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Polyclonal Antibodies against a Structure Mimicking the Covalent Linkage Unit between Picornavirus RNA and VPg: An Immunochemical Study

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Abstract—We propose that therapy of patients with anticancer drugs that poison DNA topoisomerases induces formation of covalent complexes of cellular RNAs and DNA topoisomerases. The appearance of these complexes can be detected with antibodies against a synthetic hapten mimicking the covalent linkage unit Tyr-pU(p) of picornavirus RNA and VPg. We synthesized hapten [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-(CH_2)$_6$$NH_2$, conjugated it with BSA, and immunized rabbits with the antigen obtained. The raised polyclonal antibodies were purified by successive affinity chromatography on BSA-Sepharose and hapten-Sepharose columns. Target antibodies recognized hapten and encephalomyocarditis virus RNA–VPg complex specifically as found using the dot-immunogold method. We believe that these antibodies might be useful to study mechanism of picorna and similar virus RNA synthesis. The discovery and qualitative determination of the cellular RNA–DNA topoisomerases covalent complexes with these antibodies might be useful to monitor therapy efficacy by drugs "freezing" dead-end complexes of DNA topoisomerases and nucleic acids and to understand the mechanism of DNA topoisomerase poisoning *in situ*.

Key words: antibody, RNA–VPg, covalent linkage unit, affinity chromatography, immunogold, picornavirus, DNA topoisomerase

Natural covalent complexes of RNA and proteins that are bound to each other via a phosphodiester bond are known for several groups of animal and plant viruses [1]. Most of these that have been studied structurally and functionally are covalent complexes of animal picornavirus [2, 3] and plant comovirus [4, 5] RNAs and the 5′-terminal protein VPg.

Yet, until recently cellular covalent complexes of RNA and proteins, for which the structure of the covalent linkage unit (CLU) has been proven, have not been discovered despite the following prerequisites of their existence.

First, activity of a cellular unlinking enzyme that hydrolyzes the phosphodiester bond between picornavirus RNA and VPg specifically was found in uninfected animal and in plant cells [6-8]. The role of this phosphodiesterase in picornavirus infection is not known and its cellular targets have not yet been discovered. We speculate that there are eukaryotic cellular covalent complexes of some RNA(s) and protein(s), structurally similar to picornavirus ones with the identical CLU that is a tyrosine phosphodiester of uridylic acid. These complexes are supposed to be substrates of the unlinking enzyme.

Second, it was recently found that DNA is not a unique substrate for the trans-esterification reaction catalyzed by DNA topoisomerases. *In vitro* the latter were able to promote this reaction with RNA as a substrate, as was demonstrated for bacterial topoisomerase III [9] and for human topoisomerases IIα and IIβ [10]. Ability to hydrolyze and restore an internucleotide bond of RNA means that DNA topoisomerases use the same mechanism of the enzymatic reactions as they do with the DNA substrate, so they form the catalytic transient active phos-

Abbreviations: CLU) covalent linkage unit; EMC) encephalomyocarditis; PBS) phosphate-buffered saline; PVX) potato virus X; TMV) tobacco mosaic virus.

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phodiester of tyrosine residue and 5′ (or 3′) nucleotide phosphate group [11]. Thus, the chemical structure of the covalent linkage unit of the RNA–topoisomerase covalent complex should resemble the picornavirus RNA–VPg complex.

Finally, there are cellular proteins that are involved *in vivo* in the process of the VPg-primed initiation replication of picornavirus RNA via linking of the very first nucleotide of the nascent RNA to VPg (or VPg precursor) [12-15]. We propose that antibodies against CLU of the encephalomyocarditis (EMC) virus RNA–VPg complex could be a versatile tool to search for, isolate, and study cellular tyrosine phosphodiesters of nucleic acids and proteins. We believe that these antibodies could be used for study and regulation of viral RNA replication.

The goal of this work was to synthesize a model hapten mimicking the picornavirus CLU to raise the antiserum, isolate antibodies, and characterize their specificity.

MATERIALS AND METHODS

Materials. BA 83 nitrate cellulose membranes were from Schleicher & Schuell (Germany); AuroProbe Plus GAR kit and CNBr-activated Sepharose were from Amersham–Pharmacia (Sweden); glutaric dialdehyde and sodium borohydride were from Serva (Germany); BSA (fraction V) and complete and incomplete Freund adjuvants were from Sigma (USA); poly(C) was from SKTB BAS (Novosibirsk, Russia); poly(U) and *N*-acetyltyrosine ethyl ester were from Reanal (Hungary); 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine, 1-methylimidazole, triethylamine tris-hydrofluoride, and 1-H-tetrazole were purchased from Fluka (Switzerland); *N*-methyl-2-pyrrolidinone was from Aldrich (USA); 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilyluridine-3′-*N*,*N*-diisopropyl-(2-cyanoethyl)-phosphoramidite was from Glen Research (USA); egg lysozyme and other reagents were from Reakhim (Russia).

