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CHRYSANTHEMUM VIRUS B: ANTIBODIES TO STRUCTURAL PROTEIN IN MAMMALS AND Mg^{2+} - DEPENDENT REVERSE-TRANSCRIPTASE ACTIVITY

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Summary. — Human plasma has been found to contain antibodies reacting with the structural protein of chrysanthemum virus B (CV-B) and about 10 times less intensively with the structural protein of another carlavirus, the potato virus M. It has been shown that the antibodies bind to CV-B through their $F(ab)_2$ fragments. No reaction with proteins of other plant viruses or retroviruses was observed. Antibodies reacting with CV-B protein are also present in the plasma of green monkey, goat, rabbit, and mouse, their level being somewhat lower than in man. In addition, Mg^{2+}-dependent reverse transcriptase activity reaching maximum at 37 °C was detected in the CV-B preparations. It is suggested that humans and the mammals in question developed antibodies to CV-B which could enter into some enzymatic reactions at the body temperature.

Key words: *chrysanthemum virus B*; *mammals*; *antibodies*; *reverse transcriptase*

Introduction

In our previous paper (Morozov *et al.*, 1987) we have first reported the presence of antibodies to chrysanthemum virus B (CV-B), a member of carlavirus family, in normal human plasma. It was pointed out that we might have dealt with an accidental coincidence of antigenic epitopes in CV-B proteins reacting with some antibodies present in humans and raised to other unrelated proteins. It also seemed possible that CV-B proteins might nonspecifically interact with the immunoglobulins as does the staphylococcus A-protein.

In the present investigation we searched for antibodies to CV-B among mammals and estimated the specificity of their interaction with CV-B. We also intended to verify the hypothesis of the existence of an actual relationship between CV-B and animals. The results are indicative of the existence of CV-B structural protein that can dissociate in a step-wise fashion to form two immunologically active highmolecular products. All three proteins

appeared to react specifically with human antibodies, as well as with green monkey, goat, rabbit, and mouse antibodies. In addition, CV-B preparations exhibited a reverse-transcriptase activity at 37 °C. Because CV-B is an RNA virus and because it is essentially impossible to assume the reverse transcriptase activity in terms of the common knowledge of plant viruses, a hypothesis on interaction between plant viruses and the animal organisms has been set forward.

Materials and Methods

Chrysanthemum virus B was isolated by 4 different methods from the same sample of *N. clevelandii* leaves. The isolation was carried out under the guidance and with kind assistance of A. G. Odinets (Lomonosov Moscow State University) using previously described procedures (Hollings and Stone, 1972; Hearon, 1982; Tavantzis, 1983). After homogenization of leaves the proteins were extracted from the solution using Triton X-100, n-butanol, ether, and chloroform. The virus was then sedimented by PEG, resuspended in the extraction buffer (citrate-phosphate buffer, pH 9.0) and resuspended again by ultracentrifugation. Resuspended pellets were clarified and used for further work.

Other virus preparations. Tobacco mosaic virus (TMV) was kindly supplied by M. E. Talyansky, potato virus X (PV-X) by E. N. Dobrov, potato virus M (PV-M) by O. N. Nikolayeva (all from the Deparment of Virology, Lomonosov Moscow State University). The following retroviruses were obtained in the framework of the Soviet-American Cooperation for Oncovirology: Gross Murine Leukaemia Virus (G-MuLV), Feline Leukaemia Virus (Fe-LV), Murine Mammary Tumour Virus (MMTV), and the Simian sarcoma virus (SSV). The virus samples were treated with a detergent buffer, pH 6.8, heated at 100 °C, and applied to a polyacrylamide gel.

Electrophoresis in polyacrylamide gel was carried out in vertical gradient slab gels with acrylamide concentrations 7.5—15 % supplemented with 0.1 % SDS using a buffer system designed by Laemmli (1970) at 15 mA for 4—4.5 hr. A set of marker proteins SDS-6H (Sigma, U.S.A.) including myosine (205 kD), β-galactosidase (116 kD), phosphorylase B (97.4 kD), BSA (66 kD), ovalbumine (45 kD), and carboanhydrase (29 kD) was used for gel calibration. After electrophoresis the gels were stained as described (Morozov *et al.*, 1986) or transferred directly to nitrocellulose filter after soaking in a transfer buffer.

