by the method of Kabat and Mayer (1961) using the

[1] Abbreviations used are: BSA, bovine serum albumin; RSA, rabbit serum albumin; ApApA, adenylyl-3′,5′-adenylyl-3′,5′-adenosine; ApApC, adenylyl-3′,5′-adenylyl-3′,5′-cytidine; ApUpG, adenylyl-3′,5′-uridyl-3′,5′- guanosine; ApG, adenylyl-3′,5′-guanosine; ApU, adenylyl-3′,5′-uridine; ApC, adenylyl-3′,5′-cytidine; ApA-Ova, adenylyl-3′,5′-adenosine ovalbumin;

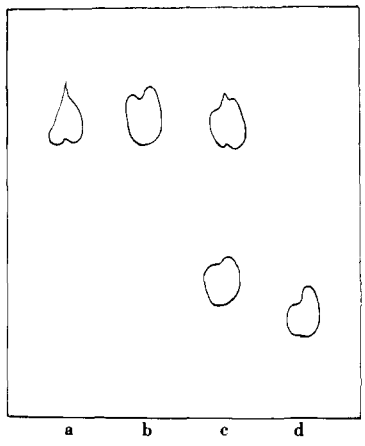

FIGURE 1: Chromatogram of (a) ApU, (b) G, (c) reaction mixture, and (d) GDP on Whatman 3MM paper developed in propanol-ammonia-water, 55:10:35, v/v/v for 18 hr.

Folin-Ciocalteau color reaction for quantification of the precipitates. Micro agar gel diffusion experiments were done as described by Lacour *et al.*, 1962.

Radiommunoassay. Globulins used for this assay were treated with charcoal prior to use. Roughly 3 g of Norit A (Fisher Scientific Co.) were boiled in 40 ml of distilled water for 10 min. After centrifugation at 4°, at 2000 rpm for 20 min, the water was decanted leaving just enough moisture to wet the charcoal; 0.5 ml of this paste was added to 10 ml of globulin and the suspension was stirred gently for 48 hr at 4°. The charcoal was removed by centrifugation and the globulin was filtered to remove all traces of charcoal.

For binding assays, the reaction mixtures contained 100 μl of [^{3}H]ApUpG (1.4 × 10^{-6} μmol, specific activity 1.29 Ci/mmol) and 100 μl of immunoglobulin. For inhibition assays, the reaction mixtures contained 50 μl of [^{3}H]ApUpG (1.4 × 10^{-6} μmol, specific activity 1.29 Ci/mmol), 50 μl of diluted, nonradioactive inhibitor, and 100 μl of immunoglobulin. In all cases, dilutions were made in saline and the immunoglobulin was added last. Reaction tubes were incubated at 37° overnight. Normal rabbit globulin carrier (100 μl) was added to all tubes and all protein was precipitated by the addition of 6 ml of 50% saturated ammonium sulfate. The tubes were kept at 4° for 2 hr and then centrifuged at 4° at 2000 rpm for 1 hr. The precipitates were washed twice with 50% saturated ammonium sulfate, solubilized in Soluene 100 (Packard), and counted in 10 ml of toluene-based scintillation fluor.

Results

Preparation and Characterization of ApUpG. The synthesis of ApUpG (Leder *et al.*, 1965) involved three reactions

$$\text{ApU} + \text{GDP} \xrightarrow{\text{polynucleotide phosphorylase}} \text{ApU(pG)}n + n\text{iP} \quad (1)$$

$$\text{ApU(pG)}n \xrightarrow{\text{T}_1\text{ ribonuclease}} \text{ApUpGp} + (n-1)\text{Gp} \quad (2)$$

$$\text{ApUpGp} \xrightarrow{\text{alkaline phosphatase}} \text{ApUpG} \quad (3)$$

In the first step, several residues of G were added to ApU, which served as a primer molecule. In the second step, the oligomer ApU(pG)$_n$ was cleaved to the trinucleoside triphosphate which was then dephosphorylated to ApUpG by alkaline phosphatase.

Figure 1 shows a chromatogram of the reaction mixture with markers of ApU, GDP, and G. A new spot, later characterized as ApUpG (see below), was easily separable from the other compounds. The reaction mixture was then chromatographed on Bio-Gel P-10 and the fractions were monitored by paper chromatography. All fractions containing the new spot were combined and purified further by paper chromatography (see Methods).

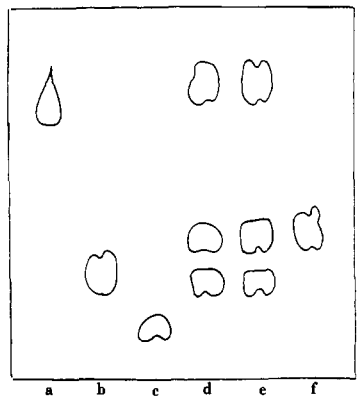

FIGURE 2: Chromatogram of the degradation products of ApUpG following snake venom phosphodiesterase digestion: (a) adenosine, (b) GMP, (c) ApUpG, (d) enzymatic hydrolysate of ApUpG, (e) artificial mixture of adenosine, UMP, and GMP treated with the enzyme, (f) UMP. The chromatography was run on Whatman 3MM paper and developed in isoamyl alcohol-tetrahydrofurfuryl alcohol-0.08 M potassium citrate (pH 3.0) for 18 hr.

