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Serological Analysis of the Deoxyribonucleic Acid Polymerase of Avian Oncornaviruses

I. Preparation and Characterization of Monospecific Antiserum with Purified Deoxyribonucleic Acid Polymerase

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Monospecific antiserum was prepared against purified deoxyribonucleic acid (DNA) polymerase from avian myeloblastosis virus (AMV). Immunodiffusion assay with purified DNA polymerase revealed that the anti-DNA polymerase serum formed one precipitation band, whereas no reaction with any of the seven major structural proteins of AMV was observed. The antiserum also demonstrated enzyme-neutralizing antibody activity that was associated with the immunoglobulin G fraction. There was no difference in the neutralization of DNA polymerase activity directed by ribonucleic acid (RNA), DNA, or RNA-DNA hybrid templates.

The ribonucleic acid (RNA) tumor or oncornaviruses (10) have been demonstrated to contain RNA-instructed deoxyribonucleic acid (DNA) polymerase activity (1, 4–6, 12–14). Serological relationships of avian C-type viral DNA polymerases "reverse transcriptase" have been described by Parks et al. (11) by using partially purified enzyme from the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) for immunization and production of antisera. These authors demonstrated that the polymerase preparations of avian oncornaviruses contained a common antigen that appeared to be group-specific (gs) since it was not found in similar preparations from mammalian oncornaviruses. Furthermore, antigens found in the polymerase appeared to be serologically distinct from the known viral gs antigens. However, limitations exist in such a serological analysis, as it is not possible with a polyvalent antiserum to distinguish adequately reactions directed against the polymerase or to other closely associated viral structural gs proteins.

Recently, Kacian et al. (7) reported the purification of the RNA-instructed DNA polymerase activity from avian myeloblastosis virus (AMV). This purified enzyme preparation was shown to contain two protein units in equimolar amounts, one of 110,000 and the other of 69,000 molecular weight. Throughout the purification procedure, the responses to homologous AMV 70S RNA, double-stranded DNA, and the synthetic homopolymer duplexes seemed to be mediated by a single protein entity. The availability of purified enzyme preparations has enabled us to prepare a monospecific precipitating antiserum against the DNA polymerase of AMV.

In this report we describe the preparation of an antiserum against highly purified DNA polymerase of AMV, with an analysis of its precipitating and neutralizing properties against the purified enzyme.

MATERIALS AND METHODS

AMV, BAI strain A, was obtained from J. W. Beard, Duke University, Durham, N.C., and purified by a method previously described (2, 7). Unlabeled deoxynucleoside triphosphates and dithiothreitol were products of P-L Biochemicals. Polydeoxythymidylate-polyriboadenylate (poly dT·poly rA) was supplied by Miles Laboratories. Tritiated thymidine triphosphate and tritiated poly rA were obtained from Schwarz-Mann. Nonidet P-40 detergent was a gift of Shell Chemical Co. Bovine serum albumin (BSA) and rabbit antisera against rat immunoglobulin G (IgG) were purchased from Miles Laboratories. "Activated" myeloblast DNA, prepared by mild deoxyribonuclease treatment, was kindly supplied by A. Kiessling, Sloan-Kettering Institute for Cancer Research, New York, N.Y. AMV 70S RNA was prepared as described previously (7). Isolated AMV proteins were kindly donated by E. Fleissner, Sloan-Kettering Institute. The proteins were separated by gel filtration from virus solubilized in 6 M guanidine hydrochloride and reducing agents (3, 9).

Purification of DNA polymerase. The polymerase

from AMV was purified as described by Kacian et al. (7); approximately 400 mg of purified, viral protein from chick plasma was used for the preparation. After the carboxymethyl-Sephadex chromatographic step, the enzyme activity was concentrated by ammonium sulfate precipitation (50% saturation) and fractionated further by glycerol gradient centrifugation. Figure 1 shows a sodium dodecyl sulfate-polyacrylamide gel of the enzyme after velocity sedimentation in a glycerol gradient. As reported previously, the enzyme contains two protein subunits in equimolar amounts and responds to homologous AMV viral RNA, double-stranded DNA, and synthetic homopolymer duplexes (7). The polymerase preparation shown in Fig. 1 was used for production of antisera and for the neutralization studies.

