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Original Research

Polyclonal antibodies against potato spindle tuber viroid RNA

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Abstract

Ribonucleic acid (RNA) can act as a hapten in the direct immunization of animals. For antigen synthesis, 65 mg of viroid RNA were obtained by *in vitro* transcription of the recombinant DNA. We received a reasonable immune response in mice and rabbits with synthesized conjugate viroid RNA-lysozyme. Analyses of polyclonal mouse and rabbit antisera as well as estimates of antibody specificity were performed by dot-Enzyme Linked Immunosorbent Assay (ELISA), sandwich ELISA, and northern immunoblotting. Antiserum obtained showed strong cross-reactions with cellular RNA. The viroid polyclonal antibody cross-reactions with cellular RNAs were depleted via titration antibodies by the plant cellular or commercial yeast RNA. We successfully used antibodies against the viroid RNA-lysozyme antigen to detect the wild-type potato viroid and diagnose potato viroid infection. We presume that intrinsic cross-reactions of RNA antibodies are potentially dangerous after nucleic acid vaccination. Research into the specificity of antibodies against viral RNAs is underway.

Keywords: potato spindle tuber viroid; dot-ELISA; immune northern blotting; diagnostics; nucleic acid vaccine

1. Introduction

Interest in antibodies specific to nucleic acids has a long history. Activity in this area was immense because antibodies to DNA and RNA were detected in the serum of patients with viral infection or diagnosed with multiple autoimmune diseases [1–7]. In the case of direct immunization of animals with nucleic acids, it became evident that natural nucleic acids act as haptens [1]. Nevertheless, antibodies to Z-DNA [8], RNA [3–5,7], synthetic and natural double-stranded RNA [9,10], modified nucleotides in RNA [11–15], and cellular RNA [16,17] were obtained.

There are few reports of antibodies' production against natural RNA [10,16–18]. A specially selected recombinant mAb against brain cytoplasmic 200RNA (BC200) with a Kd of ~7 nM could discriminate this particular RNA from other RNAs [18]. Interestingly, this mAb against BC200 tightly interacts simultaneously with two sequence stretches of the RNA at positions 76–85 and 96–104 that come closely together in the RNA tertiary structure.

In extended RNAs, antibodies recognize primary [3,4,15–18], secondary [5,9–11], and tertiary structures [18]. Hu *et al.* [19] published a comprehensive review of antibodies to synthetic and natural nucleic acids and their applications. Those antibodies specifically recognize up to six nucleotides of the RNA primary structure; the immunogenicity of dsRNA is higher than that of ssRNA, and antibodies can recognize the nucleic acid helixes' structure.

The interaction of antibodies with the RNA antigen involved formation of hydrogen bonds between polar amino acid residues in the hypervariable regions of the antibodies; phosphate, oxygen, and hydroxyl groups of the furanose ring; as well as exocyclic amino, and carbonyl groups of the nucleotides in the RNA. The formation of antigen-antibody complexes might also engage stacking interactions of aromatic rings of amino acids (Tyr, Trp) and nucleotide bases.

We studied the specificity of polyclonal antisera directed against RNA-VPg and the synthetic covalent linkage unit (CLU) Tyr-(5′P → O)Up of the encephalomyocarditis virus covalent complex RNA-VPg and found that there are antibodies that specifically recognize the CLU. Moreover, some immunoglobulins of the polyclonal sera cross-reacted with other synthetic and natural RNA structures [20–22]. Two aspects were of interest to us regarding antibodies to RNA: First, there is the possibility of using them in the early serological diagnosis of viral and viroid infections, which is traditional for plant growth. The second included studies of the cross-reaction of polyclonal antibodies against viroid RNA with host RNA.

Among the infectious nucleic acids, the most intriguing is viroid RNA (vdRNA) [23]. The lack of a protein capsid has long been the reason for the impossibility of using serological methods for the viroids detection. To date, the most common methods for the molecular diagnostics of plant virus infections are immunological. The diagnostic approaches for detecting viroid infections are based on molecular hybridization of nucleic acids mainly using DNA probes and polymerase chain reaction (PCR) [24]. Lukacs *et al.* [16] reported monoclonal mouse antibodies toward the potato viroid. They cross-reacted 25 synthetic and natural nucleic acids, and the highest affinity was for ribosomal RNA.

Despite prior unsuccessful attempts, the goal of this work was to obtain polyclonal antibodies that recognize vdRNA specifically. Notably, the immune response was expected to result in polyclonal antibodies against several ribotopes of the RNA antigen. Polyclonal antibodies' palette of recognized ribotopes of the viroid complex antigen will be broader than any monoclonal antibody; therefore, polyclonal antibodies have to display higher avidity. In this regard, it was essential to study the cross-reactions of polyclonal antibodies to viroid RNA with host cellular RNAs because the similarity of RNA molecules at all levels of structural organization is much closer than that of the protein (see Discussion).

2. Materials and methods

Deionized double distilled water (TDW) was used throughout this work; stock buffer solutions were filtered through a 0.22-μm nylon membrane. Triple recrystallized chicken egg lysozyme and yeast RNA were purchased from Reakhim (Moscow, Russian Federation). Secondary goat antibodies (Jackson Immunoresearch, Cambridgeshire, UK) conjugated with alkaline phosphatase, blocking Tween-20 solution, and RNase inhibitor (Ribolock, Thermo Fisher Scientific, Waltham, MA, USA) were used in immune assays of the RNA preparations. BCIP/NBT phosphatase chromogen substrate was purchased from KPL (Milford, MA, USA). Chloroethylenetriaminoplatinum chloride (dien)platinum was synthesized according to Watt and Cude [25] with modifications related to the micro-synthesis technique. Fresh 40% formaldehyde was obtained by boiling the paraform (Merck, Kenilworth, NJ, USA)-saturated water solution for 30 min.

