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Antibody to the RNA-Dependent DNA Polymerase of Mammalian C-Type RNA Tumor Viruses

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ABSTRACT **Sera from rats bearing transplantable tumors induced by murine C-type tumor viruses contain an inhibitor of the activity of the viral RNA-dependent DNA polymerase. The inhibitor is shown to be an immunoglobulin (IgG) directed against the enzyme. Antiserum made in rabbits against partially purified murine leukemia virus polymerase also inhibits the polymerases of other mammalian C-type RNA-containing tumor viruses. Thus, the polymerases from different mammalian tumor viruses are antigenically related.**

An RNA-dependent DNA polymerase has been described in RNA-containing tumor viruses (1, 2). Our studies showed that antiserum obtained against murine leukemia–sarcoma viruses was unable to inhibit the endogenous polymerase reaction (no added template) in intact virions (3). However, enzyme activity could be inhibited after partial disruption of the virus, indicating that the polymerase occupied a site within the virion. More recently, the use of synthetic RNA and DNA polymers as templates (4), with manganese as the divalent cation (5), has been shown to greatly enhance the sensitivity of detection of polymerase activity. With these conditions, it has been possible to study factors that stimulate or inhibit the enzymatic activity of the partially purified viral polymerase. The present studies were undertaken to characterize the inhibitor found in sera from rats with tumors. We report that the inhibitor in rat sera is an antibody to the RNA-dependent DNA polymerase activity of murine leukemia virus. The enzyme has been partially purified and antiserum has been made in rabbits. The rabbit antiserum, like the rat antiserum, inhibits the enzymatic activity in cat and hamster, as well as mouse, C-type viruses.

MATERIALS AND METHODS

Viruses and polymerase assay

The sources and the preparation of viruses have been described (6). Hamster leukemia virus was provided by Dr. R. Gilden, Flow Laboratories, Rockville, Md. The conditions of the assay and the synthetic templates used have also been described (5).

Antisera

Adult New Zealand white rabbits (2–3 kg) were immunized with partially purified Rauscher leukemia virus (MuLV) enzyme (7). An equal volume of the enzyme preparation was emulsified with complete Freund's adjuvant and injected into the footpads. 3, 5, and 7 weeks later, the animals received subcutaneous "booster" inoculations.

Sera from rats bearing either a transplantable Moloney murine sarcoma virus (MSV)-induced tumor (8) or an AKR virus-induced rat lymphoma (8) were kindly supplied by Dr. R. Wilsnak, Huntington Research Laboratory, Baltimore, Md., and Dr. R. Huebner, NIH. Both sera from individual rats and pooled rat sera, with complement-fixing activity against RNA tumor virus-associatedan tigens (9), were tested. Sera from mice that carried transplantable MSV tumors were provided by Dr. A. Gazdar, NIH, Bethesda, Md. Antiserum from rabbits immunized with repeated doses (containing about 10^{12} particles) of MuLV was obtained from Electro-Nucleonics, Inc. (Bethesda, Md.). Guinea pig serum against a purified group specific antigen (gs-1) of Rauscher leukemia virus was a gift of Dr. Gilden (10). Rabbit antiserum to Rauscher leukemia virus containing the "interspec" antibody (11) was supplied by Dr. W. Shäfer, Max-Planck-Institut fur Virusforschung, Tübingen, Germany. Rabbit antisera prepared against rat IgG and whole rat serum were purchased (Hyland Labs, Costa Mesa, Calif.; and Pentex, Inc., Kankakee, Ill.).

Immunoglobulins from control and tumored-rat sera were prepared by repeated precipitation at 4°C in 50% ammonium sulfate (v/v) in Dulbecco's phosphate-buffered saline, pH 7.0.

Antibody assays

The complement-fixation assay for detecting antigens of the murine sarcoma–leukemia complex has been described (12).

