Antibodies against EMC virus RNA-VPg recognize Tyr-(5′P→O)-pU and immunostain infected cells

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Abstract Covalent complexes of nucleic acids and proteins are widespread among viruses. Covalent complexes of RNA and proteins are proposed to exist in eukaryotic cells. The goal of this work was to obtain specific antibodies to the covalent linkage unit (CLU) between virus RNA and protein to search cellular RNA-protein complexes. Antibodies were generated by direct immunization of a rabbit with the BSA-coupled EMC virus RNA-VPg complex. By a dot-blot immunoassay and immunofluorescent microscopy it was found that the antibodies specifically recognize both EMC virus RNA-VPg and synthetic CLU-containing compounds. Thus, a fraction of the antibodies was directed to CLU.

Key words: Picornavirus; Encephalomyocarditis virus; RNA-protein covalent complex; Ribotope; Membrane immunochemical analysis; Immunofluorescent microscopy

1. Introduction

Covalent complexes of nucleic acids (NA) and proteins (P) are widespread among animal and plant viruses. The goal of this work was to obtain antibodies specific to the covalent linkage unit (CLU) between RNA and protein VPg of picornaviruses. It is well known that the 5′-end uridylic acid of picornaviral RNA is linked with VPg by a phosphodiester bond via the unique tyrosine residue [1–4].

The inter-polymer phosphodiester linkage in the complex is specifically hydrolyzed by an 'unlinking' enzyme, whose activity was discovered in animal and plant cells [5–8]. One would expect that there are unknown yet cellular RNA-protein covalent complexes – potential targets for the enzyme that structurally resemble the picornaviral RNA-VPg. This type of natural compounds is difficult to detect, and to date, few cellular covalent (presumably) complexes of RNA and proteins have been characterized [9–13]. The covalent complex of the tumor suppresser p53 protein and 5.8S rRNA was isolated from rat embryo fibroblasts [9], this being the most exciting observation. The function of this complex is not quite clear yet, but the multifunctional properties of the tumor suppresser protein are well known ([9] and references therein). Thus, raising antibodies to the RNA-VPg CLU of picornaviruses arouses considerable interest both in a search for the cellular RNA-protein covalent complexes and in a study of the mechanism of the picornavirus RNA replication.

In this work antibodies that recognize either the encephalomyocarditis (EMC) virus RNA-VPg or CLU were generated by direct immunization of a rabbit with the modified EMC virus RNA-VPg. The antibodies were purified by chromatography and characterized by a dot-blot assay and immunofluorescent microscopy. It was found that they recognized CLU in either the EMC virus RNA-VPg or synthetic *N*-Ac-Tyr(*O*-pU-2′-NH_2)OEt and *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt specifically.

2. Materials and methods

O-Phosphotyrosine and BSA (fraction V), 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite and *N*-benzoyl-L-tyrosine ethyl ester were from Sigma, pU from Calbiochem, BrCN activated Sepharose from Pharmacia, 5′-*O*-(4,4′-dimethoxytrityl)-*N*-acyl-deoxynucleoside-3′-*O*-*P*-2-cyanoethyl-*N*,*N*′-diisopropylphosphoramidites from Perkin Elmer, dichloroacetic acid, dichloromethane and 1*H*-tetrazole from Aldrich and *N*-ethyldiisopropylamine from Fluka.

EMC virus propagation and RNA-VPg isolation were done as in [3], except that the ascites cells were suspended (2×10^7/ml) and infected (multiplicity of infection was 0.1, two cycle infection for 16 h) in Eagle's minimum essential medium. Actinomycin D was omitted.

Tobacco mosaic virus was kindly provided by Dr. V.K. Novikov, TMV RNA was isolated by phenol deproteinization.

The N-terminal dodecapeptide of EMCV VPg was synthesized by Dr. Yu. Semiletov.

