Serological Analysis of Human Deoxypribonucleic Acid Polymerases

PREPARATION AND PROPERTIES OF ANTISERUM TO DEOXYRIBONUCLEIC ACID POLYMERASE I FROM HUMAN LYMPHOID CELLS

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SUMMARY

The preparation and properties of an antiserum to human DNA polymerase I (6 to 8 S) are described. Care was taken in the purification of the antigen to remove certain other DNA polymerases found in human cells. An incubation of antigen and antiserum lasting about 48 hours is necessary to achieve maximal inhibition. About 1 µg of the antipolymerase immunoglobulin G, prepared in rats, neutralizes 60% of the activity present in 54 ng of the enzyme. Titrations varying both antiserum and enzyme demonstrate clear regions of antigen and antibody excess. Inhibition of enzyme activity is about the same whether the template-primer is (dA)$_{18}$ -(dT)$_{18}$, (dC)$_{18}$ -(dG)$_{18}$ or partially digested DNA. An assay was developed which measures the remaining activity in the supernatant after precipitation of enzyme-antibody complexes with goat anti-rat immunoglobulin G. In this assay, 2.2 µg of the antipolymerase immunoglobulin G quantitatively bind 33 ng of DNA polymerase I. With use of the direct neutralization assay and the immunoprecipitation test, we found little, if any, antigenic relationship between DNA polymerase I and DNA polymerase II (3.4 S). Similarly, little, if any, relationship was found to the DNA polymerases from five RNA tumor viruses. The activities of RNA-directed DNA polymerases from the blood leukocytes of two patients with acute myelogenous leukemia and from the placentas of rhesus monkeys were not inhibited in neutralization assays which were shortened because these enzymes are thermolabile. In identically neutralized assays, the antipolymerase immunoglobulin G neutralized up to 76% of the activity of DNA polymerase I. In addition to its utility in distinguishing cellular DNA polymerases, the rat antiserum should be a useful reagent for testing of novel DNA polymerases isolated in small quantities from human tumors for contamination with DNA polymerase I. This enzyme is present in abundance in proliferating tissue and often confuses the biochemical characterization of these novel enzymes.

Two major DNA polymerases have been isolated, partially or completely purified, and characterized from proliferating mammalian cells (1–7). DNA synthesizing activities also have been isolated from mammalian cell cytoplasm (8–10) and mitochondria (11–14) which, although less adequately characterized, are probably distinct from the two major polymerases. Another DNA polymerase, which is biochemically (15, 17) and immunologically (16, 17) closely related to the RNA-directed DNA polymerases from type C RNA tumor viruses derived from primate tumors, has been isolated from human leukemic blood leukocytes. As part of an effort to distinguish or define relationships among each of these enzymes, we are preparing antisera to highly purified, biochemically distinguishable DNA polymerases from human cells. This approach is especially helpful in the absence of complete purification of these enzymes, which is often difficult because available quantities of tissue are small and some of the enzymes are labile. Serological analysis has been a useful tool in the characterization of DNA polymerases from Escherichia coli (18, 19) and from RNA tumor viruses (20–24). In this paper, we describe the preparation, characteristics, and specificity of an antiserum to DNA polymerase I from human lymphoid cells. The purified antiserum inhibits other cellular DNA polymerases and DNA polymerases from a variety of RNA tumor viruses only at concentrations at least 10 times higher than those required to inhibit DNA polymerase I.

EXPERIMENTAL PROCEDURE

Cell line RPMI 1788 (27, 28) was used for purification of DNA polymerase I to be used as antigen. This lymphoblastoid cell...
line was established in 1968 from the blood cells of a normal male donor, has a minimum doubling time of 20 to 24 hours, an aneuploid karyotype, and histocompatibility antigen specificities HL-A2, HL-A6, and HL-A7 (these markers are also present on the blood lymphocytes of the donor). These cells, supplied by Associated Biomedic Systems, Inc., Buffalo, N. Y., were grown in suspension flasks in RPMI 1640 medium containing 10% fetal calf serum and were harvested in exponential growth phase. The cells were washed and frozen at -70° prior to homogenization. Most of the cells used were tested for contamination by mycoplasma species by culture both in broth and on plate (39) and found to be free of these organisms. Physiohemagglutinin-stimulated normal human blood lymphocytes were obtained as previously described (30).