Neutralizing antibody was tested for incubation of about 100 XC-plaque-forming units (13) of MuLV with different antiserum dilutions for 30 min at 25°C. The virus-serum mixture was then inoculated onto DEAE-dextran-treated BALB/3T3 cells and assayed for inhibition of MuLV plaque formation. The neutralization titer was expressed as the reciprocal of the serum dilution that inhibited XC plaque formation by at least 67%.

Chromatography

DEAE-cellulose (Reeve Angel, Clifton, N.J.) was prepared as directed by the manufacturer. Whole serum and ammonium sulfate-precipitated serum were equilibrated by dialysis at 4°C for 18 hr with starting buffer and applied to the column (less than 50 mg of protein/g dry weight of cellulose); 3- to 5-ml fractions were collected, at a flow rate of 0.5–1 ml/min. The absorbance of the fractions was determined at 280 nm on a Gilford spectrophotometer. Fractions with peak absorbance were pooled and concentrated before testing. Rat

Abbreviations: gs, group specific; MuLV, murine leukemia virus (Rauscher strain); MSV, murine sarcoma virus (Moloney strain).

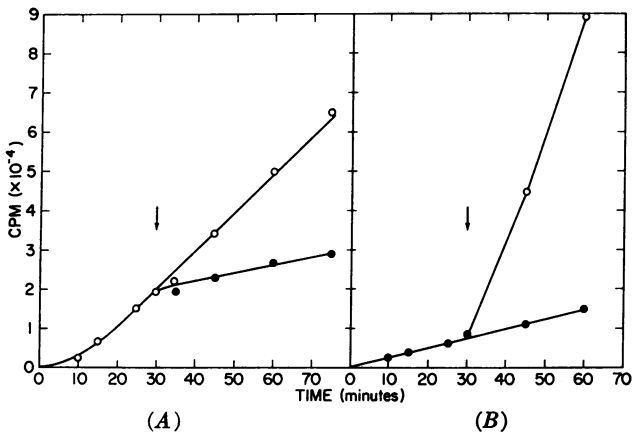

FIG. 1. Effect of antiserum and viral enzyme on the kinetics of incorporation of [^{3}H]TMP. *A*. A reaction mixture, incubated at 37°C, contained in 0.75 ml: 0.02 M Tris·HCl, pH 7.8; 2 mM dithiothreitol (DTT); 0.04 M KCl; 1×10^{-5} M [^{3}H-methyl]TTP (5000 cpm/pmol); 0.09 A_{260} poly(A·U); 1 mM $Mn(Ac)_2$; 0.1% Triton X-100; and 7.5 μg of Rauscher MuLV. At 30 min (*arrow*), the reaction mixture was split, one-half receiving 150 μg of antiserum (●) and the other half an equal volume (3 μl) of distilled water (○). 0.5-ml aliquots of the reaction mixture were assayed for Cl_3CCOOH-precipitable counts at the times indicated (5). *B*. The same as *A*, except that 250 μg of antiserum was included in the initial reaction mixture (0.55 ml). At 30 min (*arrow*), the reaction mixture was split, one-half receiving 3 μg of Rauscher MuLV (○) and the other half an equal volume (3 μl) of distilled water (●).

IgG*a*, as determined by immunoelectrophoresis, was eluted in the void volume and will be referred to as rat IgG. We did not attempt to isolate IgG*b* or other rat immunoglobulins (14).

Sephadex G-200 (Pharmacia, Inc., Uppsala, Sweden) was prepared in 0.01 M Tris·HCl, pH 8.0 on a 1×90-cm column at 4°C and the flow rate was adjusted to 10 ml/hr. The column void volume was 38 ml, as determined by a Blue Dextran 2000 marker ($> 2 \times 10^6$ daltons). 2-ml fractions were collected on an LKB fraction collector.

Immunoelectrophoresis

Immunoelectrophoresis was performed by the micromethod of Scheidegger, with 1% agarose and 0.05 M barbital buffer, pH 8.6 (15). The plates were developed for 18 hr at 25°C and then photographed.