Preparation of the F(ab)$_2$ IgG fragment. F(ab)$_2$-fragments were prepared from human IgG by papaine-digestion (Serva, F.R.G.) at 37 °C for 16 hr. The IgG was prepared from commercial plasma after previous purification on DEAE cellulose and separation on Sephadex G-200. The resulting fractions were analysed by electrophoresis and used for immunoblotting.

Immunoblotting. The proteins were transferred from PAG to nitrocellulose filter with 0.45 μm pore diameter (Towbin *et al.*, 1979) in a modified device for gel decolorization (GD-4, Pharmacia, Sweden). The proteins were transferred at 24 V for 4 hr. The filters were stained with amido black 10 B (Serva, F.R.G.). The other part of the filter was kept in 3 % ovalbumine solution (2—3 hr) in 0.01 mol/l sodium phosphate buffer, pH 7.2, containing 0.1 % Triton X-100. The filter was then rinsed with sodium phosphate buffer with 0.1 % Triton X-100. After treatment with an appropriate serum (for 3 hr) it was washed and then treated with horseradish peroxidase conjugated antibody (for 3—4 hr). After rinsing the filters the bands were visualized with diaminobenzidine (Sigma, U.S.A.). Commercial human plasma preparation, human serum, normal green monkey serum, normal rabbit, mouse, and goat sera were used, as well as antibodies conjugated with horseradish peroxidase: against human immunoglobulins (Amersham, England), against mouse immunoglobulins (the same company), against human IgG (Gamaleya Institute of Epidemiology and Microbiology), against rabbit immunoglobulins (Sigma, U.S.A.) and against goat immunoglobulins (kindly supplied by S. V. Litvinov, All-Union Oncological Centre, the U.S.S.R. Academy of Medical Sciences). All the conjugates were used in a 1 : 500 dilution.

Reverse transcriptase reaction. Reverse transcription was conducted according to Arga and Chavola (1977) using an exogenous poly(rA): oligo (dT) template. The virus preparation (10 μl, 8 μg) 10 μCi ^{3}H-TTP was added; the reaction mixture was preincubated at 0 °C for 15 min and then incubated at 20 °C, 37 °C, and 45 °C. The reaction was terminated with 10 % TCA and assayed in the liquid scintillation counter Mark-III.

Results

In some cases immunoblotting may yield either false positive or false negative results. The false positive ones can be excluded using a set of antigens to which no antibodies are present in the tested sera. It has been shown in the previous paper that CV-B virus contains a 40 kD structural protein. This protein can dissociate in a step-wise manner during storage or by heating to form proteins 37 and 34 kD (Morozov *et al.*, 1987).

Fig. 1-A shows the stained portion of the nitrocellulose filter with transferred plant virus preparations (TMV, CV-B PV-M, PV-X); Fig. 1-B shows the same filter stained with normal human plasma in the dilution 1 : 250. A significantly positive reaction was observed with the structural CV-B protein (track 2).

A similar experiment has been made with retrovirus preparations G-MuLV, FeLV, SSV, MMTV, and CV-B. Fig. 2-A shows the stained part of the nitrocellulose filter and Fig. 2-B shows the filter treated with normal human