Standard polymerase assay. Reaction mixtures (100 μliters) for homopolymer-templated reactions contained the following in micromoles: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.2 at 25 C), 5.0; potassium chloride, 10.0; magnesium chloride, 0.8; dithiothreitol, 0.04; deoxyadenosine 5′-triphosphate, 0.02; radioactive thymidine 5′-triphosphate, 0.01; synthetic poly dT·poly rA template, 1.2×10^3 pmoles of polymer phosphate per strand; and purified AMV-DNA polymerase, 0.01 μg.

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified DNA polymerase. A sample (18 μg) of purified AMV DNA polymerase used for these immunological studies was characterized as described previously* (7).

After incubation at 37 C for a specified time, the reactions were terminated at 0 C with the addition of cold 5% trichloroacetic acid. The acid-precipitable radioactivity was collected on nitrocellulose filters and counted in 0.4% 2,5-bis-2-(5-*tert*-butylbenzoaxozolyl) thiophene in toluene.

Assays using AMV 70*S* viral RNA and activated myeloblast DNA were prepared identically except that 0.02 μmole each of deoxycytidine 5′-triphosphate and deoxyguanosine 5′-triphosphate were included. Templates were added at concentrations of 1 to 2 μg per 100-μliter assay volume.

The specific activity of ^{3}H-labeled thymidine triphosphate was 300 to 400 counts per min per pmole.

Preparation of antisera. Antiserum against purified enzyme or viral proteins was prepared in (W/Fu × BN)F_1 hybrid rats. The initial immunizing material was antigen (0.3 mg of protein) emulsified with an equal volume of complete Freund's adjuvant and injected in multiple subcutaneous sites. Incomplete Freund's adjuvant was used for the subsequent inoculations (each containing 0.3 mg of protein); these were given at triweekly intervals, subcutaneously at multiple sites. Precipitating antisera against the AMV polymerase were obtained after the fifth immunization; precipitating antisera against the major viral structural proteins were obtained after the third immunization. The normal rat serum used as a control in the neutralization assays was obtained from (W/Fu × BN)F_1 hybrid rats.

For enzyme neutralization assays, rat sera were fractionated by chromatography on Sephadex G-200 in 0.1 M Tris-hydrochloride, *p*H 8.0, buffer. Figure 2 shows a typical chromatographic separation; 1.5 to 2.0 ml of serum was applied to a column (2.5 by 90 cm), and 2-ml fractions were collected at a rate of 6 ml per hr. Rat gamma globulins were identified serologically by immunodiffusion with rabbit anti-rat IgG antiserum. The relevant fractions were then concentrated by ammonium sulfate precipitation (50% saturation) and dialyzed against 0.1 M Tris-hydrochloride, *p*H 8.0. The protein concentration of IgG fractions was measured by the method of Lowry et al. (8).

Neutralization assay. In a volume of 60 μliters, 0.01 μg of purified AMV DNA polymerase was mixed with serum protein (50–150 μg) or purified IgG fraction (3–50 μg); the reaction mixture also contained 0.01 M Tris-hydrochloride (*p*H 8.2), 0.15 M potassium chloride, 20% (v/v) glycerol, and 50 μg of BSA. After 15 min at 37 C, standard polymerase assay conditions were established as described above. The acid-precipitable radioactivity was taken as a measure of the DNA polymerase activity not neutralized by added serum or IgG fraction.

Double-diffusion (Ouchterlony) tests were performed on slides in 2% Noble agar (Immunoplate, pattern C; Hyland Laboratories). The slides were left at room temperature in a humidified chamber. Optimal precipitation occurred within 24 hr.