RESULTS

Inhibition of enzyme activity by sera from rats with MSV tumors

We reported earlier that a serum pool from rats with MSV tumors, which reacted with ether-treated murine leukemia virus by complement fixation, contained an inhibitor for murine leukemia (MuLV) viral enzyme activity (3). In those studies, inhibition was seen after partial disruption of the virions caused by addition of low concentrations of detergents to expose the viral enzyme. Subsequently, the use of the synthetic RNA template, poly(A·U), and manganese, as the divalent cation, has increased the sensitivity of the polymerase assay by approximately 100-fold (5) and allowed the use of a higher concentration of Triton X-100 (0.2%) to disrupt the virions more completely. Fig. 1 shows the kinetics of incorporation of [^{3}H]TMP in response to poly(A·U) in the presence of the serum inhibitor.

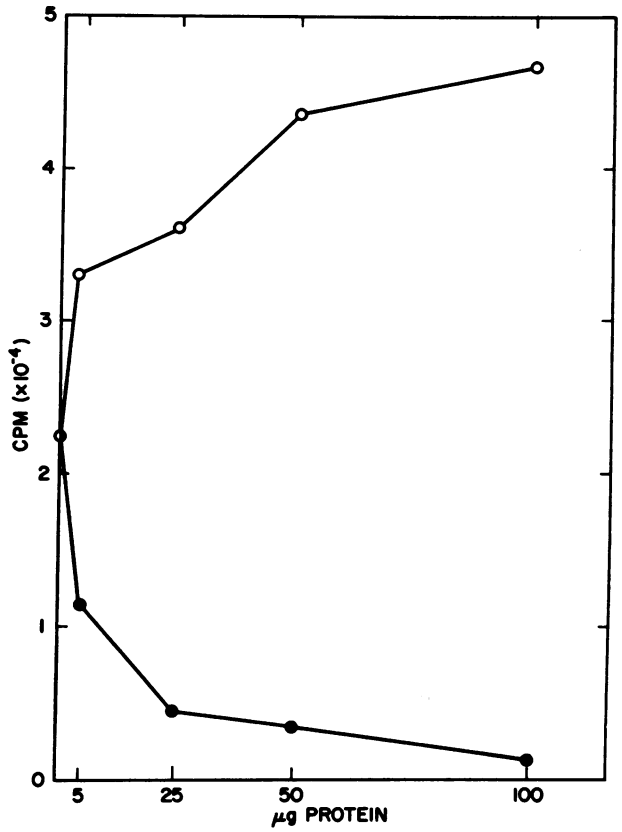

FIG. 2. Effect of increasing concentrations of normal and inhibitory rat-tumor serum on Rauscher-virus RNA-dependent DNA polymerase. Each reaction mixture, incubated at 37°C for 60 min, contained the components of Fig. 1*A* in 0.05 ml except: 0.006 A_{260} poly(A·U); 0.1 μg of Rauscher MuLV. Normal rat serum (○); serum from a rat with MSV tumor (●).

After a short lag period, synthesis increased linearly with time in the absence of inhibitor (Fig. 1*A*). The addition of antiserum at 30 min was rapidly followed by cessation of [^{3}H]-TMP incorporation. In a parallel reaction mixture, in the presence of the inhibitor from zero time (Fig. 1*B*), incorporation proceeded at a much reduced rate. However, when more viral enzyme was added during the course of the reaction, the inhibition was completely overcome.

The results of an experiment investigating the dose–response curves of a typical inhibitory and normal rat serum on the poly(A·U)-stimulated enzyme reaction of MuLV are shown in Fig. 2. Addition of increasing amounts of normal serum resulted in a stimulatory effect on the rate of the reaction. In contrast, the serum obtained from a rat with an MSV tumor inhibited the reaction, even when as little as 5 μg of protein was added. The apparent stimulatory effect of control serum was a constant finding, but the effect was partially eliminated by treating the glass tubes with silicone and by the addition of small amounts of bovine serum albumin to the reaction mixture. Previous studies have revealed a similar effect of serum on the DNA-dependent DNA polymerase activity extracted from herpes simplex-infected cells (16).