Digestion of the product by snake venom phosphodiesterase established its identity as ApUpG. Snake venom phosphodiesterase yields a nucleoside from the end of the compound bearing a free 5′-hydroxyl group and 5′-mononucleotides from all other residues. The products expected from the degradation of ApUpG are, therefore, adenosine, UMP, and GMP. These compounds as well as the hydrolysate of ApUpG were spotted on a sheet of Whatman 3MM paper. In addition, a sample containing a mixture of adenosine, UMP, and GMP was treated with the enzyme under the same conditions as ApUpG and spotted on the same paper. Chromatography was performed in isoamyl alcohol-tetrahydrofurfuryl alcohol-0.08 M potassium citrate (pH 3.0) (see Methods) and the results are shown in Figure 2. The product gave three spots with mobilities identical with the artificial mixture that had been incubated with the enzyme.

The ultraviolet spectra of ApU, GDP, and ApUpG are shown in Figure 3. The spectrum of GDP is clearly different from the others, having a peak at 260 nm and a shoulder at 275 nm. ApU and ApUpG have similar absorption maxima and minima but the spectrum of ApUpG is broader than that of

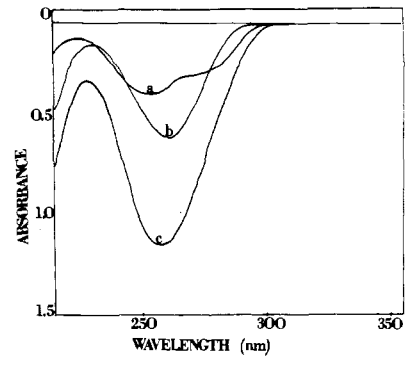

FIGURE 3: Ultraviolet spectra of (a) GDP, (b) ApU, and (c) ApUpG. All compounds are at pH 7.0 and 3 × 10^{-5} M.

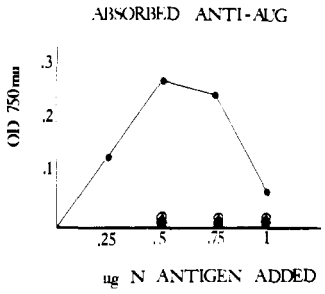

FIGURE 4: Precipitation analysis of absorbed anti-ApUpG immunoglobulin against (●) ApUpG-RSA, (○) A-RSA, (■) ApApA-RSA, (▲) ApApC-RSA.

ApU. Our results agree with published absorption data (Stanley and Bock, 1965; Aoyagi and Inoue, 1968).

Reactions of Anti-ApUpG. Anti-ApUpG reacted with ApUpG-RSA and with a number of mono- and trinucleotide conjugates (A-RSA, G-RSA, ApApA-RSA, and ApApC-RSA). The titer of the antibody *vs.* ApUpG-RSA was 196 μg of antibody protein/ml. By absorbing the immunoglobulin with A-RSA and U-RSA, it was possible to obtain a population of antibody specific for ApUpG as shown in Figure 4. The titer at this point was 76 μg of antibody protein/ml. If the globulin were absorbed with G-RSA, no anti-ApUpG antibody could be detected by precipitation with ApUpG-RSA.

Using tritiated ApUpG, it was possible to characterize the unabsorbed anti-ApUpG globulin by radioimmunoassay (Figure 5). The assay showed that only cold ApUpG inhibited the binding of tritiated ApUpG. As much as 100 pmol of adenosine, uridine, or guanosine failed to displace any radiolabeled ApUpG.

Reactions of Anti-ApApC. Figure 6 shows the immunochemical reactions of anti-ApApC immunoglobulin. As can be seen, the antibody reacted best with ApApC-RSA. The antibody also precipitated with ApC-RSA, the dinucleotide closest to the protein carrier. There is also a small but significant reaction with C-RSA. This result is in contrast to the data of Bonavida *et al.* (1972), whose preparation of anti-ApApC antibody did not react with C-BSA. There was no reaction with either A-RSA or ApA-Ova.

Reactions of Anti-ApApA. Figure 7 shows the results obtained with anti-ApApA globulin. This unabsorbed globulin reacted very well with ApApA-RSA but not at all with either A-RSA or ApA-Ova. There is, however, a small reaction with GpA-RSA and poly(A)-RSA. No reaction was observed with poly(A) (*i.e.*, unconjugated to a protein carrier).