Other Materials

Unlabeled deoxynucleoside triphosphates were obtained from Sigma; labeled substrates were from Amersham-Searle, Schwarz-Mann, or New England Nuclear. (dA) 12, (dT) 12-18, and (dC) 12 (dG) 5 were purchased from P-L Biochemicals; (A) 12 or (dT) 12-18 was a product of P-L Biochemicals or Collaborative Research. Native salmon sperm DNA, obtained from Calbiochem, was supplied by the John L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, N. J. Gibbon apo lymphosarcoma was supplied by the University of Miami. Murine leukemia virus (Rauscher) was grown in NC-37 cells, a human lymphoblastoid line established in 1966 from the blood lymphocytes of a normal male. The sources of simian sarcoma virus (SSV-1) and Mason-Pfizer virus have been described (31).

Viruses

Avian myeloblastosis virus was kindly supplied by J. W. Beard, University of Miami. Murine leukemia virus (Rauscher) was obtained from Electronucleonics, Bethesda, Md. Gibbon apo lymphosarcoma was supplied by the John L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, N. J. This virus was grown in NC-37 cells, a human lymphoblastoid line established in 1966 from the blood lymphocytes of a normal male. The sources of simian sarcoma virus (SSV-1) and Mason-Pfizer virus have been described (31).

Enzymes Assays

Cellular DNA Polymerases—Standard assays for cellular DNA polymerases were the same as described (9), except that in enzyme neutralization assays the following changes were made. The concentration and the specific activity of [H]dTTTP were generally about 18 μM and 35,000 cpm per pmol, respectively, and glass fiber filters were partially digested (activated) by treatment with pancreatic DNase I (32). DEAE-cellulose (Whatman DE52), phosphocellulose (Whatman P11), and Sephadex G-200 (Pharmacia) were prepared according to Sedwick et al. (4). Polyethylene glycol was obtained from Pierce Chemical Company. Ouchterlony double diffusion serum albumin was purchased from Miles. For use in the enzyme neutralization assay, this albumin was purified by gel filtration on Sephadex G 200. Ovalbumin and equine heart cytochrome c were purchased from Calbiochem. β-Galactosidase (Escherichia coli) was obtained from Worthington. Nonimmune serum and ascites fluids were prepared as described (5). Yeast tRNA (spheroplasts) and formaldehyde-fixed cells were prepared as described (5). For enzyme precipitation with octaoxoic acid, and agar plates for immunodiffusion were obtained from Hyland. Complete and incomplete Freund’s adjuvants were purchased from Baltimore Biological Laboratories.

Terminal Deoxynucleotidyltransferase—The assay for terminal deoxynucleotidyltransferase (30:1) was as follows. The reaction mixtures were identical except that MgCl2 was replaced with 0.5 mM MnCl2. Incubation was at 37° for 10 min. One unit of DNA polymerase activity is defined as that amount causing the incorporation of 1 pmol of labeled dNMP into acid-insoluble material per hour under the various conditions described above.

DNA Polymerase I (Fraction 3, 40 ml) was applied to a column (5 × 90 cm) of Sephadex G-200 which had been equilibrated with Buffer C + 0.35 M KCl. Fractions of 20.5 ml were collected at a flow of 15 ml per hour. The majority (80%) of the recovered activity (assayed in System B) eluted as a symmetric peak around a volume 1.15 times the void volume. A small amount of containing DNA polymerase II (9% of the recovered activity) eluted at 2.0 times the void volume and was discarded. Fractions containing the major peak of activity were pooled and dialyzed against 2.5 liters of Buffer C (0.01 M Tris-HCl, pH 7.0, 0.001 M dithiothreitol, and 10% glycerol). The dialyzed material (Fraction 4) was applied to a column (18 × 16 cm) of DEAE-cellulose which had been equilibrated with Buffer I. The column was washed with 250 ml of Buffer C + 0.08 M KCl, and DNA polymerases I and II were eluted with a linear gradient (1250 × 1250 ml) of KCl in Buffer C extending between 0.1 and 0.7 M. Fractions containing DNA polymerase I, eluting between 0.17 and 0.30 M KCl, and DNA polymerase II, eluting between 0.40 and 0.49 M KCl, were pooled and concentrated by dialysis against 30% polyethylene glycol + 0.35 M KCl in Buffer C. These activities were measured in Assay System B.

Viral DNA Polymerases—For the assay of DNA polymerases from avian myeloblastosis virus and Mason-Pfizer virus, reactions were performed in 50-μl volumes containing the following ingredients: (A) 12, (dT) 12-18, 50 μg per ml; 50 mM Tris-HCl (pH 7.8); 10 mM MgCl2; 50 mM KCl; 5 mM dithiothreitol; 80 μM dATP; 18 μM [3H]dTTTP (35,000 cpm per pmol); and 5 or 10 μl of enzyme solution. For the assay of the other viral DNA polymerases, reactions contained 10% dimethylsulfoxide instead of 0.5 mM MnCl2. Incubation was at 37° for 1 h.