Healthy and PSTVd-infected potatoes were kindly provided by Dr. N.V. Girsova (All-Russian Research Institute of Phytopathology, Moscow, Russian Federation). An Apple Scar Skin Viroid (ASSVd) preparation was presented by Dr. V. Hallan (CSIR-Institute of Himalayan Bioresource Technology, Palampur, India). The identity and purity of the PSTVd-transcript, ASSVd, RNA, RNA-antigens, and antibodies preparations obtained in this work were confirmed by the PSTVd cDNA sequencing, UV-Vis spectroscopy, gel-electrophoresis, dot-ELISA, sandwich ELISA, and immune northern blot assay. UV absorption spectra were recorded in the range 220–320 nm using Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

2.1 Isolation of Solanum tuberosum RNA

Here, 1 g of fresh or frozen *S. tuberosum* leaf tissue was thoroughly ground with a mortar and pestle in liquid nitrogen. The leaf powder was extracted with 6 mL of buffer solution (1% SDS, 100 mM NaCl, 10 mM EDTA-Na_2, 50 mM Tris-HCl, pH 7.2 for 15–20 min at 60 °C). The mixture was cooled to room temperature, and debris was removed by centrifugation. The supernatant was mixed with water-saturated phenol/chloroform (9:1 v/v, respectively) solution. Deproteinization was carried out for 3 min with vigorous vortexing. The mixture was centrifuged for 5 min at 4000 rpm; the aqueous phase was saved and repeatedly deproteinized until no protein interface was observed. Then LiCl was added to the aqueous phase to a final concentration of 2 M to precipitate high molecular weight RNA overnight at 4 °C. The precipitate mainly contained ribosomal RNA, whereas the supernatant contained most of the low molecular weight RNA according to agarose gel electrophoresis. The supernatant was aliquoted and RNA was precipitated with two volumes of ethanol and stored at –20 °C. An aliquot of low molecular weight RNA isolated from the PSTVd infected potato was used for the RT-PCR cloning of the viroid cDNA [26].

2.2 Cloning of the viroid cDNA

The cDNA copy of the Russian potato viroid isolate [26] was obtained using the following primers: (+)PSTVd 5′-atccccggggaaacctggag-3′ (forward) and (-)PSTVd 5′-ccctgaagcgctcctccgag-3′ (reverse) by cloning in the plasmid pBluescript II SK+ (Stratagene, La Jolla, CA, USA). The identity of the PSTVd cDNA was confirmed by both strands sequencing. The cloned cDNA was repeatedly used as a template for the vdRNA transcription using the RiboMAX Large Scale RNA Production T7-System (Promega) according to Gurevich *et al*. [27]. After transcription, the reaction mixture was treated with DNase I (Promega, Madison, WI, USA) 10 μg/mL for 10 min at 37 °C and deproteinized twice with a water-saturated phenol/chloroform/isoamyl alcohol mixture (25:24:1 v/v). The transcribed RNA was precipitated with 75% ethanol/0.3 M sodium acetate (pH 5.2). The RNA precipitate was washed with 70% ethanol and dried in a vacuum desiccator. In total, more than 65 mg of the PSTVd-transcript was obtained. The vdRNA sample was dissolved in water and stored at –80 °C.

2.3 Modification of PSTVd-transcript with (dien)platinum

We previously labeled RNA with (dien)platinum for multiplex determination of viruses and PSTVd in infected potatoes with nucleic acid probes [28]. To obtain a vdRNA-(dien)platinum complex, the PSTVd-transcript was denatured for 3 min at 95 °C in 1 mM $NaClO_4$. The solution was rapidly chilled, and (dien)platinum was added at a ratio of 1 Pt atom per 10 nucleotides. The mixture was then incubated for 0.5 h at 65 °C. Under these conditions, the (dien)platinum reacts quickly and quantitatively with guanine residues [29]. The mixture was used to immunize animals without further purification.

2.4 Conjugation of PSTVd RNA with lysozyme

Borate buffer (248 μL of 100 mM (pH 9.3), 208 μL of white lysozyme solution at 10 mg/mL, 40 μL of the PSTVd-transcript at 12 mg/mL, and 4 μL of 37% formaldehyde

were thoroughly mixed and incubated for 2 h at room temperature. The reaction was stopped by cooling to 0 °C and adding (3 × 150 μL) $NaBH_4$ (20 mg/mL) for 30 min to prevent a reverse reaction after dilution. The vdRNA-lyso conjugate was precipitated with 5% trichloroacetic acid and washed with 70% ethanol.

2.5 Characterization of the PSTVd-transcript, antigens, and ASSVd

Agarose gel electrophoresis was performed in 1.5% agarose gels using a buffer composed of 40 mM Tris-acetate, 2 mM EDTA (pH 7.5), and 0.5 μg/mL ethidium bromide. An ASSVd preparation was characterized by UV-spectrophotometry and 7% polyacrylamide gel-electrophoresis in the TAE buffer [30].

2.6 Immunization of laboratory animals

Laboratory outbred mice and female Chinchilla rabbits were immunized. Pre-immune sera were used as a control in the ELISA screening after immunization and stored at –70 °C. Blood was collected from the tail vein of mice and the ear vein of a rabbit. For immunization, vdRNA-(dien)Pt in PBS (1 mg/mL) and the vdRNA-lyso antigen in PBS with 0.01% SDS (~0.1 mg/mL) were prepared. Antigen synthesis was repeated several times to accumulate a sufficient quantity for the immunizations. Female mice (three for each antigen) were immunized subcutaneously seven times with the vdRNA-(dien)Pt and thirteen times with the vdRNA-lyso antigens with an interval of 10–12 days. The first two injections (50 μg/mouse and 450 μg/rabbit) were boosted with Freund's complete adjuvant (30%) and with Freund's incomplete adjuvant (50%), respectively. Subsequent immunizations were performed with the pure antigens. The last two injections were with the pure PSTVd-transcript: 300 μg/mouse and 2.9 mg/rabbit. The antibody titers were determined by dot-ELISA after each two immunizations. After 11 immunizations, the antiserum titer was over 1:3000. Aliquots of the sera were frozen at –70 °C for long-term storage.