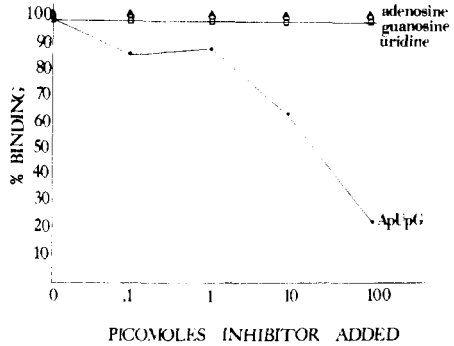

FIGURE 5: Hapten inhibition of binding of tritiated ApUpG by unabsorbed anti-ApUpG immunoglobulin.

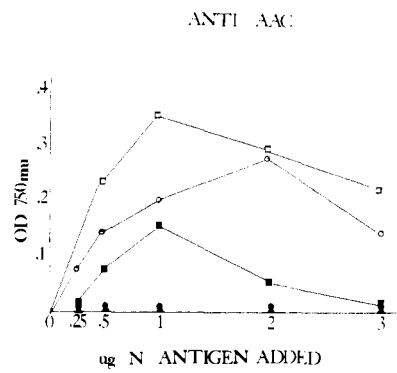

FIGURE 6: Precipitation analysis of anti-ApApC immunoglobulin against (□) ApApC-RSA, (○) ApC-RSA, (■) C-RSA, (●) A-RSA, (▲) ApA-Ova.

Discussion

Our results demonstrate that antibodies highly specific for a trinucleotide sequence can be obtained either directly from the immunized animal or after absorption with cross-reacting antigens. The most striking result was observed with antibody specific for ApApA. Without prior absorption, it reacted with ApApA-RSA but not at all with A-RSA or ApA-Ova. This same result was obtained with globulins from two animals. That this high specificity might result from a unique three-dimensional structure of the trinucleotide is indicated by recent X-ray diffraction studies on ApApA (Suck *et al.*, 1973), which showed that the ApApA molecule had an unusual structure with both a helical and a nonhelical portion. The first two adenosines formed part of a right-handed helix while the third adenosine was folded back on the phosphate group of the helical fragment. At least part of this structure might persist in our conjugate, making it possible for the antibody to recognize ApApA as a unique three-dimensional structure rather than just a sequence. The small cross-reaction with poly(A)-RSA might indicate a similarity, but not necessarily an identity, in the structure of part of the poly(A) molecule. The basis for the small cross-reaction with GpA-RSA is not readily explicable at this time but may reflect aspects of the three-dimensional structure of GpA.

The specificities of the antibodies raised to ApApC were in agreement with earlier studies with anti-dinucleotide antibodies. In the earlier studies (Wallace *et al.*, 1971), strongest reaction was with the dinucleotide determinant group but the next strongest reaction was with the conjugate containing the base closest to the linkage to the protein, *e.g.*, anti-ApG (ab-

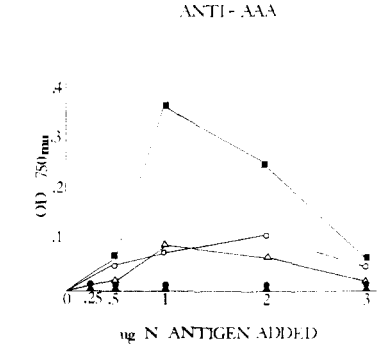

FIGURE 7: Precipitation analysis of anti-ApApA immunoglobulin against (■) ApApA-RSA, (○) GpA-RSA, (△) poly(A-RSA), (●) A-RSA, (▲) ApA-Ova.

sorbed with BSA) reacted best with the ApG conjugate and next best with G-BSA, rather than with A-BSA, as would have been predicted from the work of Landsteiner (1936). Thus, ApApC immunoglobulin reacted best with ApApC-RSA, next best with ApC-RSA, and then with C-RSA. The anti-ApApC antibodies of Bonavida *et al.* (1972) behaved the same way. One factor that appears to be responsible for the reaction with the base directly coupled to the carrier is the activity as a determinant group of the morpholine ring formed by the coupling reaction. L. Levine (personal communication) has independently established this fact experimentally.

Anti-ApUpG immunoglobulin reacted initially with a number of hapten–RSA conjugates, but it was possible to absorb the globulin to increase specificity so that reaction was observed only with ApUpG-RSA and G-RSA. Radioimmunoassay carried out with unabsorbed globulin showed high specificity for ApUpG; in fact, none of the mononucleosides tested displaced radiolabeled ApUpG. Since this method selects only the antibody with the highest affinity, the results show that there is, in the unabsorbed globulin, a population of antibody of high binding affinity which is specific for ApUpG as a sequence.

It is interesting to note that many immunizations were required to obtain antibody to ApUpG and that even then the immunoglobulin obtained was of low titer. Similar observations were made in six rabbits. That these animals were not just poor antibody producers was shown by testing their sera with BSA. All rabbits began to show considerable precipitating antibody to BSA by the third immunization. It is tempting to speculate that intracellular synthesis of antibodies specific for ApUpG can proceed only for a short time before the antibody reacts with the initiator codon sequence in mRNA and shuts off protein synthesis. Experiments are under way to determine if antibody specific for ApUpG can inhibit protein synthesis in an *in vitro* system.

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