2.1. Synthesis of RNA-VPg antigens, immunization of a rabbit and antibody isolation

The antigen RNA-VPg, conjugated with BSA (**AG I**) was obtained by glutaric dialdehyde coupling of EMC virus RNA-VPg with BSA. With this purpose 17 μl of 1% SDS, 52 μl of BSA (1 mg/ml) and 9 μl of 1 M $NaHCO_3$ (pH 10.3) were added to 500 μg of EMCV RNA-VPg. 9 μl of 5% glutaric dialdehyde was added to the cocktail obtained and the mixture was incubated for 30 min at 37°C. The reaction was stopped by fast chilling to 0°C with the addition of $NaBH_4$ (12 μl, 20 mg/ml) and subsequent incubation for 3 h at 0°C. The resulting conjugate was precipitated with 11 μl of 3 M acetate buffer (pH 4.6) and 340 μl of ethyl alcohol at −20°C. The precipitate was washed with 70% alcohol and vacuum dried. The antigen RNA-VPg (**AG II**) was obtained using an identical procedure except that BSA was omitted. A rabbit was injected intracutaneously with a 10 day interval twice with 250 μg of **AG I** dissolved in PBS and then six times with 100 μg of **AG II**.

The antiserum was fractionated by a sulfate ammonium salting out of globulins [14]. The precipitated globulins were further fractionated by DEAE chromatography [14], the immunoglobulins were characterized by UV spectrophotometry and SDS-PAG electrophoresis as in [15]. The depletion of Abs with BSA was carried out using a home-made BSA-BrCN-Sepharose column. Affinity-purified anti-BSA Abs were eluted from this column. The Abs against the N-terminal

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Abbreviations: Ab, antibody; BSA, bovine serum albumin; EMC, encephalomyocarditis; CHO, Chinese hamster ovary; CLU, covalent linkage unit; pfu, plaque-forming unit; PAG, polyacrylamide gel; RP HPLC, reverse phase high-performance liquid chromatography; TLC, thin layer chromatography

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dodecapeptide of EMCV VPg were also purified by affinity chromatography.

2.2. Analysis of antibody specificity

Nitrocellulose membranes BA 85 (Schleicher and Schuell), secondary goat anti-rabbit Abs conjugated with the colloidal gold and silver enhancement system (Amersham) were used for immunochemical detection of the substances.

Usually, 1–10 μl of the samples were spotted by a microsyringe onto membranes wetted with 20×SSC. Nucleic acid composed samples were fixed by UV light at 254 nm (3×15 W UVB lamps, the distance to the object was 7 cm, 4 min). The membranes were blocked with 2% of Tween-20/PBS and washed in 0.025% Tween-20/PBS. Then the membranes were incubated overnight at 4°C in the PBS solution of the primary Abs ($A_{280} \approx 0.1$), thereafter they were washed with 0.025% Tween-20/PBS and incubated for 2 h at room temperature with conjugates of the secondary Abs and colloidal gold. The images were obtained by silver enhancing according to the manufacturer's protocol. All immunochemical detections were repeated at least twice.

2.3. Synthesis of Tyr-(5′P→O)-pN phosphodiesters mimicking the CLU

$dT_{13}C_2$ (70 nM) was synthesized by the solid phase (35 mg LCAA-CPG-dCBz, Millipore) phosphoramidite method [16] with a Biosearch Model 8600 DNA synthesizer. After 5′O-DMTr had been cleaved, the polymer was thoroughly washed with absolute dichloromethane and acetonitrile. Subsequently, the oligodeoxynucleotide was treated with a phosphorylating mixture (0.85 mmol 2-cyanoethyl-*N*,*N*-isopropylchlorophosphoramidite and 1.1 mmol *N*-ethyldiisopropylamine in 1.5 ml of 1,2-dichloroethane) for 20 min at room temperature (21°C). The excess of reagents was washed out with absolute 1,2-dichloroethane and absolute acetonitrile. Phosphorylated oligodeoxynucleotide on a solid support reacted with 0.57 mmol *N*-benzoyl-L-tyrosine ethyl ester and 0.45 mmol of 1*H*-tetrazole in 1 ml of acetonitrile at room temperature for 40 min. The support was washed with acetonitrile and the phosphoramidite group was oxidized by a standard procedure [16]. Deprotection and cleavage of oligonucleotide were achieved by treating with concentrated aqueous NH_3 at 50°C overnight. The yield of ethyl ether of (*N*-Bz)Tyr-(5′P→O)-$dT_{13}C_2$ was 60%.