Nuclease assay. Nucleolytic activities in sera and

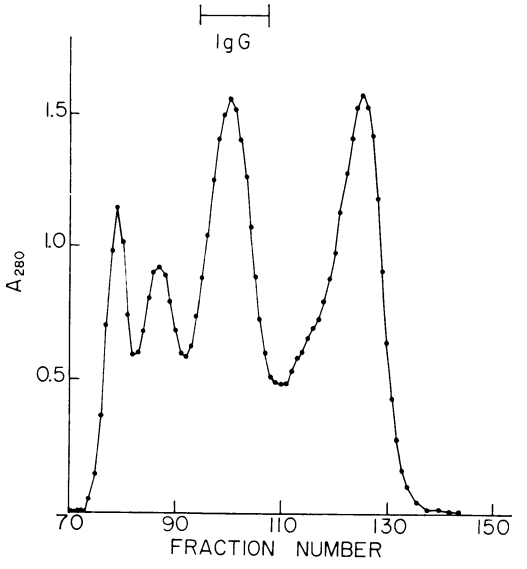

FIG. 2. *Gel filtration chromatography of rat anti-AMV DNA polymerase serum. A 1.5-ml amount of rat antiserum was applied to a Sephadex G-200 column (2.5 by 90 cm) and chromatographed at 6 ml/hr with 0.1 M Tris-hydrochloride (pH 8) elution buffer. IgG was identified serologically.*

IgG fractions were measured by the solubilization of acid-precipitable, radioactively labeled nucleic acids. ^{3}H-AMV RNA, ^{3}H-*Escherichia coli* DNA, and ^{3}H-poly rA duplex with oligo-dT were used as substrates. A 150-μg amount of serum protein or 50 μg of purified IgG was incubated for 60 min at 37 C with each labeled nucleic acid under standard polymerase assay conditions (minus deoxynucleoside triphosphates and DNA polymerase). Samples (50 μliters) of the incubation mixture were removed at 0 and 60 min and treated with 5% trichloroacetic acid, and the acid-precipitable radioactivity was determined.

RESULTS

Immunodiffusion studies with antiserum prepared against AMV DNA polymerase. Rat anti-AMV polymerase serum reacted in immunodiffusion with concentrated preparations (600 μg per ml) of purified viral DNA polymerase (Fig. 3). One major precipitation band was generally observed, although occasionally a second more rapidly diffusing minor component was also detected. As would be expected for such a large molecule, diffusion of the polymerase into 2% agar was slow, and the precipitin band often formed an arc about the antigen well.

The major structural proteins of AMV were isolated by chromatography in 6 M guanidine hydrochloride (Fig. 4) and tested by immunodiffusion with rat anti-AMV DNA polymerase serum. In all tests, these proteins did not react with the polymerase antiserum; these same proteins, however, were precipitated by their homologous antisera (*unpublished data*). Figure 3 illustrates tests with two of these isolated proteins.

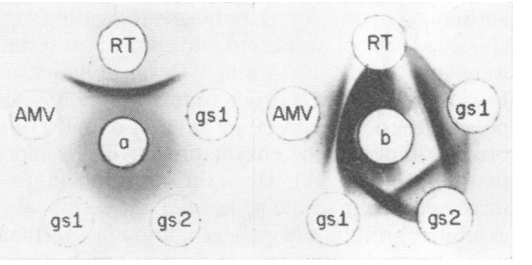

FIG. 3. *Immunodiffusion tests with rat anti-AMV DNA polymerase antiserum. Central well a, rat anti-AMV DNA polymerase antiserum; central well b, rabbit anti-AMV serum. The peripheral wells of both patterns are derived from the same antigen pools. Abbreviations: AMV, ether-treated; RT, viral reverse-transcriptase enzyme; gs1 and gs2 refer to group-specific antigens of AMV isolated by gel filtration in guanidine hydrochloride.*

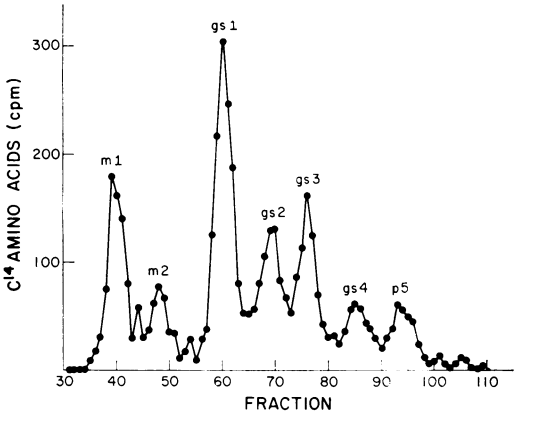

FIG. 4. *Chromatographic separation of AMV proteins by gel filtration in 6 M guanidine hydrochloride and reducing agents. A small sample of MC29 virus, radiolabeled with ^{14}C-amino acid mixture, was included with the AMV in order to identify the protein peaks. Seven distinct viral proteins are observed. Two of these polypeptides (m1 and m2) were glycoproteins and identified immunologically as viral membrane antigens. Four of the viral proteins (gs1, gs2, gs3, and gs4) are serologically distinct group-specific antigens, localized in the interior of the virus. The seventh and smallest protein, p5, has yet to be serologically defined. For details see references 3 and 9.*

Polyvalent rabbit antisera prepared against ether-degraded AMV and hamster sera from animals bearing transplants of RSV-induced sarcomas (CoFAL reference antisera) precipitated the major structural viral antigens, but did not react with preparations of purified enzyme.