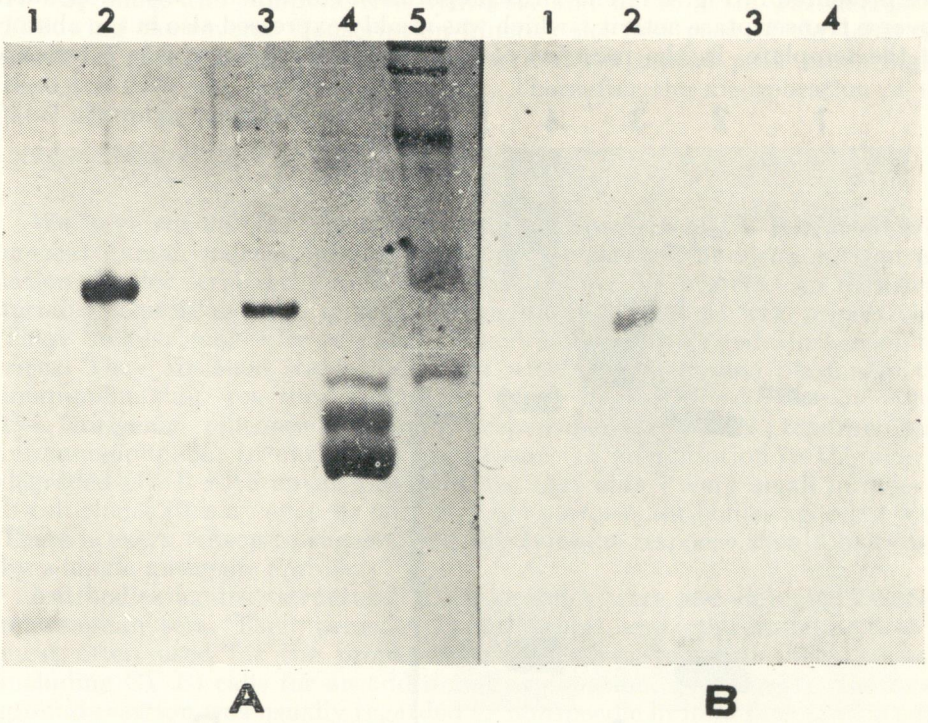

Fig. 1.

Nitrocellulose filter with transferred plant virus preparations
A — stained with amido black 10 B. B — treated with normal human plasma diluted 1 : 250 followed by horseradish peroxidase conjugated anti-human IgG 1 — TMW, 2 — CV-B, 3 — PV-M, 4 — PV-X, 5 — m.w. markers.

plasma in the 1 : 250 dilution. It can be seen from Fig. 2-A that the structural protein of CV-B underwent a step-wise degradation (as a result of prolonged storage). All the 3 proteins, however, interacted with normal human plasma (track 3) which did not react with any of the structural proteins of above mentioned retroviruses. Extensive tests with mammal sera for the presence of antibodies to CV-B structural protein demonstrated that antibodies to this protein were present in several mammals tested (green monkey, goat, rabbit, mouse). Moreover, the reaction seemed specific, since $F(ab)_2$-fragment from human IgG also reacted with CV-B (track 2) (Fig. 3).

Furthermore, we looked for the presence of reverse transcriptase activity in the CV-B preparation. The reaction was conducted under standard conditions using a poly(rA) : oligo(dT) template. The original mixture without the template or the TMV preparation serve as a negative control. The following parameters of the presumable enzyme were assessed: 1. ionic dependence (Mg^{2+} or Mn^{2+}); 2. optimal temperature; 3. kinetics of the reaction. The results of the kinetic studies of reverse transcriptase reaction are presented in Fig. 4-I. The virus preparation contained a Mg^{2+}-dependent reverse transcriptase activity, which was weakly expressed also in the absence of the template. In the presence of Mg^{2+} the incorporation was 5 times as

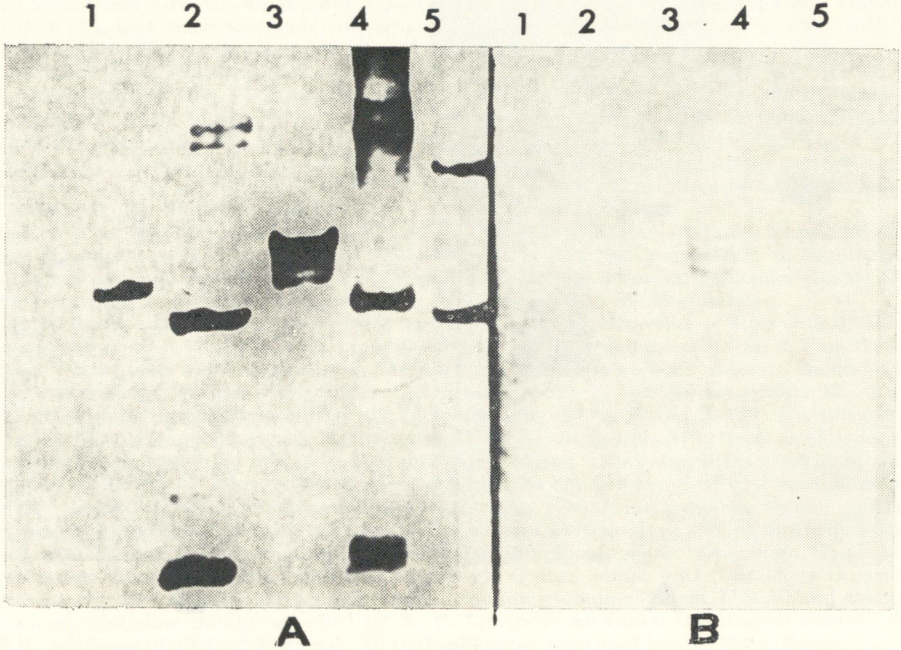

Fig. 2.