2.7 Isolation of antibodies to the PSTVd-transcript

The globulin fraction of the antiserum was obtained by fractionation with ammonium sulfate [31]. Immunoglobulins against the vdRNA-lyso antigen were isolated from the globulin fraction by affinity chromatography on the Hi-Trap Protein G column (GE Healthcare, Boston, MA, USA) according to the manufacturer.

2.8 Conjugation of the primary antibodies with horseradish peroxidase

The concentration of horseradish peroxidase was calculated according to A_{403}* 0.44 = C (mg/mL); 0.2 mL of 0.1 M $NaIO_4$ was added with stirring to 6 mg of HRP in 1.1 mL of 1 mM sodium acetate buffer, pH 4.4 [32]. The reaction proceeded at room temperature for 20 min and was followed by exhaustive dialysis against 1 mM sodium acetate buffer (pH 4.4 overnight at 4 °C). The pH of the HRP solution was adjusted to pH 9–9.5 with sodium bicarbonate, and then 1 mL of the primary antibody preparation (9–12 mg/mL) in 10 mM sodium bicarbonate was added. The reaction mixture was incubated for 2 h at room temperature. The reaction was stopped by adding 0.1 mL of 4 mg/mL sodium borohydride solution in portions within 0.5 h at 0 °C followed by extensive dialysis against PBS.

2.9 dot-ELISA of antisera and antibodies

Antigens and RNA samples were adsorbed and cross-linked to 0.22 μm nitrocellulose membrane impregnated with 4 M LiCl by UV irradiation for 7 min at 120,000 $\mu J/cm^2$ in a UV-Stratalinker 1800 (Stratagene, USA). Membranes with adsorbed RNA were washed (3 × 5 min) with TBS-T buffer (25 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.5), blocked with 2% Tween-20 for 1 h at room temperature, and washed again with TBS-T. The antigen-antibody reaction with antisera diluted 3000-fold was run overnight at 4 °C. To deplete antibodies cross-reacting with the vdRNA-lyso antigen, lysozyme and total yeast RNA were added to the antisera (antibody) solution up to 1.5 mg/mL and 0.15 mg/mL, respectively. Membranes were washed as above, incubated with secondary antibodies conjugated with alkaline phosphatase, and then developed with the BCIP/NBT phosphatase chromogen solution (KPL, Milford, MA, USA) according to the manufacturer's protocol.

2.10 Sandwich ELISA of antisera and antibodies

MaxiSorp Nunc-Immuno plates (Swedesboro, NJ, USA) were coated with the capture primary polyclonal antibodies against antigens (1 μg/mL) overnight at 4 °C in 20 mM carbonate/bicarbonate buffer with pH 9.6 followed by blocking with 1.0% BSA solution in PBS-T buffer (PBS with 0.02% Tween-20) for 30 min at 37 °C; 200 μL of two-fold consecutive dilutions of antigen and RNA samples were run in duplicate and proceeded overnight at 4 °C. After intensive washing with PBS-T buffer, immune plates with bound RNAs were incubated with primary antibodies (0.5 μg/mL) and conjugated with horseradish peroxidase in PBS-T overnight at 4 °C. Finally, immune plates were washed, and the bound HRP-conjugate was detected with a peroxidase substrate ABTS (Biochemica AppliChem, Billingham, UK) in the phosphate-citrate buffer, pH 4.4 at 37 °C for 30 min. We used the sample standard deviation to quantify the spread of a set of data.

2.11 Immune northern blot analysis

Antigens and RNA samples in water were electrophoresed in 2% agarose gels in TAE buffer with ethidium bromide (0.5 μg/mL). Sample blotting onto 0.22-μm nitrate cellulose membrane (BioRad, Hercules, CA, USA) was proceeded overnight using 20 × SSC as the trans-

fer solution [30]. After UV cross-linking the samples as above, the membrane was blocked with 2% Tween-20 (Helicon) in TBS-T (25 mM Tris, 137 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h at room temperature and then incubated with polyclonal antibodies against the PSTVd-transcript (1 μg/mL) in TBS-T solution overnight at 4 °C. After washing with TBS-T, membranes were incubated with the phosphatase-conjugated secondary antibody (Jackson ImmunoResearch, Cambridgeshire, UK) diluted 1:7000 in TBS-T solution for 1 h at room temperature. The bound conjugate was visualized with BCIP/NBT phosphatase chromogen substrate.

3. Results and discussion

3.1 PSTVd-transcript, the antigens, and their characteristics

It is challenging to extract sufficient viroid from infected potatoes. PSTVd accumulates in the infected potato to less than 1 μg per 1 kg of plant mass [23]. Therefore, we used the plasmid with the cloned PSTVd cDNA [26] as a template in the RiboMax system for obtaining the linear viroid RNA. The secondary structures of the natural and the *in vitro* transcribed viroid RNA are expected to be very similar [33]. The PSTVd-transcript had a UV spectrum typical of RNA (Fig. 1A) and the predicted electrophoretic mobility (Fig. 1B). PSTVd-antigens: viroid RNA-(dien)platinum (vdRNA-(dien)Pt) and viroid RNA-lysozyme (vdRNA-lyso) were prepared by modification of the *in vitro* synthesized PSTVd-transcript with (dien)platinum or by formaldehyde cross-linking to egg white lysozyme, respectively.