Neutralization of DNA polymerase activity by

antisera. To test for enzyme neutralization, increasing levels of antiserum and control rat serum were incubated with purified DNA polymerase. Enzyme inhibition was determined by the loss of polymerase activity. A 50-μg amount of antiserum protein inhibited the enzymatic activity by more than 90% (Fig. 5A). However, as a function of increased serum protein, normal rat serum also exhibited an inhibitory effect, although less than that observed with the specific antiserum. These results suggest the presence of both a specific antibody against the enzyme and a nonimmunological inhibitor of DNA polymerase activity.

To eliminate this inhibitor, all sera were chromatographed on Sephadex G-200, and the IgG fraction was isolated. Figure 5B shows enzyme neutralization assays with isolated IgG of normal rat serum tested against the AMV DNA polymerase. After this purification, no inhibition of the polymerase activity was observed in the presence of up to 50 μg of normal rat IgG.

To examine the nature of the nonspecific inhibitory factor, normal rat serum, antipolymerase serum, and their corresponding IgG fractions were tested for their effect on components of the DNA polymerase assay. Since nucleolytic activity could account for the observed inhibition, assays detecting degradation of nucleic acid templates and DNA product were performed. Incubation of normal rat serum or antipolymerase serum with radioactively labeled AMV 70S RNA resulted in a 40% loss of acid-precipitable radioactivity; however, nuclease tests of serum with labeled *E. coli* DNA or with the hybrid template-labeled poly rA annealed with oligo-dT did not result in a loss of acid-precipitable template. Thus, whole rat serum had ribonuclease activity, but no appreciable nuclease activity for DNA or hybrid nucleic acids. Similar tests with isolated IgG from normal rat serum and rat antisera were consistently negative for ribonuclease activity, indicating the effectiveness of the isolation procedure.

Conditions for neutralization of DNA polymerase activity. Addition of purified rat anti-AMV DNA polymerase IgG fraction to a standard polymerase assay (at zero time) resulted in no significant neutralization of DNA polymerase activity (Fig. 6). This observation suggested that a preincubation of antibody with antigen may

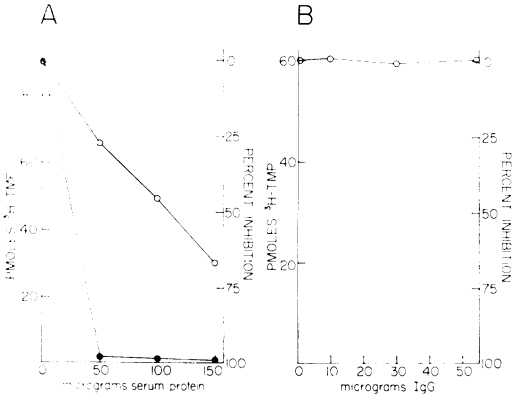

FIG. 5. *Enzyme neutralization assays with whole serum. A, Standard neutralization assay mixtures were prepared containing 0.01 μg of purified DNA polymerase and increasing amounts of rat anti-AMV DNA polymerase serum (closed circles) or normal rat serum (open circles). Preincubation of the serum and enzyme was for 15 min at 37 C, and the polymerase activity assay was performed at 37 C for 60 min. B, Standard neutralization assays were prepared as described above with 0.01 μg of purified DNA polymerase and increasing amounts of normal rat serum IgG fraction.*

