

ANTIBODIES TO REVERSE TRANSCRIPTASE OF AVIAN ONCOVIRUSES IN SERA OF SPECIFIC-PATHOGEN-FREE CHICKENS

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Summary. — Inhibitors of reverse transcriptase of the avian myeloblastosis virus have been found in immunoglobulins isolated from sera of normal specific-pathogen-free chickens. These inhibitors were shown to represent antibodies reacting with the enzyme.

Key words: reverse transcriptase; avian myeloblastosis virus; antibody; immunoglobulins

Introduction

Inhibitors of RNA dependent DNA polymerase in sera from rats with AKR virus induced sarcoma were first found by Gervin *et al.* (1970). They were also the first who suggested that these inhibitors were most likely antibodies. This was soon confirmed (Oroszlan *et al.*, 1971; Aaronson, 1972; Wu *et al.*, 1977). Antibodies to reverse transcriptase of murine oncoviruses were found in eluates from renal glomeruli of AKR mice where they were present as complexes with the antigen (Hollis *et al.*, 1974).

Antibodies to reverse transcriptase of avian myeloblastosis virus were found in sera of chickens infected experimentally with this virus and in sera from normal commercial broilers, natural carriers of the virus (Graevskaya and Sito, 1977).

In this study we tried to detect antibodies to reverse transcriptase of avian oncoviruses in sera from specific-pathogen-free chickens.

Materials and Methods

Sera. Sera from chickens of Dahmsdorf poultry farm free from specific pathogens were tested. This poultry farm is under permanent control of the Avian Diseases Unit, Zooveterinary Section of Humboldt University. The fowl herds are regularly examined for the presence of 25 species of viral and microbial agents and antibodies to them. The presence of the viruses of chicken leukaemia-sarcoma complex is determined by COFAL-test (Heider *et al.*, 1973) and by immunofluorescence (Krogner and Knöpke, 1972); antibodies to them by neutralization test (Glathe *et al.*, 1971). All sera used in this study were obtained from chickens negative by these three tests.

Isolation of serum immunoglobulins. Serum immunoglobulins (Ig) were salted out with a semi-saturated ammonium sulphate solution and dialysed against cold physiologic solution for 24 hr;

Table 1. Examination of Ig in sera of specific-pathogen-free chickens for the presence of inhibitors to reverse transcriptase of avian myeloblastosis virus

Generation of chickens	No. of sera tested	No. of sera lacking inhibitors	No. of sera inhibiting the enzyme activity	
			in the range from 20 to 50%	over 50%
4th	41	26	14	1
5th	25	7	11	7

the Ig obtained were separated by gel filtration through Sephadex G-200 and concentrated on Amicon filter. Protein concentrations were determined by the method of Lowry. The resulting Ig were tested for their capacity to inhibit the purified reverse transcriptase.

Virus. Avian myeloblastosis virus, strain BAI-A, was used. The conditions of chicken inoculation and harvesting of the virus-containing plasma were described previously (Graevskaya *et al.*, 1975). The methods of virus purification and reverse transcriptase isolation were also reported elsewhere (Graevskaya *et al.*, 1975).

The activity of the enzyme was determined in 50 μ l volume of a reaction system containing 0.06 mol/l Tris-HCl (pH 8.0), 0.005 mol/l MgCl₂, 0.04 mol/l KCl, 0.025 U of exogenic matrix poly(rA)-oligo(dT), 5 mol/l dithiothreitol, 0.1 mol/l TTP, and 5 pmol/l ³H-TTP (specific radioactivity 100–200 cpm/pmol). The mixture was incubated for 15 min at 37 °C. The reaction was stopped by placing the specimens into an ice bath and adding 0.2 ml of a solution containing 1 mg/ml ATP and 0.6 mg/ml RNA. Then 0.2 ml of 25% CCl₃COOH was added and the mixture was kept at 0 °C for 10 min. The precipitates were collected on Millipore filters. Radioactivity was determined in a toluene scintillator.