(Dien)platinum reacts with RNA [28,34]. We previously modified the viroid RNAs with (dien)platinum under conditions where predominantly guanine bases were modified [29] for diagnostics of virus infections [28]. We considered the chemical modification of the PSTVd-transcript with both (dien)platinum and lysozyme to be stochastic. Regarding the antigens, we assumed that modification of the PSTVd-transcript with (dien)platinum causes minimal structure distortion because the melting temperature of the modified RNA probes, UV-spectrum, and electrophoretic mobility in the agarose gel (Fig. 1B) changed only insignificantly.

(Dien)platinum also reacts with DNA, and this modification enhanced its antigenic properties [29]. Moreover, we assumed that, along with antibodies to the adduct of (dien)platinum and guanine residues, part of antibodies would also recognize nucleotides adjacent to this adduct. However, the titer of mice antiserum against the vdRNA-(dien)Pt antigen was significantly lower than that against the vdRNA-lyso antigen (Fig. 2); therefore, we stopped working with this antigen.

The choice of lysozyme as a booster protein for the synthesis of the RNA-antigen was made earlier [22]. This basic protein (pI 9.3) readily forms complexes with RNA

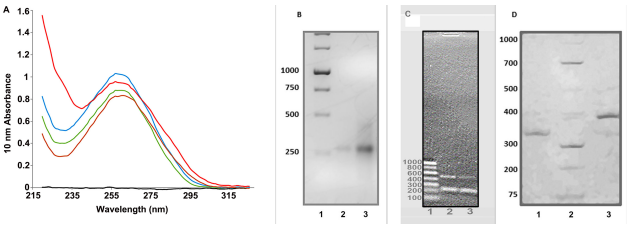

Fig. 1. Characteristics of the PSTVd-transcript, its antigens, and Apple Scar Skin Viroid (ASSVd). (A) UV-spectra; colored curves: black—baseline. Green—PSTVd-transcript: A_{260} 0.879, A_{260}/A_{230} 2.18, A_{260}/A_{280} 2.38. Blue—vdRNA-(dien)Pt: A_{260} 1.010, A_{260}/A_{230} 1.98, A_{260}/A_{280} 2.18. Red—vdRNA-lyso: A_{260} 0.946, A_{260}/A_{230} 0.99, A_{260}/A_{280} 1.68. Brown—ASSVd: A_{260} 0.832, A_{260}/A_{230} 2.93, A_{260}/A_{280} 1.92.

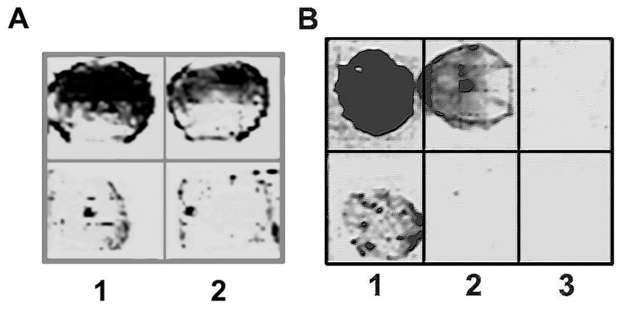

Fig. 2. Comparison of mice sera and antisera against antigens vdRNA-(dien)Pt and vdRNA-lyso. Top line: (A) Antiserum after seven immunizations with vdRNA-(dien)Pt antigen. (B) Antiserum after 3 immunizations with vdRNA-lyso antigen. From left to right, quantities of antigens applied to the membranes: (1) 100 ng; (2) 20 ng; (3) 4 ng. Bottom line: (A,B)—mice pre-immune sera.

and partially protects RNA from nucleases. Linking with lysozyme produced more visible changes; most products of the reaction smeared close to the gel load wells far from the position of the vdRNA (Fig. 1C). There are both fast and slow kinetics of the formaldehyde reaction with nucleic acid bases [35] and proteins [36–39] above neutral pH. Depending on the reaction conditions, reactions with formaldehyde occur in a few minutes whereas the slow reactions take from hours to days [35,39]. We assumed that subsequent protein and nucleic acid modification reactions change their conformation bringing together (and moving aside) the functional groups that form the methylene cross-links between the protein and the nucleic acid.

Three main parameters play a critical role during formaldehyde cross-linking: reaction temperature, incubation time, and formaldehyde concentration. Room temperature is advantageous because it presents the most pertinent condition. Considering the ionization constants of the amino acids [40] and nucleic base functional groups, the conjugation reaction between lysozyme and vdRNA was

performed at pH 9.3. In this mild alkali medium, the RNA is stable, and all heterocyclic bases readily interact with formaldehyde. The reaction proceeds through the formation of methylol derivatives and reactive Schiff bases [35]. We assumed that formaldehyde could potentially react with several amino acid residues of the egg white lysozyme [41]: The first is the primary amino group of N-terminal lysine and the ε-amino groups of six lysine residues. This then proceeds much slower with the amide groups of asparagine and glutamine, the guanidyl group of arginine, the hydroxy groups of threonine and serine, the sulphydryl group of cysteine, the phenol group of tyrosine, the phenyl group of phenylalanine, the indole group of tryptophan, and the imidazole group of histidine [36–39], thus suggesting 70 total potentially reactive amino acid residues in the lysozyme.

The Russian isolate of PSTVd used in the present work consists of 357 nucleotides (71A, 78 U, 108 C, and 100 G) and has a molecular mass of ~114,733 [26]. We selected stoichiometric binding conditions of (dien)platinum and lysozyme to RNA in both cases considering reacting chemical groups, and we used reaction conditions that minimize profound RNA modification (Fig. 1B,C). Under mild antigen-preparation conditions, part of the transcript remained unconjugated to lysozyme. Therefore, the concentration of the vdRNA-lyso antigen preparation was determined by the content of its main component, the transcript.