Nitrocellulose filter with transferred CV-B and animal retrovirus preparations
A — stained with amido black 10 B. B — treatment with normal human plasma diluted 1 : 200 and anti-human conjugate 1 — G-MuLV, 2 — FeLV, 3 — CV-B, 4 — SSV, 5 — MMTV.

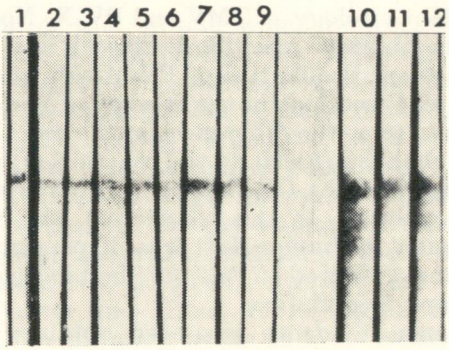

Fig. 3.
Nitrocellulose filters with transferred CV-B were treated with: 1 — normal human plasma, 2 — $F(ab)_2$-fragment of human IgG; 3-5 and 10-12 with individual human sera, 6 — green monkey serum, 7 — rabbit serum, 8 — goat serum, and 9 — mouse serum. All the sera were diluted 1 : 250.

high as that with Mn^{2+}. Temperature studies (Fig. 4-II) have shown that no reaction occurred at 20 °C, at 37 °C the incorporation of the label exceeded 10—20 times the template-free synthesis and at 46 °C it was decreased. Following the reverse transcription kinetics the first peak of the label was observed 10 min after the onset of reaction; the second peak much higher than the first one, occurred after 2 hr. Thereafter the incorporation of the label abruptly decreased.

Discussion

We have shown that the 40 kD structural protein of CV-B reacted with normal human plasma IgG regardless of the method of virus antigen isolation. Under stringent conditions of isolation, this protein can dissociate forming 2 proteins with molecular weights of 37 and 34 kD, respectively. These smaller molecules are also formed after a prolonged storage of the virus. They are then stable and react with normal human plasma in the immunoblotting reaction indicating that degradation did not affect the antigenic epitopes (epitope) responsible for their binding with immunoglobulins. In our belief, the absence of precipitation in the agar of degraded CV-B with antibodies indicates that only a very small number of B-cell clones (it may even be only 1 clone) produce antibodies against CV-B. There is every reason to suggest that this immune response should be caused by a single antigenic epitope.

Antibodies against structural CV-B proteins were also detected in some mammalian sera. Their presence in the rabbit sera (the animal which is most often used for the preparation of antisera to plant virus proteins including CV-B) calls for an additional explanation. Presumably, the background reaction was usually regarded as nonspecific in immunological studies of CV-B. It should be noted that the immunoblotting is more sensitive than the methods used previously.

A number of experiments using the immunoblotting procedure with different animal and plant viruses has been carried out. No reaction was observed with the animal retroviruses or with 2 viruses traditionally used

in plant virology — TMV and PV-X. Nor did PV-M react with the sera tested as the observed reaction was weak and irreproducible. Because we only had a very small quantity of PV-M this question will be handled in the nearest future. Variations in the intesity of reaction of CV-B protein with individual human sera, the differences in the reaction among human and animal immunoglobulins, as well as the reaction of human, goat, and rabbit immunoglobulins with the CV-B protein provide strong evidence for the specificity of this reaction. In this connection, the result of the treatment with $F(ab)_2$-fragment of human IgG was of paramount importance. The positive result obtained allowed to rule out the binding of CV-B protein to the Fc fragment of immunoglobulins.

Hence, evidence has been obtained for the existence of antibodies in humans and in some mammals reacting with CV-B protein (or the whole virus). The mechanism of this antibody formation is unclear at present. The fact of immunization with CV-B itself has not yet been clearly established. Some alternative explanations of the phenomenon may be put forward.