Time course of appearance of serum inhibitor in animals with tumors

The time course of appearance of serum inhibitor in rats inoculated with a transplantable MSV tumor is illustrated in Fig. 3.

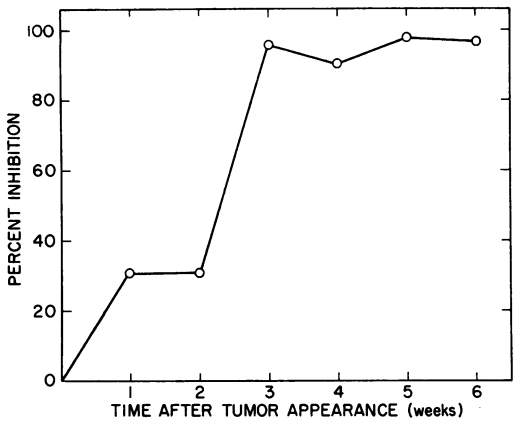

FIG. 3. Time course of appearance of serum inhibitor. Sera obtained at weekly intervals after inoculation of a Fischer rat with MSV tumor (each serum contained 25 ± 5 μg of protein) were added to the reaction mixture as in the legend to Fig. 2, except that 25 μg of bovine serum albumin was also included in each reaction mixture. The incorporation in the absence of rat serum was 50,000 cpm.

Animals were bled prior to tumor inoculation, then at weekly intervals. An equal quantity of serum (25 ± 5 μg of protein) from each bleeding of an individual animal was assayed for its inhibitory effect on poly(A·U)-stimulated MuLV enzyme activity. There was a time-dependent development of inhibitory activity. While sera obtained prior to tumor inoculation showed no inhibitory effect, sera obtained 3 weeks or more after the development of a palpable tumor in responding animals inhibited enzymatic activity by more than 90%.

Purification of the serum inhibitor

The time course of appearance of the inhibitor was consistent with that of a primary response to an antigenic stimulus; this observation suggested that the inhibitor might be an antibody. Alternatively, it might be some other specific or nonspecific inhibitor in the sera of animals with tumors. It was important, therefore, to purify the serum inhibitor. Some of its characteristics are shown in Table 1. It was stable to dialysis against 0.01 M Tris·HCl, pH 7.0, for 15 hr at 4°C. When the inhibitory serum was fractionated with ammonium sulfate, the activity was retained in the globulin fraction. On a DEAE-cellulose column, the majority of the inhibitory activity was eluted at 0.01 M potassium phosphate, pH 8.0; this is the characteristic elution pattern of rat IgG (14). The ratio of the absorbance of this fraction at 280–260 nm was 1.48; such a ratio is consistent with that of protein. The immunoelectrophoretic pattern of the fraction showed a single detectable serum protein, IgG, when reacted with anti-(whole rat) serum. The results of gel filtration of serum on Sephadex G-200 are

TABLE 1. *Characteristics of antiserum inhibitor*

1. Not dialyzable.
2. Precipitated by 50% ammonium sulfate.
3. Elutes at 0.01 M phosphate from DEAE-cellulose.
4. E_{280}/E_{260} ratio of 1.48 after DEAE-cellulose chromatography.
5. Included in Sephadex G-200; peak of inhibitory activity corresponds with IgG (see Fig. 4). MW about 160,000.