The following enzyme preparations were also used in the study: nuclease P1 (EC 3.1.30.1) from *Penicillium citrinium* (100 U/ml) and snake venom phosphodiesterase (EC 3.1.4.1) from *Crotalus atrox* (0.9 U/ml) (Sigma, USA); alkaline phosphatase from *Escherichia coli* (EC 3.1.3.1) (34 U/mg) (NPO Biolar, Russia).

^{31}P-NMR spectra (161.978 MHz, external standard 85% H_3PO_4) and ^{1}H-NMR spectra (400.135 MHz) were obtained using an AM-400 spectrometer (Bruker, Germany).

[*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$, the phosphodiester of protected tyrosine and derivative of uridylic acid, was synthesized by phosphoramidite chemistry on modified polymer support CPG-500 bearing 1,3-dihydro-1,3-dioxo-2-[6-[bis(4-methoxyphenyl)-phenylmethoxy]hex-1-yl]isoindol group [16]. The specific polymer capacity was 30 μmol of the ligand phosphodiester per gram polymer according to dimethoxytrityl cation determination.

The polymer (200 mg) was treated with 3% $CHCl_2COOH$ solution in CH_2Cl_2 and then washed with CH_2Cl_2 and CH_3CN successively. A mixture of 0.3 ml of 0.1 M 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilyluridine-3′-*N*,*N*-diisopropyl-(2-cyanoethyl)-phosphoramidite in anhydrous CH_3CN and 0.3 ml of condensing reagent (0.45 M tetrazole and 0.1 M 1-methylimidazole in anhydrous CH_3CN) was added to the polymer support and stirred for 15 min. The condensation step was repeated. The procedures of capping, oxidation, and removal of 5′-*O*-dimethoxytrityl group were performed under standard conditions [17]. A mixture of 0.6 ml of *N*,*N*-diisopropylethylamine, 2 ml of CH_2Cl_2, and 0.4 ml of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite was added to the polymer with bound uridine, and the reaction mixture was kept for 1 h with agitating at regular intervals. The polymer was washed with anhydrous CH_3CN, then a mixture of 0.5 ml of 0.4 M *N*-acetyl-tyrosine ethyl ester in anhydrous CH_3CN and 0.5 ml of condensing reagent was added to the polymer. Condensation was brought about for 40 min with agitation by a stirrer. After oxidation under standard conditions [17] the reaction product was removed from the polymer support after treating with NH_4OH–EtOH (3 : 1) at 56°C for 16 h. The protecting 2′-*O*-tBDMSi groups were removed with a mixture (0.5 ml) of triethylamine tris-hydrofluoride–*N*-methyl-2-pyrrolidinone–triethylamine (4 : 6 : 3) for 1.5 h at 65°C. Then, 10 ml of 0.1 M NH_4HCO_3 was added, and the target product was isolated by reverse phase high-performance liquid phase chromatography (RP HPLC) on a column (4.6 × 250 mm; Waters, USA) with LiChrosorb RP-18 (Merck, Germany) using a linear acetonitrile gradient (0-20%) in 0.05 M NH_4HCO_3. The yield of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ was 4 OD_{260} (NH_4^+-salt). The homogeneity was 93% according to RP HPLC data. The spectral ratios: 250 : 260 = 0.77; 270 : 260 = 0.85; 280 : 260 = 0.37; 290 : 260 = 0.05. ^{31}P-NMR spectra (D_2O) δ_P (ppm): –4.32 [$C_6H_4OP(O)O$], 0.49 [$NH_2(CH_2)_6OP(O)O$]. ^{1}H-NMR spectra (D_2O) δ_H (ppm): 7.76 (1 H, d, H6, J = 8 Hz), 7.28 and 7.20 (4 H, d, d, aromatic H, Tyr J = 8 Hz), 6.02 (1 H, d, H1′, J = 7 Hz), 5.78 (1 H, d, H5, J = 8 Hz), 4.70-4.15 (6 H, m, H2′, H3′, H4′, H5′ H5″, C*H* Tyr), 3.97 [2 H, m, $OCH_2(CH_2)_5NH_2$], 3.20-2.95 [4 H, m, $O(CH_2)_5CH_2NH_2$, CH_2 Tyr], 2.00 (1 H, s, CH_3CO), 1.71 (4 H, m, $OCH_2CH_2CH_2CH_2CH_2CH_2NH_2$), 1.46 (4 H, m, $OCH_2CH_2CH_2CH_2CH_2CH_2NH_2$).