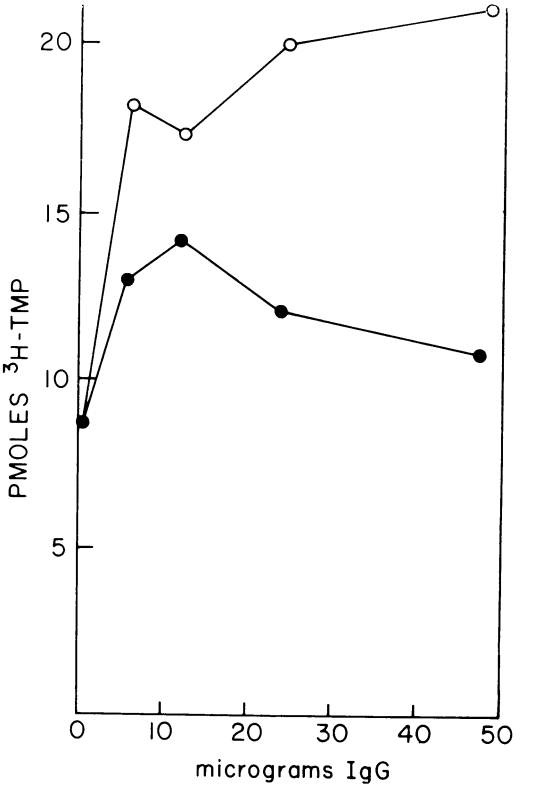

FIG. 6. *Effect of anti-AMV DNA polymerase IgG and normal rat IgG on DNA polymerase activity without preincubation. Standard polymerase assay mixtures were prepared containing 0.01 μg of purified DNA polymerase and increasing concentrations of normal rat serum IgG (open circles) or anti-AMV DNA polymerase IgG (closed circles). The reaction mixtures were incubated 40 min at 37 C and terminated as described in Materials and Methods. Bovine serum albumin and 20% (v/v) glycerol were not present in the incubation mixtures.*

be required for neutralization to occur. If such were the case, it would be necessary to monitor the stability of the enzyme during the preincubation and to determine the time of preincubation for optimal neutralization.

Enzyme stability. Incubation of the purified viral polymerase at 37 C in the absence of the components required for DNA synthesis resulted in rapid loss of activity. A search for conditions that would ameliorate this loss was therefore instituted. It had been noted previously (7) that glycerol and BSA conserved enzyme activity on storage. BSA proved to be more effective, and a detailed study was made of its inclusion during the preincubation period on the subsequent activity. Increasing amounts of BSA were mixed with purified polymerase, and after 15 min at 37 C standard polymerase assays were performed (Fig. 7). Optimal response to BSA was achieved at levels of 50 to 100 μg of BSA per reaction mixture. The addition of BSA to the incubation mixture also had the advantage of eliminating the stimulation of DNA polymerase activity by low levels of normal serum or IgG (11).

Preincubation time. Having established the conditions for stabilization of the enzyme in the absence of DNA synthesis conditions, the preincubation time necessary for neutralization of DNA polymerase by the anti-AMV DNA polymerase IgG was then determined. Two fivefold standard neutralization assay mixtures (300 μliters) were prepared containing purified DNA polymerase and anti-AMV DNA polymerase IgG or normal rat IgG. At 0, 15, 30, 60, and 90 min, 50-μliter samples were removed and added to standard polymerase assay mixtures for determination of percent neutralization. After 15 min of preincubation, more than 80% of the enzyme activity was neutralized (Fig. 8). Extending the preincubation step to longer times resulted in only a 10% increase in percent neutralization.

Titration of enzyme and antibody. To determine the optimal levels of antibody and enzyme required for enzyme neutralization, each component was tested by titration against a constant amount of the other. Poly dT·poly rA was used in these studies because of its superior effectiveness as a template. Figure 9 shows the incorporation of radioactive substrate into polymerized