Determination of the extent of enzyme activity inhibition. 0.01–0.001 μ g of purified reverse transcriptase and 10–100 μ g of Ig of tested serum were mixed. The reaction mixture in a volume of 50–70 μ l contained 0.01 mol/l Tris-HCl buffer, pH 8.0, 0.015 mol/l KCl, 20% glycerin and 50 μ g bovine serum albumin. After incubation for 15 min at 37 °C, 50 μ l of standard polymerase mixture was added to each specimen containing 0.06 mol/l Tris-HCl buffer, pH 8.3, 0.04 mol/l KCl, 0.005 mol/l MgCl₂, 5 mmol/l dithiothreitol, 5 pmol/l ³H-TTP (specific radioactivity 100–200 cpm/pmol- and exogenous matrix poly (rA)–(dT)₁₂, 0.02 optic units per specimen. The reaction was stopped and read as mentioned above. The degree of inhibition of the enzyme activity in specimens containing Ig was expressed in per cent of activity of control specimens.

Table 2. Inhibition of avian myeloblastosis virus reverse transcriptase by serum specimens collected at a 6 month interval

Number of chickens	Level of inhibition (%)	
	1st specimen	2nd specimen
3	0	0
7	0	0
2	0	16
13	0	27
19	0	42
5	24	30
11	28	35
20	19	35
22	34	50
23	40	27
25	32	21
26	42	58

Table 3. Relationship between the amount of Ig used in neutralization test and the level of inhibition of the reverse transcriptase

Ig fraction prepared from	Amount of Ig (μg)	Level of the enzyme inhibition (%)	
Rabbit serum to the reverse transcriptase of avian myeloblastosis virus	17.5	8.0	
	35.0	51.0	
	52.5	80.0	
Sera of normal leukemia-free chicken	No. 4514	6.0	32.2
		10.0	43.3
	No. 3605	25.0	40.0
		30.0	58.0
		35.0	75.0
	No. 3563	35.0	46.0
		47.0	67.0
		95.0	80.0

Results

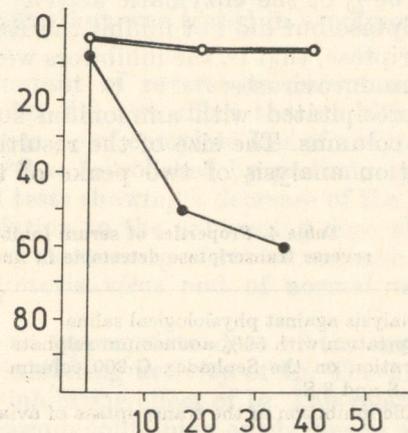
Sixty-six chicken sera were tested: 41 sera from chickens of the 4th generation and 25 of the 5th generation in this poultry farm. From 12 chickens of the 4th generation the sera were collected twice at a 6 month interval. It may be seen from Tables 1 and 2 that 50% of the chickens examined had inhibitors to avian myeloblastosis virus reverse transcriptase; in most of them the inhibition level did not exceed 50%. Noteworthy is the fact that sera from chickens of the 4th generation contained the inhibitors less frequently than chickens of the 5th generation and the level of inhibition by their antibodies was lower.

Fig. 1.

Determination of IgG specificity against reverse transcriptase of avian myeloblastosis virus

Rauscher murine virus was concentrated by differential centrifugation, disrupted with 0.01% NP-40 at 0 °C for 30 min and IgG to reverse transcriptase of avian myeloblastosis virus was added (reverse transcriptase activity of the disrupted virus lacking IgG served as control). The IgG was preincubated for 15 min at 37 °C, then added to the standard polymerase mixture and further incubated for 15 min at 37 °C. (○—○). In parallel, reverse transcriptase of avian myeloblastosis virus was inhibited by antibodies to the enzyme (●—●).

Abscissa: amount of Ig (μg); ordinate: enzyme inhibition (per cent).



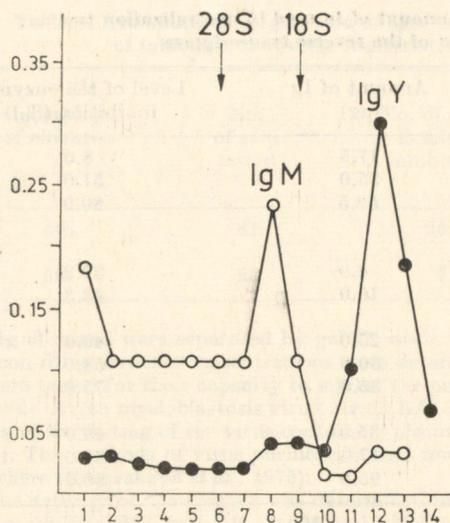


Fig. 2.