Enzymatic hydrolysis of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$. To 0.5 OD_{260} [*N*(Ac), *C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ in 15 μl of buffer (0.03 M NaOAc, 1 mM $ZnSO_4$, pH 5.2), 5 μl of nuclease P1 in 0.03 M NaOAc (pH 5.2) was added, and

the mixture was incubated for 6 h at 37°C. Then 5 μl of buffer (1 M Tris-HCl, 0.05 M $MgCl_2$, pH 7.8), 0.1 mg of alkaline phosphatase from *Escherichia coli*, and 10 μl of phosphodiesterase in buffer (0.1 M Tris-HCl, 5 mM $MgCl_2$, pH 7.8) were added. The reaction cocktail was incubated for 6 h at 37°C, heated for 2 min at 100°C, and analyzed by RP HPLC (Milichrom, Russia) on microcolumn (2 × 62 mm) with Nucleosil C-18 (5 μm, Macherey-Nagel, Germany). Linear acetonitrile gradient (0-25%) in 0.05 M $LiClO_4$ was applied, and RP HPLC was followed by quantitative analysis at six UV wavelengths. The hydrolyzate contained two products, one of them being uridine. The second product was identified as *N*-acetyl-tyrosine amide due to its spectral characteristics and the retention time (analytical RP HPLC) with a product obtained after treating *N*-acetyl-tyrosine ethyl ester by a mixture NH_4OH–EtOH (3 : 1) for 16 h at 56°C. Uridine and *N*-acetyl-tyrosine amide were in 1 to 0.9 proportion.

Synthesis of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ and BSA conjugate. To 8 μl of 62 mM [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$, 11.3 μl of 0.4 M (pH 9.3) borate buffer and 24 μl (17.65 nmol) of BSA solution were added, and reaction was started after addition of 5% glutaric dialdehyde solution (2 μl); the reaction cocktail was incubated for 3 h at 40°C. The reaction was stopped at 0°C by the subsequent addition of 6.3 μl $NaBH_4$ (15 mg/ml) in three equal portions with 15 min intervals. Reduction was continued for 1 h at 0°C and stopped by addition of glacial acetic acid (250 μl). Conjugate of hapten and BSA was precipitated by isopropanol (1 ml) at –20°C overnight, washed by 96% alcohol, collected by centrifugation, and dried in a desiccator. Unbound and bound [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ was determined by UV spectrophotometry after OD_{260} measurements of supernatant and dissolved precipitate.

Rabbit immunization with conjugate of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ and BSA. Antiserum against [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ was raised in 2-kg rabbits by injecting antigen (50 nmol in 1 ml of phosphate-buffered saline (PBS)) subcutaneously at multiple sites in the back. First and second immunizations were carried out with complete and incomplete Freund adjuvant, respectively. Following four injections over a period of eight weeks, blood and sera were collected by standard procedures. Preimmune and immune antisera were kept at –70°C.

Isolation of immunoglobulins. Fraction of immunoglobulins was obtained by ammonium sulfate fractionation followed by DEAE-chromatography from antisera against [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ as described [18].

BSA coupling to BrCN-Sepharose. BrCN-activated Sepharose was added to 10 ml of BSA solution (7 mg/ml) in bicarbonate buffer (0.1 M sodium bicarbonate, pH 8.5, 0.5 M NaCl). Coupling was for 2 h at room temperature with occasional shaking. Unbound protein was washed out with bicarbonate buffer. To block the remaining unreacted active groups, a slurry of the adsorbent was equilibrated with 0.1 M Tris-HCl, pH 8.0, and kept again for 2 h at room temperature. The affinity matrix was thoroughly washed with 0.1 M sodium acetate buffer, pH 4.0, and then with PBS. Through all steps of the adsorbent preparation, OD_{280} of solutions was monitored. Up to 84% of the BSA was coupled to the matrix.

Coupling of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ with BrCN-activated Sepharose. To BrCN-Sepharose equilibrated with bicarbonate buffer, pH 8.3, 310 nmol of ligand in 700 μl of the same buffer was added, and the reaction mixture was incubated for 2 h at room temperature with occasional shaking. Excess liquid was decanted and the unbound ligand was washed out by bicarbonate buffer. Excess of BrCN-activated groups was blocked as above. Seventy percent of the [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ was tightly bound to the Sepharose matrix.