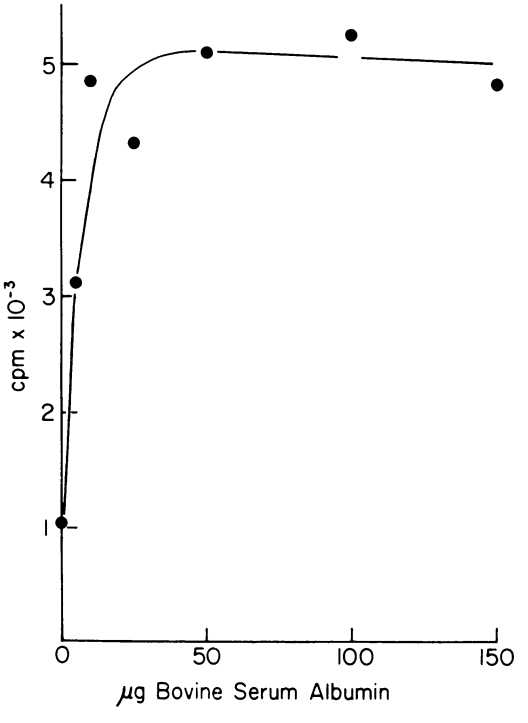

FIG. 7. *Stabilizing effect of bovine serum albumin on AMV DNA polymerase activity during preincubation. Standard neutralization assay mixtures were prepared containing 0.01 μg of purified DNA polymerase with increasing amounts of bovine serum albumin (50 mg per ml in 0.01 M Tris-hydrochloride, pH 8.2). After 15 min at 37 C, standard polymerase assay conditions were established. Upon incubation at 37 C, 20-μliter samples of the reaction mixture were removed at 0, 15, 30, 60, and 90 min. The activity described in the figure represents the 60-min sample.*

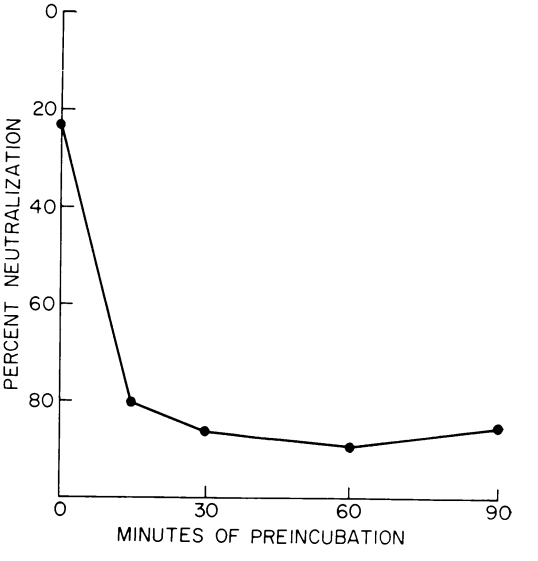

FIG. 8. *Effect of time of preincubation on neutralization of DNA polymerase activity. Two fivefold standard neutralization assay mixtures (300 μliters) were prepared containing 0.05 μg of purified DNA polymerase and 36 μg of anti-AMV DNA polymerase IgG or normal rat serum IgG. Samples (50 μliters) of reaction mixture were removed after 0, 15, 30, 60, and 90 min at 37 C and placed in standard polymerase assay mixtures. After an additional incubation of 37 C for 30 min, the reactions were terminated.*

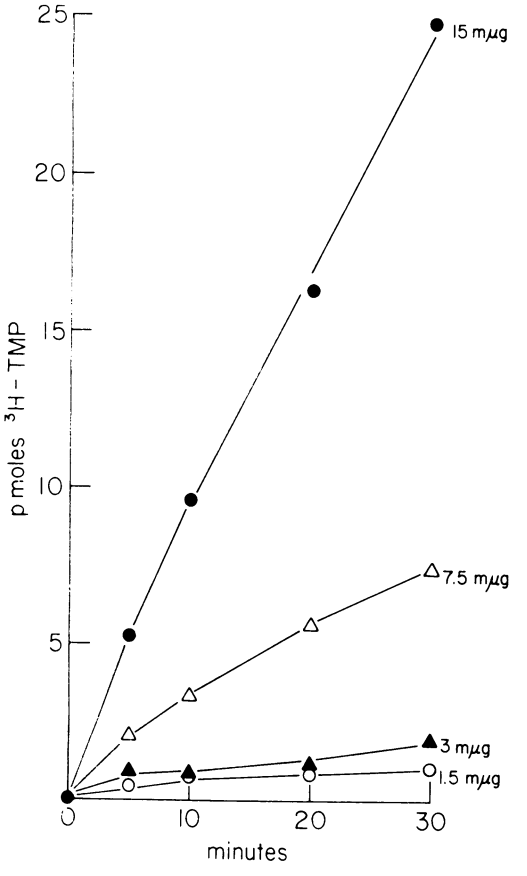

FIG. 9. *Titration of purified DNA polymerase in the standard polymerase assay. Standard polymerase assays were prepared with increasing concentrations of purified DNA polymerase present. Samples of 20 μliters were removed at the designated times, and the acid-precipitable radioactivity was determined. The activity in picomoles represents a 20-μliter sample of the 100-μliter reaction mixture.*