Electrophoresis in 1.5% agarose: (B) (1) the vdRNA-(dien)Pt antigen. GeneRuler, 1kb DNA Ladder (Thermo Fisher Scientific); (2) the PSTVd-transcript; (3) the vdRNA-(dien)Pt antigen; (C) (1) the vdRNA-lyso antigen. RiboRuler low range RNA ladder (Thermo Fisher Sci., bases); (2) the PSTVd-transcript modified with lysozyme; (3) the PSTVd-transcript. The upper band is the viroid dimer. (D) (1) ASSVd, 7% polyacrylamide gel electrophoresis. Trace amount of the PSTVd-transcript was added; (2) DNA ladder (base bairs, Thermo Fisher Scientific GeneRuler 1kb Plus); (3) the PSTVd-transcript.

3.2 Comparison by dot-ELISA of mice antisera to vdRNA-(dien)Pt and vdRNA-lyso antigens

We found that the vdRNA-lyso antigen was much more immunogenic than the vdRNA-(dien)Pt antigen (Fig. 2). The immune response was weak even after seven immunizations of mice with the vdRNA-(dien)Pt antigen (Fig. 2A). A more robust response was observed after the third immunization of mice with the vdRNA-lyso antigen (Fig. 2B). Therefore, we immunized a rabbit with this antigen only.

The results indicated that immunization with RNA preparations is more challenging than immunization with protein antigens. According to our protocol, at least eleven injections were required to obtain a viroid antibody titer sufficient for reliable detection. A comparison of the immune responses suggests that lysozyme act as an internal booster of the immune system that significantly accelerates the immune response to the antigen's RNA component.

3.3 Cross-reactions of antiserum and polyclonal antibodies against the vdRNA-lyso antigen and their suppression

Dr. Lukacs reported that monoclonal antibodies against PSTVd cross-react with cellular RNA—particularly ribosomal RNA [16]. Notably, in an infected cell, the mass of cellular rRNA is hundreds of times superior to the mass of PSTVd. The dot-ELISA assay of specificity of antiserum and purified antibodies against the vdRNA-lyso antigen (Figs. 3C,4) showed that both cross-react with lysozyme and potato cellular RNA. In the polyclonal antiserum, complete blocking of antibodies to lysozyme was achieved by adding 1.5 mg/mL lysozyme. To completely block cross-reacting cellular antibodies, we added commercially available yeast RNA or cellular potato RNA to the polyclonal antiserum or the antibodies (Fig. 3). The minimal required amount of the yeast and cellular RNA was determined experimentally. As a result, a linear diagnostical signal intensity dependence in the range from 1 μg to 60 ng was observed both for the antigen-conjugate and the unmodified PSTVd-transcript (Fig. 3A,B).

We hypothesized that antibodies to the PSTVd-lyso antigen recognize not only nucleotide(s) covalently associated with lysozyme but (oligo)nucleotides adjacent to the points of modification. The results in Fig. 4 confirm this supposition.

3.4 Polyclonal antibodies to the vdRNA-lyso antigen as a tool of the viroid infection diagnostics

To detect PSTVd in the infected potato, we removed high-molecular-weight cellular rRNAs from the cleared lysate of the viroid-infected plant cells (see Methods) by 2 M LiCl precipitation. We then concentrated the low-molecular-weight RNA through precipitation from the LiCl supernatant using ethanol. Dot-ELISA of the antiserum to the vdRNA-lyso antigen confirmed that it contains antibodies interacting with lysozyme, the antigen, and the unmodified PSTVd-transcript (Figs. 3,4). The immunogenic reactivity towards lysozyme was neutralized by adding 1.5 mg/mL lysozyme. We confirmed that the primary source of cross-reactions is ribosomal RNAs [16]. The cross-reaction with the plant's cellular RNAs was suppressed by saturation cross-reactive antibodies with 0.15 mg/mL yeast RNA. We found that the sensitivity of detecting the viroid RNA-transcript (up to 60 ng/mL, Fig. 5) was sufficient to determine natural viroids in a sample of low-molecular-weight RNA from infected potatoes. The data above are supported by quantitative methods of the sandwich-ELISA with the isolated antibodies against the vdRNA-lyso antigen (Fig. 5).

Isolated antibodies. Blue (round black markers)—the low-molecular-weight RNA of the uninfected potato; Green (triangular black markers)—the low-molecular-weight RNA of uninfected potatoes with the addition of the

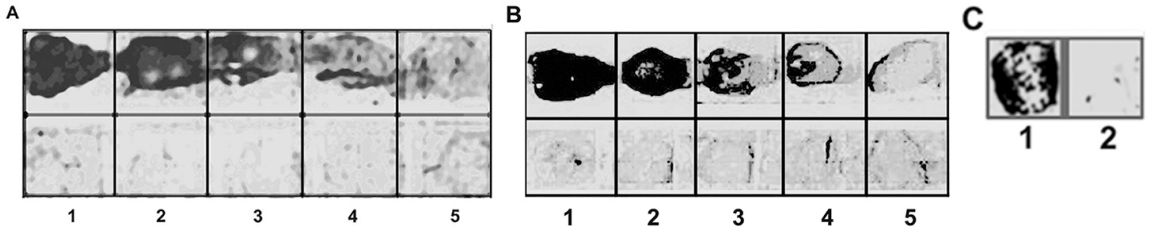

Fig. 3. Dot-ELISA of the rabbit antiserum to the vdRNA-lyso antigen after suppressing the antiserum cross-reaction by lysozyme (A and B) and yeast RNA (C). Membranes with samples were incubated in the immune (upper line) and pre-immune (bottom line) sera with added 1.5 mg/mL lysozyme: (A) the vdRNA-lyso antigen; (B) PSTVd-transcript. Both A and B membranes were spotted with 1.0 μg (1); 500 ng (2); 250 ng (3); 125 ng (4); and 62.5 ng (5) of the PSTVd RNA. (C) Antiserum cross-reaction with 1 μg potato cellular RNA (1) and its suppression with added 0.15 mg/mL commercial yeast RNA (2).