Synthesis of 5′P→O phosphodiester between Urd(2′-NH_2) coupled with the resin and *N*-benzoyl-L-tyrosine ethyl ester was carried out analogously. The yield was about 30%. The structure of tyrosine derivatives of (oligo)nucleotides was proved by spectrophotometry, RP HPLC, TLC and amino acid analysis.

2.4. Immunofluorescent microscopy of EMCV infected cells

CHO cells were plated in F10 medium supplemented with 10% fetal calf serum and gentamicin (100 mg/ml) at 37°C. The EMC virus suspension was added (30 pfu/cell), the virus adsorption proceeded at room temperature for 30 min, and the unsorbed virus was washed out. The cells were incubated for 7 h at 37°C and then fixed with methanol at −20°C. Immunofluorescence staining was performed as described in [14]. Secondary goat anti-rabbit immunoglobulins conjugated with tetramethylrhodamine isothiocyanate (Sigma) were used. The preparations were examined with Photomicroscope-3 (Opton).

3. Results and discussion

3.1. Synthesis of antigens, rabbit immunization and antibody isolation

Little or nothing has been reported regarding immunization of animals with RNA directly, though autoimmune Abs against cellular RNAs produced after some virus infections are extended over many animal species (for review see [17]). It is a matter of general experience that the immune response to cell nucleic acids is complex and weak.

To increase the antigenic power of the RNA-VPg compound and its resistance to cellular nucleases [18], and to suppress its infectiousness which is important in the case of mouse immunization (unpublished data), the natural virus RNA-VPg complex was modified with dialdehyde and then coupled with BSA. Aliphatic amino groups of VPg and amino groups of heterocyclic nucleic bases are the first to interact with aldehydes [19] and become cross-linked. To render modification irreversible, the reaction of RNA-VPg with dialdehyde was stopped by sodium borohydride treatment. As a result of the coupling, the electrophoretic mobility of RNA-VPg in 1% agarose dropped appreciably (data not shown). At the last stages of the immunization, to increase the immune response against RNA-VPg itself, **AG II** (see Section 2.1) was used whereas BSA was omitted.

We have found that the resulting immunogenicity of **AG I** and **AG II** was very low: even by the immunogold method a specific reaction with EMCV RNA-VPg was displayed by 1:25 dilution of the final antiserum only (data not shown). Using immunofluorescent microscopy, specific immunostaining of the EMCV infected cell was discovered with the same serum diluted up to 500 times. Even though the first two immunizations were carried out with **AG I** (in which RNA-VPg is coupled with BSA) only, we found that the titer of the BSA-specific immunoglobulins in the serum was higher than that of the RNA-VPg one by a factor of 25.

An electrophoretically pure fraction of IgG was obtained (apparently, admixture of IgM was also detected) after sulfate ammonium fractionation and DEAE cellulose chromatography of immune serum. Then this preparation was depleted by chromatography on the BSA Sepharose column to remove immunoglobulins specific to BSA. According to absorption at 280 nm, at this step 96% of IgG was adsorbed onto the BSA affi-column. The resulting preparation of Abs (**Ab-1**) (those that were purified on DEAE cellulose and depleted with BSA) was further used in the study.

3.2. Analysis of antibody specificity

Evidently, an immune response against the complex antigen like EMC virus RNA-VPg results in the formation of Abs with a wide range of specificity, among which are immunoglobulins recognizing viral RNA ribotopes, epitopes of EMCV VPg and, apparently, those of the CLU. To examine the specificity of the Abs, two methods were chosen: membrane immunochemical analysis and immunofluorescent microscopy. The analysis of complex compounds like RNA-VPg by dot immunochemistry on a membrane filter represents a certain technical problem because of the restrictions imposed by the capacity of the membrane. Our experience suggests the use of the BA-85 (0.45 μm) nitrocellulose membrane (up to 3 pmol of EMCV RNA-VPg can be reproducibly fixed by UV light at 254 nm onto ~ 3 mm^2 of the membrane) and the immunogold detection system.