Affinity chromatography of target immunoglobulins. Two successive affinity chromatography steps were performed to purify target antibodies. At first, immunoglobulins against BSA~[*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ (~30 mg) were freed on BSA-Sepharose column from most of the antibodies against the bulky carrier antigen, that is BSA. For this purpose preparation of immunoglobulins, dissolved in a minimal volume of PBS, was applied on 0.8 × 9 cm column, equilibrated with PBS, and chromatography was repeated three times with flow rate 3 ml/h at 4°C. According to absorbance at 280 nm of the effluent and solution of desorbed antibodies (pH 2.8), approximately 65% of the immunoglobulins were adsorbed on the column at this step.

Second, the rest of eluted immunoglobulins that were depleted of the BSA recognizing antibodies, after equilibration with 0.1 M phosphate buffer (pH 8.0) were successively adsorbed on a column with [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$-Sepharose adsorbent (1 ml, 0.4 × 8 cm). Then the column was washed with 1 ml of 0.5 M NaCl to remove nonspecifically bound proteins. The column was equilibrated with PBS, and specific antibodies were eluted with a minimal volume of 50 mM glycine buffer (pH 2.8) and immediately neutralized (pH 7.0-7.3) by addition of 0.1 M phosphate buffer (pH 8.0). The required minimal volume of glycine buffer was found by staining on nitrocellulose membrane of the spotted eluate with Coomassie G-250. These antibodies were kept on ice.

Synthesis of conjugate of [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$ with egg lysozyme. To 1 μl of 4.55 mM [*N*(Ac),*C*O(NH$_2$)]Tyr-(5′P → O)Up-O-(CH$_2$)$_6$NH$_2$, 0.97 μl of 0.4 M sodium borate buffer (pH 9.3) and 1 μl of egg lysozyme (9.3 mg/ml) were added. Then 0.91 μl of 0.1% glutaric dialdehyde was

added, and the reaction mixture was incubated for 3 h at 40°C. The coupling reaction was arrested by chilling to 0°C and addition of 1.4 μl $NaBH_4$ (0.5 mg/ml, in three equal portions) at intervals of 15 min. Reduction was continued for 1 h at 0°C and stopped by addition of glacial acetic acid (4 μl).

Complete hydrolysis of RNA–VPg by ribonuclease T1. EMC virus RNA–VPg (9.44 μl, 0.53 μM) was completely digested for 1 h at 4°C by added ribonuclease T1 (EC 3.1.27.3) (0.97 μl, 5 U/μl).

Immunogold detection of antigens with silver enhancement. Colloidal gold conjugated secondary antibodies with silver enhancer of AuroProbe Plus GAR kit was used for immunochemical visual detection of target antigens on nitrocellulose membranes by a spot technique essentially as recommended by the manufacturer.

BA-83 or BA-85 nitrocellulose membranes were rinsed in water (for analysis of protein-free nucleic acids with 20× SSC buffer) and air-dried. Experimental and control specimens (1-7 μl) were spotted onto nitrocellulose membrane. Protein-free nucleic acids were fixed by 2-4 min UV-radiation (254 nm, 45 W) from a standard TL-20 C UV-illuminator (Vilber Lourmat) for fluorescent nucleic acid–ethidium bromide complex visualization.

Membrane strips were subsequently washed for 3 min in water, incubated in solution of 5% BSA/PBS for 30 min at 37°C, washed in 0.1% BSA/PBS three times in intervals of 5 min, and treated with primary antibodies (0.025 mg/ml) in 300-1000 μl of 0.1% BSA solution for 2 h at room temperature with gentle shaking. If lower concentrations of the primary antibodies were used, incubation time was increased to overnight at 4°C.

The Amersham-Pharmacia antigen detection protocol includes a membrane blocking procedure with BSA. This is inappropriate for analysis of antibody preparations that contained cross-reacting anti BSA immunoglobulins. In this case membranes were blocked with 0.2% Tween-20 for 20 min and instead of 0.1% BSA/PBS washing solution we used 0.025% Tween-20/PBS.

Amino acid analysis. Complete hydrolysis of proteins and conjugates was carried out in CF_3COOH–HCl (2 : 1 v/v) for 1 h at 155°C. Hydrolyzates were analyzed with a Hitachi 835 (Hitachi, Japan) amino acid analyzer.