Sedimentation analysis of chicken IgM and IgY fractions after gel filtration through Sephadex G-200 were concentrated in Amicon apparatus and fractionated in 10–30% sucrose density gradient with 0.1% sodium dodecylsulphate at 18,000 rev/min for 17 hr at 20 °C. 28S and 18 S ribosomal RNAs were used as markers
Abseissa: fraction number; ordinate: optical density (260 nm)

Examinations of sera collected at a 6 month interval showed that the inhibitors appeared in 3 chickens during the observation period; previously these birds had been negative. In other cases, the observed small variations in the levels of inhibitors were in the range of normal experimental error.

The discovery of reverse transcriptase inhibitors in sera of normal leukemia-free chickens demanded to study the immunological nature of these inhibitors. A direct relationship between the amount of immunoglobulin used in the experiment and the level of inhibition produced by it was established. The results are shown in Table 3.

The specificity of Ig for reverse transcriptase of both murine and avian oncoviruses was compared (Fig. 1). The Ig of normal chickens inhibited about 60% of the enzymatic activity of avian myeloblastosis virus reverse transcriptase but did not inhibit the Rauscher murine leukemia virus reverse transcriptase, that is, the inhibitors were specific for the reverse transcriptase of avian oncoviruses.

Ig precipitated with ammonium sulphate were fractionated on Sephadex G-200 columns. The size of the resulting globulins was determined by sedimentation analysis of two peaks of the inhibiting activity obtained. The

Table 4. Properties of serum inhibitors of avian myeloblastosis virus reverse transcriptase detectable in normal specific-pathogen-free chickens

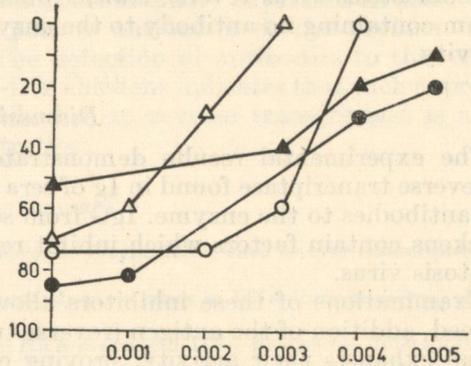
1. No dialysis against physiological saline
2. Precipitation with 50% ammonium sulphate
3. Separation on the Sephadex G-200 column into two peaks with sedimentation constants of 21 S and 8 S
4. Specific inhibition of the transcriptase of avian oncoviruses

Fig. 3.

Inhibition effect of Ig from sera of specific-pathogen-free chickens after incubation with different doses of antigen (reverse transcriptase)

35 μg of Ig and increasing doses (from 0.001 to 0.005 μg) of reverse transcriptase were mixed. The antigen-Ig contact proceeded for 1 hr at 37 °C and then for 18 hr at 5 °C; the resulting precipitate was removed by centrifugation for 10 min at 7000 rev/min, and polymerase activity of the supernatant was tested by inhibition test. The control consisted of Ig from rabbit antiserum to reverse transcriptase (●—●).

IgG from the chicken Nr 3605 (○—○)
 IgG from the chicken Nr 3663 (▲—▲)
 IgG from the chicken Nr 3517 (△—△)



results are presented on Fig. 2. The Igs were found to sediment at a rate of 21 and 8 Svedberg units, respectively, which corresponds to the sizes of chicken IgM and IgY classes (Leslie and Klem, 1969). The main properties of the discovered inhibitors are summarized in Table 4. All the presented characteristics allow the discovered inhibitors of reverse transcriptase to be classified as antibodies.

The test which gives the most direct result from our point of view was used for final elucidation of the nature of the discovered inhibitors. We proceeded from the fact that if the Ig under study are antibodies, their contact with reverse transcriptase (antigen) will result in their binding and decrease or complete elimination (depending on the adsorbing dose of the antigen of the inhibiting effect.