The results of analysis of the **Ab-1** specificity are demonstrated in Fig. 1. Data taken from different experiments are accumulated in one picture. It is evident that the **Ab-1** recognized in any case EMCV RNA-VPg, TMV RNA, *N*-Ac-Tyr(*O*-pU-2′-NH_2)OEt, *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt, $dT_{13}C_2$ and BSA (Fig. 1, 1st line). The reaction of **Ab-1** with EMCV RNA-VPg, TMV RNA and BSA was stronger than with *N*-Ac-Tyr(*O*-pU-2′-NH_2)OEt and *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt. The reaction of **Ab-1** with $dT_{13}C_2$ was weaker than with *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt. One can notice that affinity purified Abs to BSA did not recognize any compounds examined except BSA (Fig. 1, 4th line). The preimmune immunoglobulins also exhibited no specific reaction with the

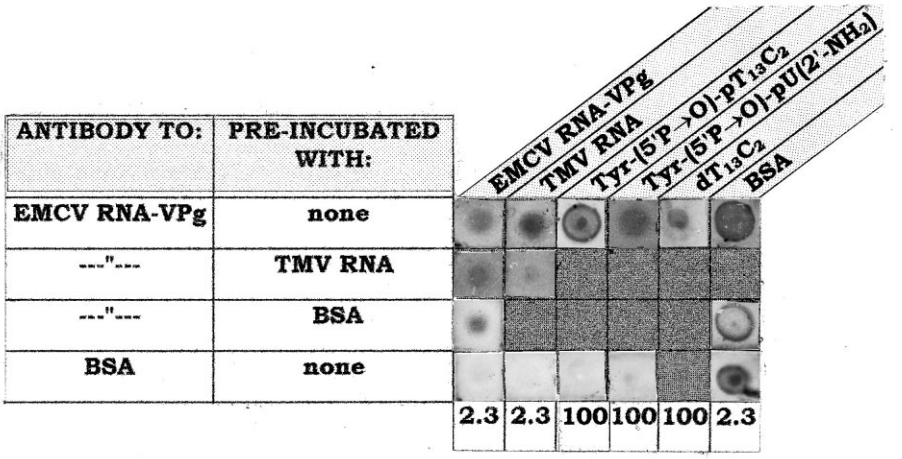

Fig. 1. Interaction of Abs against EMCV RNA-VPg or against BSA with different ligands spotted onto the membrane. Horizontal lines of the grid represent different antibodies, vertical rows represent different ligands, as indicated. Numbers under the spots are pmol of the ligands. Empty squares are shaded.

antigens and ligands used (data not shown). Thus, **Ab-1** interacts specifically with the viral nucleic acids and synthetic CLU-containing compounds.

As mentioned earlier, the antiserum contained high quantities of Abs interacting with BSA. In spite of purification of the Ab preparation against EMCV RNA-VPg by affinity chromatography on the BSA Sepharose column, reaction of the purified Abs with BSA still remained visible (Fig. 1). An addition (up to 20 μg/ml) of BSA to this Ab preparation substantially reduced its reaction with BSA, but not with EMCV RNA-VPg (Fig. 1, 3rd line).

The reaction of **Ab-1** with pure TMV RNA (Fig. 1) indicates that **Ab-1** contains anti-ribotope immunoglobulins. We found, however, that titration of the Ab preparation by an addition of TMV RNA up to 20 μg/ml drastically restrained the immune reaction with TMV RNA, but not with EMCV RNA (Fig. 1, 2nd line). So, **Ab-1** contained not only broadly specific anti-virus RNA Abs, but also Abs to either specific EMC virus RNA ribotopes or to VPg, or to CLU.