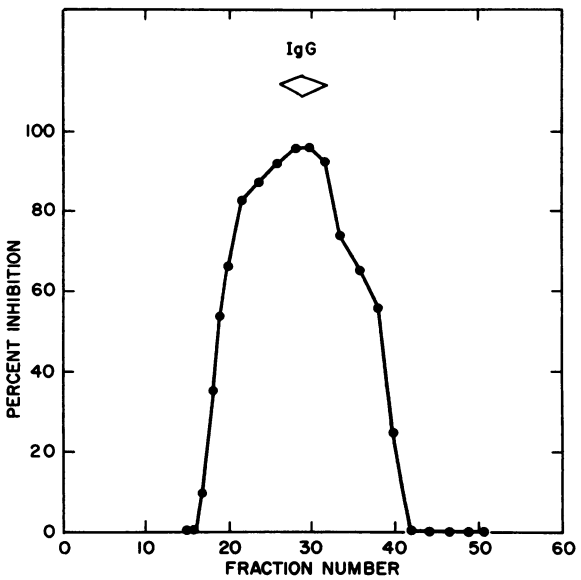

FIG. 4. Elution pattern of serum inhibitory activity from Sephadex G-200. Each fraction (0.02 ml) was assayed for its ability to inhibit the Rauscher MuLV polymerase, with poly-(A·U) as template. The reaction mixtures were as indicated in Fig. 2. The incorporation in the absence of Sephadex fractions was 30,000 cpm. The *diamond* indicates the peak tubes of IgG, as determined by immunoelectrophoresis.

illustrated in Fig. 4. The peak of inhibitory activity coincided with the peak concentration of IgG as determined by immunoelectrophoresis (fraction 28). In comparison with aldolase (156,000 daltons) as a marker, the inhibitor was estimated to be slightly larger than 156,000 daltons. From the above results, we concluded that the inhibitor in serum from rats with MSV tumors was an immunoglobulin, IgG.

Properties of the purified inhibitory immunoglobulin

The DEAE-cellulose-purified antibody was tested for activity against MuLV polymerase that had been partially purified by column chromatography on Sephadex G-200 (7). This enzyme preparation was completely separated from intact virions by detergent treatment before chromatography. The partially purified enzyme was very sensitive to inhibition by the IgG fraction; less than 10% of its activity remained after addition of 0.3 μg of protein. That the partially purified enzyme (molecular weight <100,000) was inhibited rules

TABLE 2. *Enzyme inhibition by rabbit antiserum to partially purified Rauscher MuLV polymerase*

Serum protein added (μg)	Cpm of [^{3}H]TMP Incorporated	
	Before immunization	After immunization (8th Week)
10	115,105	25,310
25	98,335	17,320
50	101,645	9,780
100	78,465	4,580

Reaction mixture as described in the legend to Fig. 2. Incorporation in the absence of serum was 51,000 cpm.

out the possibility of an indirect effect; for example, with less-purified enzyme preparations, the immunogobulin might have acted by causing agglutination of virus particles so as to remove them from the reaction. Mizutani *et al.* (17) have detected an endonuclease, and possibly a ligase, activity in Rous sarcoma virus. Which components of the MuLV polymerase system the antibody is directed against is, at present, unresolved.

An important property of an antibody is its ability to bind specifically to an antigen and for the complex to remain stable upon dilution (18). As shown in Fig. 5, when virus and antiserum were incubated together prior to dilution to a non-inhibitory antibody concentration, the inhibition increased with the time of incubation, reaching 70% by 30 min. In contrast, when the antiserum and virus were maintained separately prior to dilution into the reaction mixture, only a 10% inhibition was seen.

In other experiments, there was no inhibition when the synthetic template poly(A·U) was incubated with the IgG fraction prior to dilution. This result demonstrates that the antibody activity was directed against the viral enzyme, but not against the template. No exonuclease activity could be detected in active antibody preparations when [^{3}H]poly (A) (single-stranded RNA), or the product(s) of the poly-(A·U)-stimulated reaction were tested as substrates.

Rabbits were inoculated with partially purified MuLV polymerase prepared as described (7). Serum obtained 8 weeks after immunization clearly inhibited the viral enzyme activity. At comparable concentrations of protein, control (before immunization) serum had no effect (Table 2). In other experiments, sera obtained at the 2nd through 5th week also showed no inhibitory activity. The rabbit serum inhibitor was shown to be an IgG immunoglobulin by the methods described above.