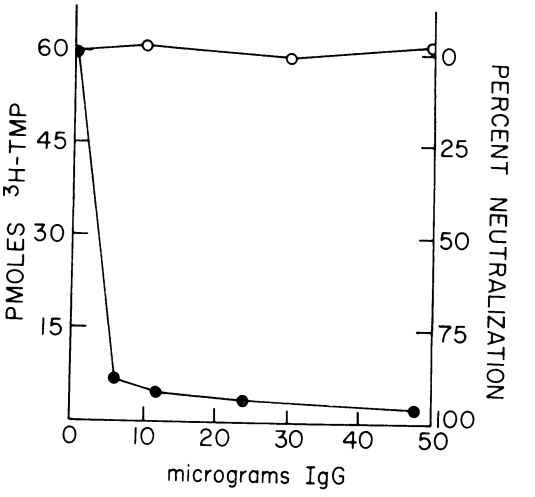

FIG. 10. *Titration of anti-AMV DNA polymerase IgG in the standard neutralization assay. Standard neutralization assay mixtures were prepared containing 0.01 μg of purified DNA polymerase protein and increasing concentrations of anti-AMV DNA polymerase IgG (closed circles) or normal rat serum IgG (open circles). After 15 min at 37 C, polymerase assay conditions were established, and 60 min later the reactions were terminated. Acid-precipitable radioactivity represents the DNA polymerase activity not neutralized by IgG protein.*

product with increased DNA polymerase and time. Less than 7.5 ng of enzyme protein per standard reaction mixture was detectable within the limits of the assay. Employing 10 ng of enzyme protein in neutralization assays with increasing amounts of anti-AMV DNA polymerase IgG, it could be shown that 90% of the polymerase activity was neutralized with 6 μg of IgG protein, whereas normal rat IgG had no detectable neutralizing activity (Fig. 10).

To illustrate further the effect of antigen concentration on the neutralizing ability of the antibody, a series of assay mixtures containing increasing amounts of polymerase protein were incubated with 6 μg of anti-AMV DNA polymerase IgG fraction (Fig. 11). Although the antiserum neutralized lower concentrations of enzyme, it was rendered ineffective by a concentration of 250 ng of polymerase protein.

Neutralization of RNA- and DNA-instructed DNA polymerase activity. IgG from antiserum prepared against the AMV polymerase was tested for neutralization of enzyme activity on three classes of templates—RNA, DNA, and hybrid nucleic acids. The antibody neutralizes to a comparable extent the activity of AMV DNA polymerase directed by AMV 70*S* RNA, "activated" myeloblast cell DNA, and poly dT·poly rA (Fig. 12).

DISCUSSION

Immunization of rats with the highly purified DNA polymerase of AMV resulted in the formation of monospecific antisera having both precipitating and neutralizing activity. By immunodiffusion, these antisera revealed no serological relationship between the viral DNA polymerase and any of the seven major structural proteins of AMV. In some immunodiffusion tests, a second minor precipitin arc was observed in the reaction when antipolymerase serum was tested, with purified enzyme that had been stored for prolonged time. This component migrated more rapidly in agar than the intact enzyme and likely