To identify the population of the immunoglobulins recognizing the CLU, the interaction between the **Ab-1** and *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt and *N*-Ac-Tyr(*O*-pU-2′-NH_2)OEt was examined. A positive signal was obtained in all cases and, as mentioned above, it was distinctly weaker when obtained with $dT_{13}C_2$ (Fig. 1, 1st line). In the case of the CLU-mimicking compound *N*-Ac-Tyr(*O*-pU-2′-NH_2)OEt, the strength of the signal correlated linearly with the ligand quantity in the range of 3–300 pmol (data not shown). It is important to note that the only common antigenic determinants shared by the RNA-VPg complex and the synthetic model phosphodiesters of tyrosine and nucleotides are those which belong to the CLU. An addition of *O*-pTyr or pU in ~100 times molar excess over *N*-Ac-Tyr(*O*-pU-2′-NH2)OEt to the **Ab-1** preparation showed no interference (data not shown). Thus, in the **Ab-1** preparation obtained there is a fraction of immunoglobulins which interact preferentially with the CLU.

To reveal conclusively the immunoglobulin population against EMC virus RNA-VPg, CHO cells were infected with the EMC virus and virus propagation was examined by immunofluorescent microscopy (Fig. 2). It is interesting that the background immunostaining of the mock-infected cells, as well as early-infected ones (up to 3 h) was weak and diffuse,

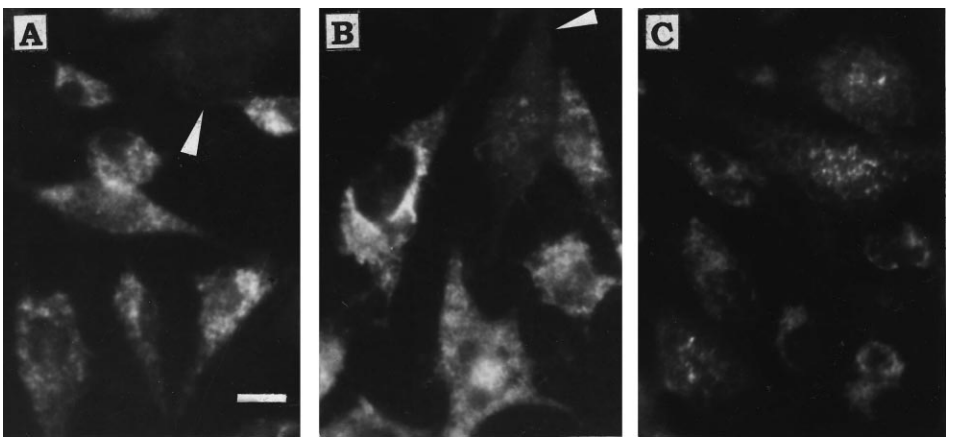

Fig. 2. Immunofluorescent microscopy of CHO cells infected with the EMC virus and immunostained either with antibodies against EMCV RNA-VPg (A) or with antibodies to the synthetic N-terminal dodecapeptide of EMCV VPg (B) and preimmune serum (C). Uninfected cells are indicated by arrows. Bar, 10 μm.

regardless of a high content of cellular RNAs in the cytoplasm. It confirms that **Ab-1** were not broadly specific anti-RNA Abs. Maximal immunostaining of infected CHO cells was attained after 7 h infection. Similar results were obtained during a study of the EMC virus infection dynamics in mouse L fibroblasts [20]. As a control, the infected cells were also displayed with the Abs specific to the synthetic N-terminal dodecapeptide of EMC virus VPg (Fig. 2B), and a similar immunostaining pattern was obtained. No specific immunostaining of the infected cells was revealed with the preimmune antiserum or the immunoglobulins (Fig. 2C). Taking into consideration the data obtained, we conclude that in the Ab preparation under study there is a population of immunoglobulins specifically recognizing EMC virus RNA-VPg.

In connection with the above, it is of interest to obtain Abs against the mentioned synthetic models, mimicking the CLU, and to study all cross-reactions between these Abs and the ligands. The preliminary studies showed that antiserum to *N*-Ac-Tyr(*O*-p$T_{13}C_2$)OEt cross-reacts with EMC virus RNA-VPg [20]. We believe that the Abs against EMC virus RNA-VPg and the above synthetic models might be useful in a search for yet unknown cellular RNA-protein covalent complexes and in studying the mechanism of the EMC virus infection.

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