Antiserum specificity against viral enzymes

The antiserum was tested for its ability to inhibit the RNA-dependent DNA polymerase of several different leukemia and sarcoma viruses. Viral enzyme preparations were assayed under conditions where the incorporation of [^{3}H]TMP was proportional to time and the amount of polymerase added; the amount of each enzyme was adjusted to incorporate an equal amount (pmol) of [^{3}H]TMP in the absence of antibody. With antibody obtained from rats with MSV tumors, the following viruses were inhibited to a comparable degree: murine leukemia virus (Rauscher and Moloney strains); murine sarcoma virus (Moloney and Kirsten strains); hamster leukemia virus; feline leukemia virus (Rickard strain); and feline sarcoma virus (Gardner strain). These results imply a high degree of relatedness of *mammalian* C-type viral RNA-dependent DNA polymerases. In contrast, the antibody was much less effective against two avian tumor viruses, Rous sarcoma (Schmidt-Ruppin strain) and avian myeloblastosis virus, and against the mouse mammary-tumor virus. Rabbit serum prepared against partially purified MuLV polymerase showed the same pattern of inhibition.

Inhibitory sera: lack of correlation with known antiviral antibodies

Individual sera from a total of more than 30 normal rats and mice showed no detectable antibody to the MuLV polymerase. Among animals with tumors, Fischer rats inoculated with a transplantable AKR lymphoma were particularly active, with 15 out of 15 sera showing more than 95% inhibition of

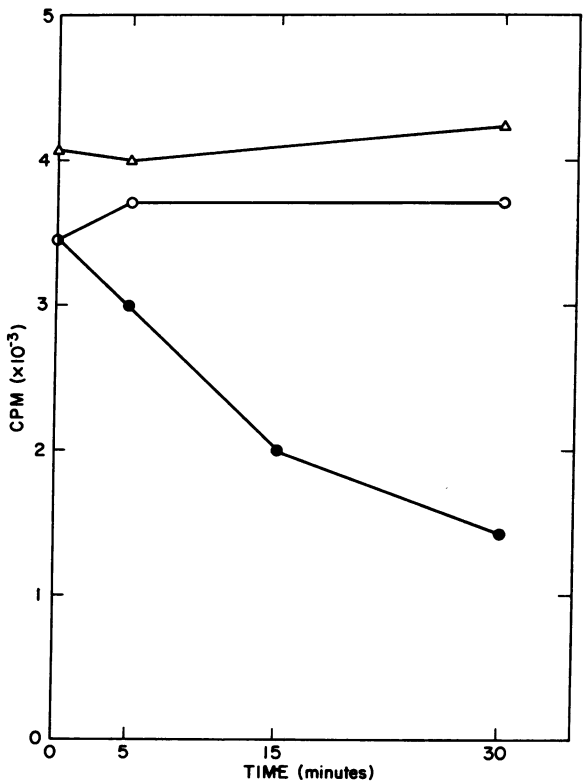

FIG. 5. Ability of antibody to specifically inhibit Rauscher leukemia viral polymerase. Three reaction mixtures, *A*, *B*, and *C*, were incubated at 25°C; each contained in 0.15 ml: 2 mM DTT; 0.1% Triton X-100; 0.4 μg of bovine serum albumin. In addition, incubation mixture *A* contained 15 μg of Rauscher MuLV; incubation mixture *B* contained 14 μg of purified immunoglobulin; incubation mixture *C* contained 15 μg of Rauscher MuLV and 14 μg of purified immunoglobulin. After different times of incubation, 5 μl of these reactions was transferred to new 0.05-ml reaction mixtures and assayed for polymerase activity as described in the legend to Fig. 2. Δ—Δ, 5μl of *A*; ●—●, 5 μl of *C*; O—O, 5 μl of *A* + 5 μl of *B*.

viral enzyme activity when 70 μg of serum protein was added to the reaction. Only 20% of a total of 20 serum samples obtained from rats of the same strain inoculated with a transplantable MSV tumor inhibited the viral enzyme. In addition, 18 sera from BALB/c and NZB mice that carried transplantable MSV tumors failed to show inhibitory activity. The absence of antibodies in the majority of these sera indicates that a murine leukemia–sarcoma virus-releasing tumor was not in itself sufficient for inducing detectable amounts of anti-polymerase activity.