RESULTS AND DISCUSSION

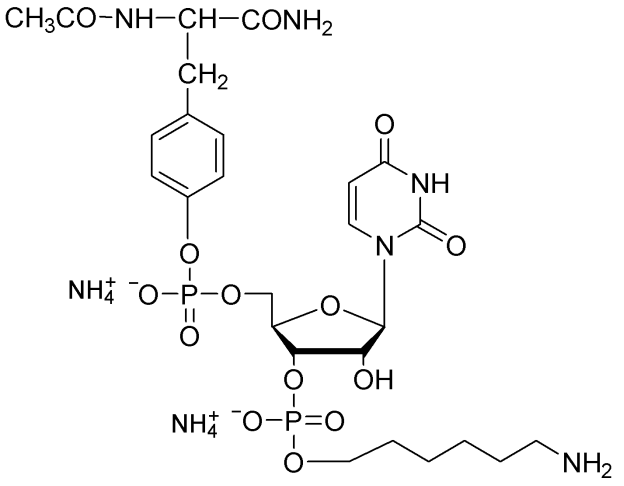
Earlier we obtained antisera against the EMC virus RNA–VPg complex and succeeded in isolating a fraction of immunoglobulins enriched with antibodies against CLU. The chemical structure of the EMC virus RNA–VPg complex is represented schematically below (the covalent linkage unit, CLU, is indicated in bold italic):

O-pU-pU-pG-pN_{7500}
|
NH_2-Gly-Pro-***Tyr***-Asn-Glu-Thr-Thr-Arg-Val-Lys-

-Pro-Lys-Thr-Leu-Gln-Leu-Leu-Asp-Ile-Gln-COOH.

Although the final antibody preparation cross-reacted with EMC virus RNA–VPg and RNA–Kpep, tobacco mosaic virus (TMV) RNA, and oligodeoxynucleotide $dT_{13}C_2$, the target immunoglobulins interacted 3-10 times stronger with tyrosine phosphodiesters of oligodeoxynucleotides than with control oligo- and polynucleotides. Moreover, we succeeded in examining the EMC virus infection development in murine L-, Chinese hamster CHO-, and human HeLa cells with these antibodies by immunofluorescent microscopy [19].

Synthesis of [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ and its conjugate with BSA. To obtain antibodies directly to the covalent linkage structure of the picornavirus RNA–VPg complex, we synthesized a model phosphodiester of tyrosine and the very first nucleoside diphosphate of EMC virus RNA, i.e., pUp. Aminohexanol spacer of the model compound [*N*(Ac), *C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ was used as a linker both for preparing antigen and for synthesis of the affinity adsorbent (see below).



The structure of the desired hapten was confirmed by ^1H- and ^{31}P-NMR spectroscopy and biochemically. ^{31}P-NMR spectra revealed the presence of two distinct resonances, one of which (at –4.3 ppm) is characteristic for the presence of the phosphate diester between the phenolic hydroxyl group of protected tyrosine and the 5′-end of the uridine moiety. A resonance at 0.49 ppm was related to the phosphate diester between 3′-end of uridine and hexamethylene amino linker. Amino acid analysis showed the expected presence of tyrosine in the model compound as well. Treatment of the synthesized hapten with nuclease P1, snake venom phosphodiesterase, and phosphatase

and amino acid analysis confirmed the expected proportion of uridylate and tyrosine residues in the model CLU.

To obtain antigen, hapten mimicking CLU of the EMC RNA–VPg complex was conjugated with BSA. According to NCBI data (NP_851335), BSA contains 60 lysine residues and approximately half of them, as found by us, are exposed for chemical modifications. Therefore hapten and BSA were conjugated in molar ratios 28 : 1 by glutaric dialdehyde. Unstable Schiff bases and excess of dialdehyde were reduced by sodium borohydride. We calculated that the molar extinction coefficient of the model hapten at 260 nm is 10,500. In our experiment 495 nmol of hapten was mixed with 17.65 nmol of BSA. After reaction and precipitation of the desired conjugate, we found 126.27 nmol of hapten in unbound form (in supernatant and washes). The yield of the nucleotidylated BSA was estimated by spectrophotometry of conjugate solution after subtraction of BSA adsorption at 260 nm; it was 74.5%. Thus, about 20 molecules of hapten were linked with each molecule of BSA.

Immunogold analysis of antisera and antibody preparations on nitrocellulose membrane. Immobilization of small molecules, like hapten, onto nitrocellulose membrane is a challenge to membrane immunochemical analysis. [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ is photochemically destroyed during UV-radiation, which gained widespread acceptance in nucleic acids and polynucleotides immobilization. Taking into consideration that proteins are strongly adsorbed onto nitrocellulose, we coupled hapten to a carrier protein. As one might expect, immune antiserum contained antibodies against BSA in high concentration (see Fig. 1).