The Ig under study was put in contact with reverse transcriptase for 1 hr at 37 °C followed by 18 hr at 5 °C after which the precipitate formed was removed by centrifugation and the supernatant was tested in a polymerase activity inhibition test.

The possible presence in the supernatant of reverse transcriptase not bound into the antigen-antibody complex did not affect the inhibition of newly added enzyme in the specimen because of complete loss of enzymatic activity after 1 hr of incubation at 37 °C as established by preliminary experiments. Fig. 3 presents the results of tests showing a decrease of the inhibiting effect of 3 Ig under study in relation to the exhausting dose of the antigen (reverse transcriptase). The controls were Ig of rabbit antiserum to reverse transcriptase of avian myeloblastosis virus and of normal rabbit serum.

As seen in this Figure, selection of an appropriate dose of the antigen results in complete elimination of the inhibiting activity of Ig. The higher the antigen dose, the lower the residual inhibiting effect of Ig. This relationship is analogous to that observed with serum containing antibodies to avian

myeloblastosis virus reverse transcriptase. In contrast, Ig of normal rabbit serum containing no antibody to the enzyme do not inhibit DNA-polymerase activity.

Discussion

The experimental results demonstrate convincingly that the inhibitors of reverse transcriptase found in Ig of sera from normal leukemia-free chickens are antibodies to the enzyme. IgG from sera of normal specific-pathogen-free chickens contain factors which inhibit reverse transcriptase of avian myeloblastosis virus.

Examinations of these inhibitors allowed to classify them as antibodies. Indeed, addition of the antigen (reverse transcriptase of avian myeloblastosis virus) exhausts their activity, proving convincingly their capacity to bind to antigen. Moreover, the discovered inhibitors inhibited only the reverse transcriptase of avian oncoviruses, but not the enzyme of Rauscher murine leukemia virus. Another inhibitor would not have such selective properties.

Recently Bauer and Temin (1980) demonstrated rabbit, goat, rat, dog, and human immunoglobulins to possess the inhibiting activity for DNA-polymerase of reticuloendoteliosis virus and some oncogenic viruses type C of mammals. Discussing the nature of the discovered inhibitors, the authors present some prooves permitting their classification as antibodies.

Previously we had found antibodies to reverse transcriptase in sera of normal commercial chickens. We explained their occurrence by wide prevalence of leukemia-sarcoma complex viruses in birds.

It is more difficult to explain the nature of antibodies to reverse transcriptase in sera of chickens free from specific pathogens in which the conventional methods detect neither viruses of the leukemia-sarcoma complex nor antibodies to them. In this regard, two suggestions may be made. Bauer and Hofschneider (1976) found RNA dependent DNA polymerase different from the known viral reverse transcriptase in embryos of normal leukemia-free chickens. Antibodies to reverse transcriptase of avian myeloblastosis virus partially inhibited this enzyme and almost completely their own reverse transcriptase. It is possible that birds in the Dahmsdorf poultry farm also possess such RNA dependent DNA polymerase and we have found antibodies to it which partially inhibited reverse transcriptase of avian myeloblastosis virus. On the other hand, as far back as in 1973 Baluda showed that in normal chicken cells the expression of viral genome may range from the presence of gs antigen alone to complete virus production. With development of radio-immunoassay it has been demonstrated that the expression of gs antigen may be observed in the absence of detectable leukemic viruses in chicken populations. Examinations of embryonic extracts from chickens of the 4th generation in the Dahmsdorf poultry farm by the radioimmunoassay (kindly performed by E. G. Picker in the laboratory of Dr. A. D. Altshtein) showed that most of them contain endogenous gs antigen of chicken leukemia-sarcoma complex virus, that is, the expression of at least a part of viral genome has been observed in chickens from this poultry farm. If not only the "gag"

gene but also the "pol" gene becomes occasionally expressed in some of the chickens examined, these might possess antibodies to the product of this gene, the reverse transcriptase. The detection of antibodies to this enzyme in serum Ig from normal leukemia-free chickens indicates that such expression actually occurs; detection of antibodies to reverse transcriptase is a very sensitive method of their demonstration.

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