The results in Table 3 show the lack of correlation between the level of enzyme inhibitory activity of selected sera and their reactivity against known viral antigens of the murine tumor viruses. For example, serum from a rat with an AKR tumor showed very low complement-fixation activity against ether-disrupted MuLV, as has been noted previously (10); yet the serum was highly inhibitory to the viral enzyme. Rabbit antibody to ether-disrupted MuLV, which had very high complement-fixation reactivity against gs antigens, had no effect on the enzyme. Guinea pig antiserum to purified murine gs-1 antigen (10), rabbit antiserum against the "interspecies" antigen (11), and serum from rats with MSV tumors

TABLE 3. *Lack of association between known Rauscher viral antibodies and anti-polymerase antibody*

Serum source	CF Titer* against ether-treated virus (gs antigen)	Neutralization titer†	Inhibition of enzyme activity (%)‡
Rat no. 1 (AKR Lymphoma)	4	<20	>95
Rat no. 2 (MSV Sarcoma)	80	>400	>95
Rat no. 3 (MSV Sarcoma)	160	>400	<10
Rabbit anti-Rauscher MuLV	160	<20	<10
Guinea pig anti-gs 1 (Rauscher MuLV)	64	<20	<10
Rabbit anti-interspec antigen (Rauscher MuLV)	80	...	<10
Rabbit anti-Rauscher MuLV polymerase	>4	<20	>90

* Expressed as the reciprocal of the serum dilution that gave a 3+ reaction with two units of antigen (CF, complement fixation).

† Expressed as the reciprocal of the serum dilution that gave over 67% reduction in XC plaque formation of 100 plaque-forming units of Rauscher MuLV.

‡ % inhibition with 70 μg of serum protein added to the reaction mixture, which was otherwise identical to that of Fig. 2.

that reacts with MSV-associated antigens (9) all failed to show anti-polymerase activity. There was also no apparent correlation between the titer of neutralizing antibody of a given serum and the level of anti-polymerase activity (Table 3).

DISCUSSION

In the present studies, sera from rats with murine leukemia–sarcoma virus-induced tumors have been shown to contain an inhibitor to the viral RNA-dependent DNA polymerase. This inhibitor is an antibody, as defined by its time course of appearance in sera from animals with tumors and by its physicochemical properties, as revealed by chromatography and gel filtration. From the ability of purified IgG molecules to bind to the polymerase and not to the template, we conclude that the antibody is directed against the viral enzyme.

Previous studies suggested that the polymerase is located within the virion (3). Therefore, it was possible that the enzyme represented a previously described group-specific (gs) antigen for the murine viruses. The present findings, however, suggest that this is not the case. There was no correlation between the gs antibody titer of a given serum and the activity of that serum against the viral polymerase. Antibody to purified gs-1 antigen [the major murine gs antigen (10)] had no effect on viral polymerase activity. This result is in agreement with Hatanaka *et al.* (19) who, using unpurified viral enzyme, concluded that anti-(gs-1) antibody did not inhibit polymerase activity. Since the known gs antigens comprise the great majority of the total viral protein (10, 20, 21), the polymerase is probably a minor component of the virion. More direct evidence of this point comes from enzyme purification, which indicates that the polymerase constitutes less than 4% of the total viral protein (manuscript in preparation and P. Duesberg, personal communication).

The finding that antibody against the murine viral enzyme was active against the RNA-dependent DNA polymerases of other mammalian C-type viruses indicates that these viral enzymes are closely related. After the report by Geering *et al.* (22) that mammalian leukemia viruses share a common antigen, termed "gs-3", others noted similar reactivities (9, 10). The inability of sera containing a high titer of anti-"gs-3" activity to inhibit the viral enzyme suggests that the enzyme represents another internal antigen common to mammalian C-type viruses.

The present studies show that antiserum to the viral DNA polymerase can be produced by immunization of rabbits with partially purified enzymes. The antibody can also be found in the sera of animals that bear tumors induced by C-type viruses. Such antibodies might be useful in distinguishing the mammalian viral enzymes from the recently discovered RNA-dependent DNA polymerase found in normal (7) and tumor cells (23).

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