It was mentioned above that the Amersham-Pharmacia immunogold antigen detection protocol is not appropriate for analysis of antibody preparations that contain cross-reacting anti-BSA immunoglobulins. We examined cross-reacting of carrier proteins that are markedly distinct from BSA in isoelectric point with the immune antisera. As follows from Fig. 1, a minimal cross-reaction was revealed both for egg lysozyme and soybean inhibitor. Reliable and neutral in relation to immune antiserum, egg lysozyme was chosen as a carrier protein for coupling with hapten. Egg lysozyme contains seven

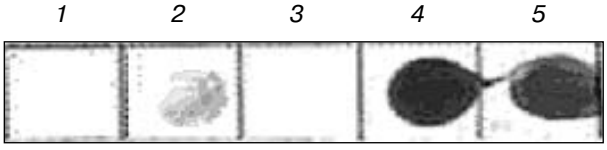


Fig. 1. Cross-reaction of some basic proteins with antiserum against [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$. Five picomoles of each protein was spotted onto BA-85 nitrocellulose membrane: *1*) egg lysozyme; *2*) cytochrome *c*; *3*) soybean inhibitor; *4*) BSA; *5*) BSA treated with glutaric dialdehyde at conditions of the hapten–BSA conjugate preparation.

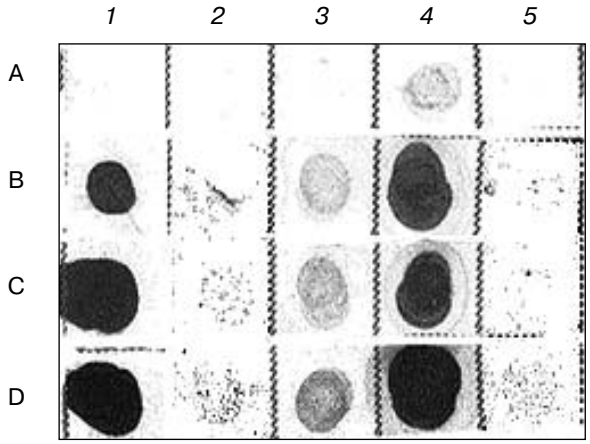


Fig. 2. Immune antisera recognize [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ specifically. Rows, antisera: A) pre-immune serum; B, C, D) immune sera against [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$~BSA diluted 5, 25, and 125-fold, respectively. Columns, proteins and antigen aliquots spotted on nitrocellulose membrane: *1*) BSA (5 pmol); *2-4*) conjugate of [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ and lysozyme (5, 25, and 125 pmol); *5*) lysozyme (125 pmol).

residues of lysine (P00703, NCBI data). Therefore hapten (~1.2 mM) was conjugated with egg lysozyme in molar ratio of 7 : 1 using glutaric dialdehyde. Amino acid analysis showed that ~6.5 molecules of hapten were bound to 1 molecule of lysozyme after 3 h incubation at 37°C with ~0.025% glutaric dialdehyde.

Immunogenicity of the hapten–BSA conjugate was estimated immunochemically using colloidal gold with silver enhancement technique (Fig. 2). As found, immune serum contained antibodies that recognized model hapten mimicking CLU of EMC virus RNA–VPg complex (Fig. 2, compare columns *4* and *5*). It is particularly remarkable that the positive signal grows as dilution of the antiserum increases (rows B-D, column *4*). It seems that reaction of the target antibodies with hapten is masked in the less diluted antiserum. Surprisingly, the pre-immune antiserum recognizes ~800 pmol of [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$. There are two explanations for this phenomenon: some immunoglobulins of the pre-immune rabbit antiserum cross-react with hapten, or this antiserum contains antibodies that recognizing hapten specifically.

As mentioned above, the immune serum contained a number of antibodies to BSA (Fig. 2, column *1*). After the immune serum was depleted of these immunoglobulins (see "Materials and Methods"), specific antibodies were isolated by two successive affinity chromatography steps. At the first step of the procedure, 65% of the total protein was adsorbed onto a BSA-Sepharose column, and immunoglobulins specific to BSA were completely removed from the immunoglobulins preparation, while

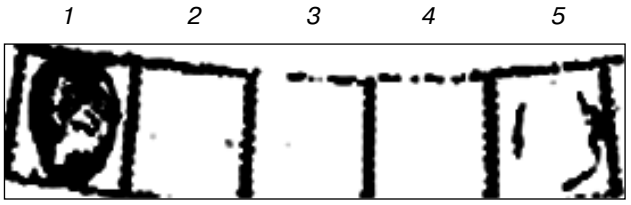


Fig. 3. Antibodies against [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ recognize the EMC virus RNA–VPg complex (concentration of antibodies purified by affinity chromatography is 0.025 mg/ml according to optical absorption at 280 nm). *1-5*) Substances spotted on nitrocellulose membrane: *1*) RNA–VPg of EMC virus (3 pmol); *2*) PVX RNA (3 pmol); *3*) poly(U) (30 pmol); *4*) BSA (100 pmol); *5*) TMV RNA (3 pmol).

eluted protein was enriched in the target antibodies (data not shown). The following affinity chromatography proceeded on Sepharose with bound [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ ligand. To get maximal yield of the target antibodies, the solution of immunoglobulins was passed through the adsorbent 3-4 times repeatedly.