Mammals, including man, may develop antibodies upon contact with CV-B protein (or with some other carlavirus immunologically closely related

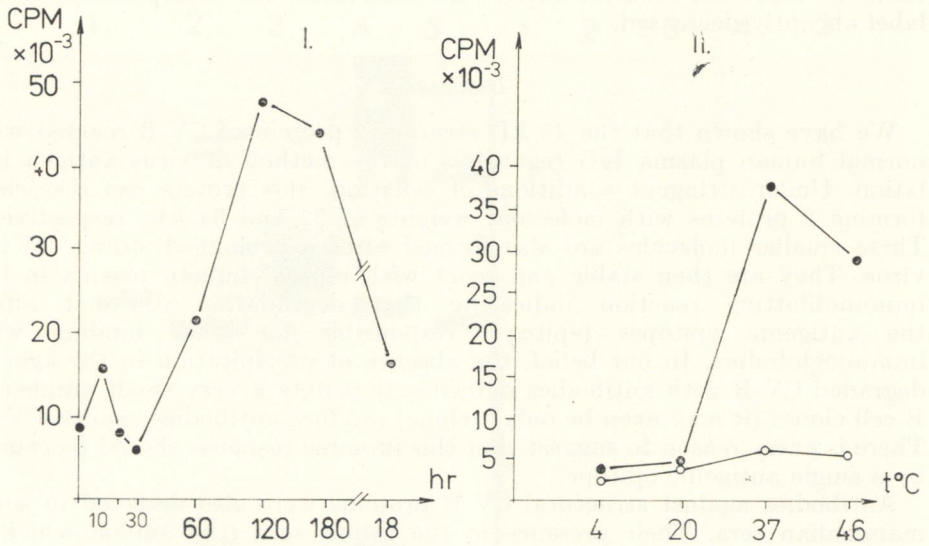

Fig. 4.

Characterization of reverse transcriptase activity detected in the CV-B preparation
I — course of reverse transcriptase reaction in the presence of exogenous template poly(rA): oligo(dT). II — temperature-dependence of reverse transcriptase activity in CV-B preparation, incubation time 40 min. 1 — with exogenous poly(rA): oligo(dT), ●———● 2 — without exogenous template (○———○).
Abscissae: 4-I: time in min or hr, respectively
4-II: temperature (°C)

to CV-B) or against a small peptide of this protein which represents the antigenic epitope. Such immunization could proceed by oral ingestion which is quite likely in view of rather a high thermostability of the viruses in question. What is implied here is the consumption of plant products infected with CV-B (or an immunologically related carlavirus). Such immunization can be primarily mediated by one particular peptide of CV-B protein. This idea is strengthened by the results of agar immunoprecipitation. In this case basic concern should be focused on the vegetables that are regularly consumed raw (cabbage, carrot, etc.), as well as on potatoes which are generally infected with carlaviruses. It is believed, however, that CV-B has rather a narrow host range (Wetter and Milne, 1981). Alternatively, immunization could also occur via microinjuries of the skin on hands and fingers. In such case infected plants may be the source of antigen.

Fortuitous coincidence of antigenic epitopes of CV-B protein with an epitope of structural elements of other viruses, bacteria or fungi cannot be completely ruled out. Antibodies to synbiotic microorganisms often occur in mammals and are normally present in rather a high titre during their life-time.

The strong difference between the titre of antibodies against CV-B in the normal donor plasma and the titre in individual human serum also has to be explained. Antibodies may be produced more intensively in regular blood donors than in the randomly selected individuals. Furthermore, antibodies may be better preserved in lyophilized commercial plasma preparations than in the individual sera which underwent a few freezing and thawing cycles.

Another result of great importance is the detection of Mg^{2+}-dependent reverse transcriptase with optimal temperature of 37 °C within the CV-B preparation. This suggests that the enzyme is active in mammals, but not in plants. Because this activity increases in the least intact virus preparations (according to the state of structural protein) it can be concluded that the enzyme responsible for this activity was present in the virion. Neither the association of enzymatic activity with structural virus elements can be ruled out. Molecular hybridization of CV-B cDNA should be performed with the retrovirus revertase (pol) genes. The presence of reverse transcriptase (homologous to that of retrovirus) in plant viruses has been reported for cauliflower mosaic virus (Pfeiffer and Hohn, 1983; Volovitch *et al.*, 1983). This virus is, however, not a typical plant virus; it contains DNA and not RNA like carlaviruses.

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