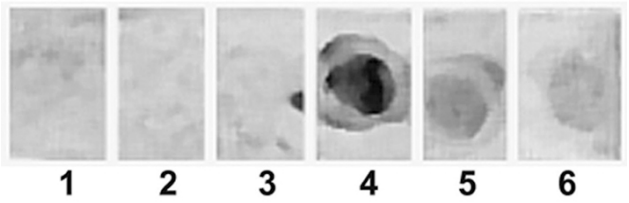

Fig. 4. Recognition of the PSTVd-transcript by antibodies to vdRNA-lyso antigen. Lysozyme (1.5 mg/mL) and the cellular yeast RNA (0.15 mg/mL) were added to the polyclonal antibody preparation to suppress cross-reactions with lysozyme and cellular RNAs. Samples from left to right: 1.0, 0.3, and 0.1 μg of low-molecular-weight potato RNA (1–3) and the same quantities of the free PSTVd-transcript (4–6) were spotted onto the membrane, respectively.

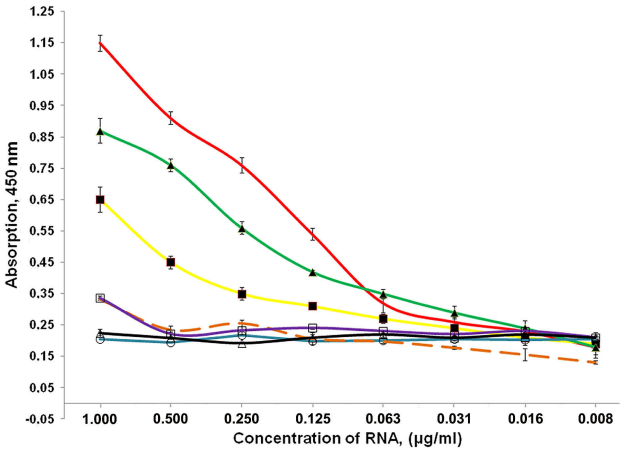

Fig. 5. Sandwich ELISA of the RNA preparations with pre-immune serum and isolated antibodies against the vdRNA-lyso antigen. Pre-immune serum. Colored curves: Brown (dotted line)—low-molecular-weight RNA of the uninfected potato. Black (triangular blank markers)—low-molecular-weight potato RNA with the addition of the free PSTVd-transcript (10 ng/mL). Violet (square blank markers)—PSTVd-transcript. Yellow (square black markers)—low-molecular-weight RNA from the PSTVd-infected potatoes; and Red (solid line without markers)—the free PSTVd-transcript.

The dependence of the signal intensity of the immunochemical reaction on the concentration of vdRNA in the low-molecular-weight fraction of the total RNA of infected potatoes (yellow line in Fig. 5) is linear from 30 to 250 ng/mL with a sample standard deviation of 0.02–0.04. Thus, the results suggest that vdRNA is immunogenic; however, the antibody-specific titer is moderate partially because the RNA might undergo intense hydrolysis in physiological fluids.

Diagnosis of viroid infections has largely been solved with the invention of the polymerase chain reaction. However, the PCR reaction requires sample preparation. Sample preparation, PCR, and RT-PCR require laboratory equipment and trained personnel. In agriculture, PCR technology is used only in specialized laboratories in highly developed countries. Historically, most of the mass diagnostic tests in practice are carried out by immunochemical methods in agriculture. Moreover, current trends in the development of molecular diagnostics of infections are focused on bringing these methods closer to their use by non-professionals or ordinary manufacturers of products; thus, they are used directly in the field for culling infected plants or animals. The use of immune chromatography test strips facilitates this. Even in the field, immune chromatography methods can diagnose pandemic, epizootic, and epiphytotic diseases [42]. This method's simplicity, speed, and scalability make it practical and convenient for analyzing many samples.

3.5 On the specificity of antibodies against the PSTVd-lyso antigen

The specificity of antibodies against RNA was determined by comparing them with antibodies to proteins. Proteins consist of 20 different amino acids, and thus there are very many combinations. There are also many ways that an antibody can recognize an epitope on a protein. It does not need to be a consecutive sequence of amino acids, i.e., it can

be part of two or more patches of the three-dimensional architecture of the amino acid sequences—this is done simply by forming the recognized part. Polyclonal antibodies can react with several but slightly differing epitopes. A similar is observed with ribotopes.

While the building blocks of RNA are limited to four bases, post-transcriptional modifications can dramatically extend the chemical diversity of RNA with up to 170 identified modifications [13,15]. Moreover, the tertiary structure of RNA is also specifically recognized by anti-RNA antibodies along with the primary and secondary structures [18]. Although antibody binding sites span a 7–10 linear nucleotide sequence, their avidity was found to increase for elongating nucleotide sequence [16,17,19]. In single-stranded DNA, the antibody's avidity for a hexanucleotide increases up to 300 nucleotides [17,19].

Nevertheless, the antibodies' specificity for nucleic acids is lower than that of antibodies to proteins—the polyclonal antibodies to RNA manifest cross-reactions with other nucleic acids. The RNA's ordinary primary structure degeneration versus the protein's primary structure explains the bulk of cross-reactions of these antibodies with other RNAs. The target ribotopes can be identified using blocking (subtracting) cross-reacting ribotopes by appropriate RNA. This access is conceptually similar to subtractive hybridization. Thus, neutralized polyclonal antibodies against the PSTVd-lyso antigen that cross-reacts with cellular RNA can detect PSTVd RNA in infected cells (Figs. 3,4,5,6). Specific immunoglobulins using ribotope subtraction can selectively recognize a closely related apple viroid (Fig. 7) and can be discovered among those raised against the PSTVd-lyso antigen.

The immune response against even simple viroid RNA is pleiotropic due to this macromolecule's plurality of structural ribotopes. The advantage of polyclonal antibodies obtained in this work over monoclonal antibodies (used by Dr. Lukacs) is their higher overall avidity towards the antigen due to binding to all ribotopes at once. Notably, immunoglobulins recognizing the target RNA, despite intrinsic cross-reactions, can be used in immune analysis.