Referring to Fig. 3, one may conclude that purified antibodies against hapten recognize specifically 3 pmol of the EMC virus RNA–VPg complex that has the same CLU as [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$. No reaction with potato virus X (PVX) RNA and poly U, despite of its 10-fold molar excess over EMC virus RNA–VPg, or 100 pmol of BSA was observed, although a slight cross-reaction with TMV RNA was observed. It should be mentioned that the same cross-reaction with TMV RNA was earlier detected by us with affinity chromatography purified antibodies against the EMC virus RNA–VPg complex. Those were enriched with the CLU recognizing immunoglobulins on affinity columns carrying Tyr-(5′P → O)d$T_{13}C_2$ or Tyr-(5′P → O)dTCC ligands. The cross-reaction was suppressed by adding TMV RNA (20 μg/ml) to the antibody solution without any detectable effect on recognition of CLU [19].

We proved that the target antibodies against [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ recognize the chemical structure of CLU in the EMC virus RNA–VPg complex rather than some RNA ribotopes. As follows from the primary structure of EMC virus RNA–VPg [20], it consists of ~7700 nucleotides and contains poly(C) sequence [21] and the first G residue takes position 3. Complete hydrolysis of EMC virus RNA–VPg by T1 ribonuclease has to result in VPg–pUpUpGp, T1-oligonucleotides, and poly(A) and poly(C) that consists of 127 cytidylic acid residues [20]. We took into consideration that free oligoribonucleotides are not retained by nitrocellulose membrane without special immobilization, whereas covalent complexes of proteins and nucleic acids (oligonucleotides) are sorbed on this membrane firmly [1]. Thus VPg–pUpUpGp has to adsorb on nitrocellulose membrane, while free oligo- and polynucleotides do not. According to the logic above, VPg–pUpUpGp has to be the only product of the T1 ribonuclease hydrolysis that has to be retained on nitrocellulose membrane. To be sure that poly- and oligonucleotides were washed out completely during immunochemical analysis, we spotted onto the same membrane poly(C) of ~600 nucleotides length in a molar equivalent to 5 pmol of 127-mer "C" sequence in the EMC virus RNA. As seen from Fig. 4, intensity of signals of 5 pmol of EMC virus hydrolyzate after washing out of free oligo- and polynucleotide RNA constituents is almost the same as of 5 pmol of the whole non-hydrolyzed RNA–VPg complex. Moreover, 5 pmol of the EMC virus RNA–Kpep complex gives similar intensity signal as well. Thus, both VPg and Kpep do not hinder recognition of the covalent linkage unit structure with antibodies. Figure 4 shows that 600-mer of "C" is not "stained" with immunogold antibodies, and we conclude that the signal seen is developed due to the firmly adsorbed VPg–pUpUpGp, more precisely due to the Kpep–pUp unit.

As the covalent linkage unit consists of the uridylic acid and tyrosine residues bound via a phosphodiester bond, we analyzed reaction of antibodies obtained with these constituents. We found no influence after addition of large quantities (up to 2 μM) of uridylic acid or *O*-phosphotyrosine to the antibody solution (Fig. 5, compare A and B). The purified antibodies also do not react with N-terminal dodecapeptide of the EMC virus VPg and, consequently, with Kpep and VPg (B in Fig. 5).

Summarizing the data obtained, we conclude that polyclonal purified antibodies recognize CLU in picornavirus RNA–VPg complex and in the mimicking model, i.e. [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6NH_2$ specifically. We believe these antibodies might be useful for searching for cellular RNA–protein covalent complexes that are structurally similar to the picornavirus structure.

These complexes are of great chemical and biological interest. They must have unique properties, because a covalent bond determines the limit of specificity and strength of the complex formation.

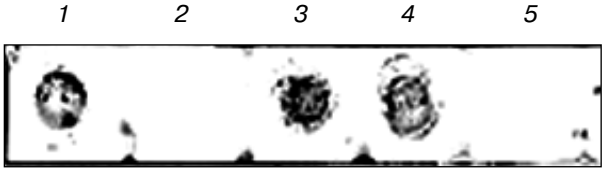


Fig. 4. Affinity purified antibodies recognize the CLU chemical structure specifically (all substances were spotted on nitrocellulose membrane without fixation with UV-light or baking; concentration of antibodies is 0.025 mg/ml): *1*) EMC virus RNA–VPg (5 pmol) after complete hydrolysis with T1 ribonuclease (4.83 U); *2*) ribonuclease T1 (4.83 U); *3*) EMC virus RNA–VPg (5 pmol); *4*) EMC virus RNA–Kpep (5 pmol); *5*) poly(C) (5 pmol relative to 127-mer of "C" in the EMC virus RNA–VPg).