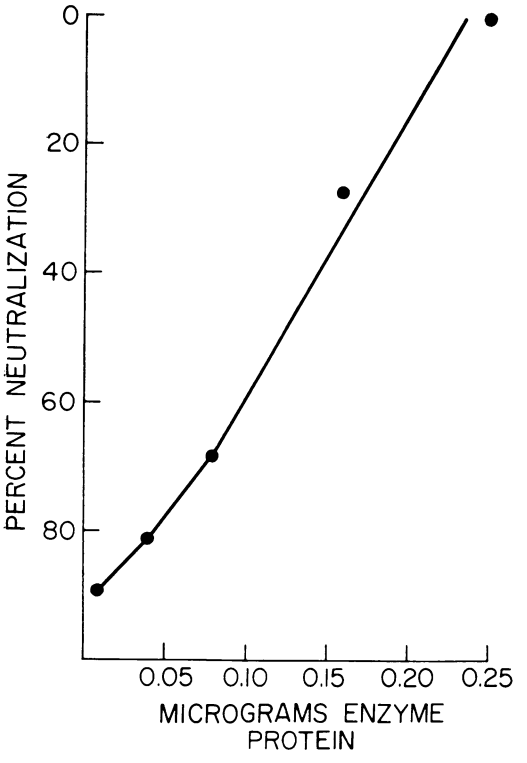

FIG. 11. *Titration of DNA polymerase in the enzyme neutralization assay. Neutralization assay mixtures were prepared containing 6 μg of anti-AMV DNA polymerase IgG or normal rat serum IgG and increasing amounts of purified DNA polymerase. After 15 min at 37 C, the polymerase assay conditions were established, and the reaction mixtures were incubated 60 min at 37 C.*

represented one of the enzyme subunits. Precipitation was not observed when the antisera were tested against ether-treated AMV, but this would be expected, as the polymerase represents only 0.3 to 1.0% of the total viral protein.

Neutralization of DNA polymerase activity by the anti-AMV DNA polymerase antisera was observed. However, normal rat serum also exhibited some inhibitory effect. Since nucleolytic activity could account for the observed inhibition, assays for detecting degradation of ^{3}H-AMV RNA, ^{3}H-poly rA·oligo-dT (template), and ^{3}H-DNA (product) were performed. The results indicated the presence of ribonuclease activity, but no degradation of ^{3}H-poly rA·oligo-dT or ^{3}H-DNA was detected. Although it is not clear at this point what the nature of the inhibitory factor is, these results do not support nucleolytic activity as an explanation for the observations in Fig. 5. It is possible that the inhibitory effect may be directed to the DNA polymerase itself, with a proteolytic activity as a possible explanation.

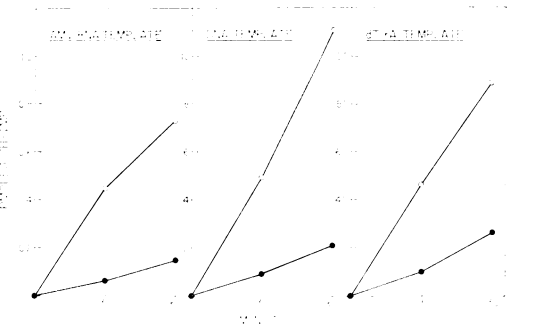

FIG. 12. *Effect of anti-AMV DNA polymerase IgG on DNA polymerase reactions instructed by various templates. Neutralization assays were performed containing 3 μg of anti-AMV DNA polymerase IgG (closed circles) or 3 μg of normal rat serum IgG (open circles) and 0.01 μg of purified DNA polymerase. After the 15-min preincubation period at 37 C, polymerase assay conditions were established; 50-μliter samples were removed at the designated times. AMV 70S RNA (2 μg), activated myeloblast DNA (1.2 μg), and poly dT·poly rA (0.4 μg) were the nucleic acid templates.*

Support for the nonimmunological nature of the inhibitory factor in normal rat serum was provided upon purification of the IgG fraction. No inhibition of DNA polymerase activity was observed in the presence of increasing concentrations of normal rat serum IgG, whereas potent neutralizing antibody activity was found with IgG from the anti-AMV DNA polymerase serum.

To demonstrate neutralization of enzyme activity by anti-AMV DNA polymerase antibody, a preincubation of antigen and antibody was necessary. BSA was shown to stabilize the enzyme activity during the preincubation step. It was also observed that by including 50 μg of BSA in the neutralization assay mixtures, enhancement of the DNA polymerase activity in the presence of low levels of normal IgG was no longer evident.

Anti-AMV DNA polymerase was equally effective in neutralizing DNA polymerase activity directed by RNA, DNA, or RNA-DNA hybrids as templates. However, as is evident from the activities expressed in Fig. 11, the template efficiency of the natural RNA was approximately 100 times less, making detection of neutralization of RNA-directed DNA polymerase activity much more difficult under the previously established assay conditions. Improving the RNA-directed polymerase activity would require an increase in DNA polymerase that would concomitantly require additional antibody for maintaining the above level of neutralization.

Having demonstrated the preparation of a monospecific precipitating antiserum against

AMV DNA polymerase and established neutralization assay conditions, it is now possible to extend these studies to include a serological comparison of the AMV DNA polymerase to DNA polymerases from other avian and mammalian tumor viruses.

ACKNOWLEDGMENTS

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