3.6 Immune northern blot analysis

The specificity of the antibodies was investigated through northern immunoblotting. Fig. 6 shows that the antibodies react specifically with the PSTVd-transcript obtained *in vitro* and the potato spindle tuber viroid in the low-molecular-weight RNA preparation of infected potato.

We currently obtained a population of polyclonal immunoglobulins with different specificities, and each recognizes its ribotope. The antibodies were raised against stable RNA structures. Notably, even small viroid RNA is a large macromolecule (>115 K) versus ordinary proteins (~30 K). Thus, RNA has many antigenic determinants, and most of them are stable at defined conditions. We were interested in determining the selectivity of the antibodies for the potato

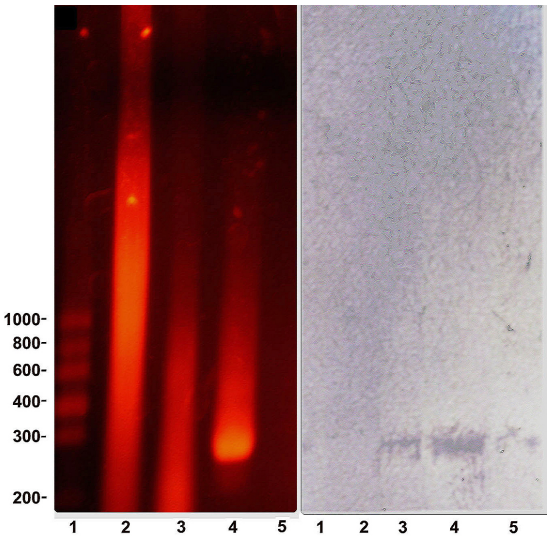

Fig. 6. Electrophoretic and immune northern blot analyses of the PSTVd-transcript and the PSTVd-infected potato. Left—electrophoresis of RNA samples in a 1.5% agarose gel in the presence of ethidium bromide. Left row: numbers show the length of the marker RNAs in nucleotides. (1) Marker RNAs of the Thermo Fisher Scientific RiboRuler Low Range RNA Ladder; (2) low molecular RNA isolated from healthy potato (10 μg); (3) low-molecular-weight RNA isolated from the PSTVd-infected potato (10 μg); (4) the PSTVd transcript (2 μg); and (5) the PSTVd transcript (0.2 μg). Right—immune northern blot analysis. Concentration of antibody preparation 1 μg/mL.

viroid ribotopes and used the Apple Skar Skin Viroid at our disposal. ASSVd belongs to the same Pospiviroidae family as the PSTVd. It is known that viroid structures are conservative within one family.

For comparative analysis, the same quantitative dilutions of the PSTVd-transcript and apple viroid were applied to the membrane (Fig. 7).

Fig. 7 shows that the antibodies against PSTVd-lyso interacted differently with the PSTVd-transcript and ASSVd. They recognized the PSTVd-transcript but not the apple viroid. Moreover, the polyclonal antibody preparation appears to contain a fraction of immunoglobulins that cannot be saturated with the PSTVd-transcript, but these recognize ASSVd. Thus, despite their close relationship, these viroids appear to have similar and different ribotopes recognized by different immunoglobulins in the preparation of polyclonal antibodies against the PSTVd-lyso antigen.

Both viroids (PSTVd and ASSVd) belong to the Pospiviroidae family and have similar secondary structures (Fig. 8, Ref. [23,43]).

The apple scar skin viroid (ASSVd) consists of 329 nucleotides. It differs strikingly from all potato spindle tuber viroid (PSTVd)-related viroids in that its central domain shows no sequence similarity with the central conserved region of the latter [43]. Thus, there is different recognition

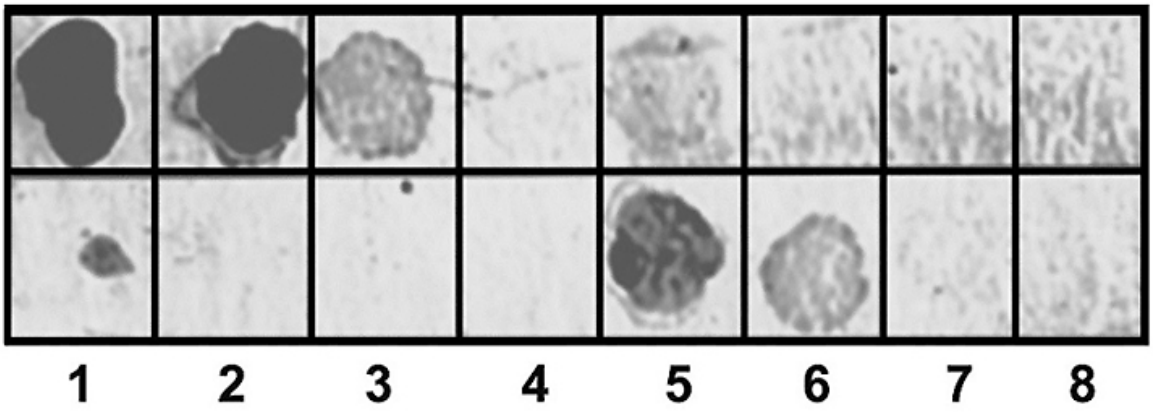

Fig. 7. Dot-ELISA of the specificity of antibodies against the PSTVd-lyso antigen. Similar quantities: Upper line—1, 0.3, 0.1, and 0.03 μg of the PSTVd-transcript and the apple viroid ASSVd (1–4 and 5–8, respectively) were applied onto a nitrocellulose membrane. Bottom line—the exact quantities of the PSTVd-transcript and apple viroid were applied onto the membrane, but 3 μg/mL of the PSTVd transcript was added to the primary antibody solution. The membranes proceeded with antibodies as described in Materials and methods.