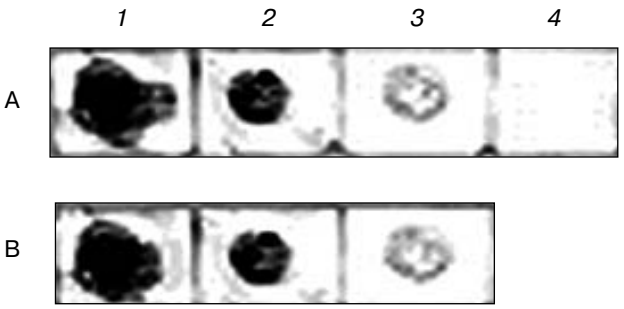


Fig. 5. Uridylic acid and phosphotyrosine do not interfere during recognition of [*N*(Ac),*C*O(NH_2)]Tyr-(5′P → O)Up-O-$(CH_2)_6$ NH_2 by the purified antibodies. A) Concentration of antibodies is 0.025 mg/ml; B) uridylic acid and O-phosphotyrosine (1 nmol of each) were added to 300 µl of antibody solution). Ligands spotted onto nitrocellulose membrane: *1-3*) 500, 150, and 50 pmol, respectively, of the hapten–lysozyme conjugate; *4*) N-terminal dodecapeptide of the EMC virus VPg (500 pmol).

The search for RNA–protein covalent complexes of cellular origin has a prolonged history [22]. Authors have deduced that peptides and ribosomal RNA are bound covalently because of isolated nucleotide–peptides from the total cellular RNA preparation were stable in alkaline medium.

In 1992, R. Carrol and colleagues reported that multifunctional p53 isolated from mouse and human cells is covalently bound to 5.8S rRNA [23]. Although they did not identify nucleotide residue that linked directly with Ser389 residue of p53, this presumably covalent complex is a very attractive candidate for further investigation.

It was recently found that DNA is not the unique substrate of DNA topoisomerases for the trans-esterification reaction. *In vitro* these enzymes were able to catalyze trans-esterification reaction with RNA, as was demonstrated for bacterial DNA topoisomerase III [9] and for human DNA topoisomerases IIα and IIβ [10]: *E. coli* DNA topoisomerase III catalyzed catenanes and knot formation of RNA, whereas human DNA topoisomerases worked with double-stranded RNA more efficiently than with DNA. Ability to hydrolyze and restore an internucleotide bond of RNA means that topoisomerases use the same mechanism of the enzymatic reactions as with the DNA substrate, so they form the catalytic transient active phosphodiester of tyrosine residue and 5′ (or 3′) nucleotide phosphate group [15].

Specific complex of DNA and topoisomerase is related to so-called relaxation complexes that are transformed into the DNA-*topo* dead-end covalent complexes after denaturation, proteolysis, or poisoning of the enzyme with antibiotics and drugs or inhibitors. Because some inhibitors of DNA topoisomerases have gained widespread acceptance in therapy of oncological diseases, one can propose that the covalent complexes of the cellular RNAs and DNA topoisomerases will be formed in patients during the drug (DNA topoisomerase inhibitor) treatment. If this prediction is correct, then side effect of the therapy has to lead to accumulation of these complexes. This brings up an interest to identification of RNA molecule(s) in the covalent dead-end complexes of DNA topoisomerases and cellular RNAs from the cancer cells of patients after a course of the DNA topoisomerases poisoning therapy. It is likely that isolation of the eukaryotic RNA–DNA topoisomerase covalent complex is not simple task. In this connection, it should be emphasized that chemical structure of the covalent linkage unit of the RNA–DNA topoisomerase covalent complex resembles the picornavirus RNA–VPg one. We suggest that antibodies obtained in this work will be an appropriate tool for the search for the RNA–DNA topoisomerase(s) complexes.

Finally, we speculate that some complexes of RNA and DNA topoisomerases might be substrates for the cellular VPg-unlinking enzyme that catalyzes hydrolysis of the phosphodiester bond between picornavirus RNA and VPg [6-8]. This enzyme could repair "dead-end" complexes of RNA and topoisomerases and diminish the therapeutic effect of the drug. If this is the case, poisoning of the unlinking enzyme may be desirable for efficient therapy.

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