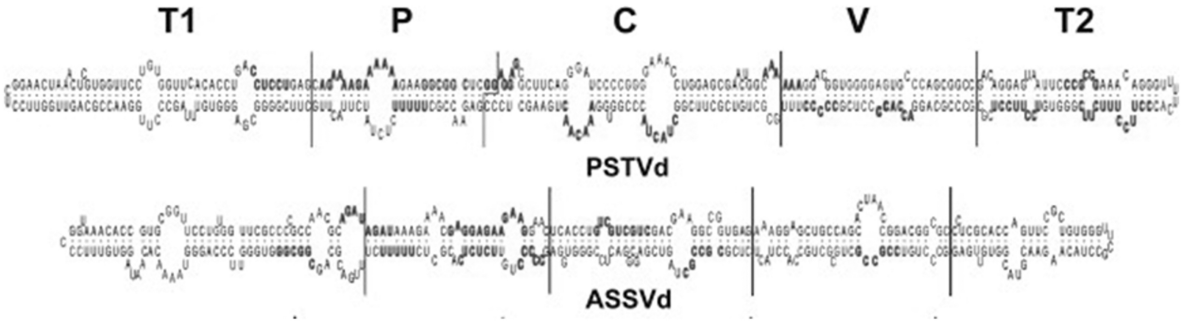

Fig. 8. Primary and secondary structures of PSTVd and ASSVd. Sequentially, the viroid domains for PSTVd and ASSVd are presented according to references [23] and [43]. T1, left terminal domain; P, pathogenicity domain; C, central domain; V, variable domain; and T2, right terminal domain. Vertical dashes show domain boundaries.

of PSTVd and ASSVd (see Fig. 7); this is mainly explained by the differences in their primary structure. We assume that the specificity of antibodies to the target RNA can be significantly increased if, instead of RNA, one uses the ribotopes with the specific primary structure as RNA antigens. Thus, it would be interesting to know the differences in the ribotopes of viral and cellular RNAs.

Furthermore, we observed that the resulting polyclonal antibodies have cross-reactions with cellular RNAs—mainly with rRNA. This polyclonal antibody property is critical to the recently increased interest in vaccine mRNAs. The developers' primary attention is on the vaccine properties of viral proteins encoded by the vector mRNA. Studies that assessed the immunogenicity of messenger RNA and vector DNA vaccines showed that the vaccines were safe with no evidence of short-term allograft-related adverse effects in either cohort.

One might wonder if an mRNA (DNA) vaccine would lead to an overactive immune response. Humans have innate immunogenicity against foreign RNA [44,45] and DNA [1,2,7], but the specificity of antibodies for nucleic acids is much lower than that of antibodies for proteins. RNAs are usually weaker antigens than proteins but not their complexes with proteins. RNAs form complexes with proteins in cells. In this regard, the likelihood of undesirable formation of antibodies to RNA increases sharply. This is undesirable due to the more remarkable similarity of the RNA composition of the pathogen and the host versus proteins.

We speculate that antibodies to mRNA and DNA can cross-interact with cellular RNAs and host DNA because of their chemically close PAMPs (Pathogen-Associated Molecular Pattern). Furthermore, foreign high molecular weight mRNAs are exposed to nucleases and the RNA interference system during vaccination; they are then degraded as many RNA complexes in the cell. Degradation occurs to those mRNAs in which natural nucleotides have been partially replaced with nuclease-resistant analogs and even those bound to polyamines, lipids, dextran, and placed into liposomes. The resulting mRNA fragments that lost their ability to translate are still immunogenic.

Our preliminary data with the plant and animal virus RNAs support this supposition. Thus, this is a likely pathway to mRNA and vector DNA vaccination's unfortunate consequences—an autoimmune disease [44,45]. The cause of the disease is that antibodies produced in response to bacterial or viral nucleic acid during infection (vaccination) begin to bind to host nucleic acids, their regulatory sequences, and even proteins due to molecular mimicry [46,47]. In most cases, this leads to a disruption of the cellular mechanisms that control our bodies. Autoimmune diseases develop slowly but are relatively common. We suspect that the current practice of vaccination using mRNA and DNA vaccines as well as inactivated and live viruses have not had sufficient time (in the case of mRNA) to study the long-term consequences of nucleic acid vaccination. The long-term side effect of the nucleic acid vaccination requires additional study in our opinion [48].

Finally, diverse functional RNAs participate in a wide range of cellular processes. The RNA structure is critical for function—both for RNA on its own and as a complex with proteins and other ligands. Therefore, analysis of RNA conformation in cells is essential for understanding their functional mechanisms. However, no appropriate method has yet been established, and there are few practical tools for recognizing the conformation of structured RNA *in vivo*. Antibodies against RNA can fill this gap, thus helping to recognize specific RNA conformations. To be closer to this purpose, the definition and identification of the PSTVd and some virus RNA' ribotopes are underway. Furthermore, antibodies against target ribotopes can potentially be a magic bullet that regulates *in vivo* RNA activity.

Abbreviations

ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ASSVd, apple scar skin viroid; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; Dien, diethylenetriamine; HRP, horse radish peroxides; Lyso, lysozyme; PBS, phosphate buffer saline; PSTVd, potato spindle tuber viroid; TDW, deionized double distilled water; vdRNA, viroid RNA.

Author contributions

YD, TG, and KB conceived and designed the experiments; TG and KB performed the experiments; TG and KB contributed reagents and materials; YD and KB analyzed the data.

Ethics approval and consent to participate

All experiments on animals (mice, rabbits) were carried out in accordance with the animal care regulations of the Moscow State University by Lomonosov. The protocol was approved by the Bioethics Committee of the Faculty of Biology, Moscow State University by Lomonosov.

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Conflict of interest

The authors declare no conflict of interest.

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