One unit of DNA polymerase activity is defined as that amount causing the incorporation of 1 pmol of labeled dNMP into acid-insoluble material per hour under the various conditions described above.

Enzyme Purification

Cellular DNA Polymerases—Purification of DNA polymerases I and II from RPMI 1788 cells proceeded from 70 to 100 times the amount of washed frozen cells. The procedure was a modification of previously described techniques (5). All operations were performed at 0–4°. The cell suspension (1 ml) was homogenized and stirred for 1 h with 250 ml of a buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, and 1 mM EDTA. 10% glycerol. After centrifugation, the supernatant was centrifuged at 37,000 × g for 60 min, and the supernatant was saved. The pellet was homogenized and stirred for 1 h with 250 ml of a buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, and 1 mM EDTA + 10% glycerol. This suspension was centrifuged as above; the supernatants were combined and dialyzed for 8 h against 36 liters of Buffer C. This crude extract (Fraction 1) was adjusted to 0.275 M KCl and added to 1 liter (gravity packed wet volume) of DEAE-cellulose which had been equilibrated with Buffer C + 0.25 M KCl. The resulting slurry was stirred for 1 h and centrifuged at 4100 × g for 10 min. The supernatant was saved and the packed DEAE-cellulose was resuspended and stirred for 1 h in 1 liter of Buffer C + 0.3 M KCl. After centrifugation, the supernatants were combined and dialyzed for 8 h against 36 liters of Buffer C. The resulting solution (Fraction 2) was applied to a column (25 × 50 cm) of phosphocellulose which had been equilibrated with Buffer C + 0.08 M KCl. The column was washed with 250 ml of Buffer C + 0.08 M KCl, and DNA polymerases I and II were eluted with a linear gradient (1250 × 1250 ml) of KCl in Buffer C extending between 0.1 and 0.7 M. Fractions containing DNA polymerase I, eluting between 0.17 and 0.30 M KCl, and DNA polymerase II, eluting between 0.40 and 0.49 M KCl, were pooled and concentrated by dialysis against 30% polyethylene glycol + 0.35 M KCl in Buffer C. These activities were measured in Assay System B.

DNA polymerase I (Fraction 3, 40 ml) was applied to a column (5 × 90 cm) of Sephadex G-200 which had been equilibrated with Buffer C + 0.35 M KCl. Fractions of 20.5 ml were collected at a flow of 15 ml per hour. The majority (80%) of the recovered activity (assayed in System B) eluted as a symmetric peak around a volume 1.15 times the void volume. A small amount of containing DNA polymerase II (9% of the recovered activity) eluted at 2.0 times the void volume and was discarded. Fractions containing the major peak of activity were pooled and dialyzed against 3.5 liters of Buffer I (0.01 M imidazole-HCl, pH 7.0, 0.001 M dithiothreitol, 0.001 M EDTA, and 10% glycerol). The dialyzed material (Fraction 4) was applied to a column (200 × 200 ml) of DEAE-cellulose which had been equilibrated with Buffer I. The column was washed with 100 ml of Buffer I + 0.01 M KCl and the polymerase eluted with a linear gradient (200 × 200 ml) of KCl in Buffer I extending between 0.01 and 0.3 M. Active fractions were pooled, concentrated by dialysis against 30% polyethylene glycol in Buffer I, and dialyzed against Buffer C. This purification (DNA polymerase I; Fraction 5, 13 ml) was used as antigen.

DNA polymerase II from RPMI 1788 cells was further purified as follows. The activity eluting from phosphocellulose between 0.40 and 0.49 M KCl was pooled, concentrated as described above, and applied either in batches to a column (2.5 × 30 cm) of Sephadex G-200, or in toto to the larger column used for purification of DNA polymerase I. At least 90% of the activity (asayed in System B) eluted as a symmetric peak around a volume approximately 1.7 to 2.0 times the void volume. The active fractions...
were pooled, concentrated as described above, and dialyzed against Buffer I. This material was then passed over a column (1.8 × 8 cm) of DEAE-cellulose equilibrated with Buffer I; the enzyme was not bound under these conditions. The material passing through the column was concentrated and dialyzed as described above.

DNA polymerase II from phytohemagglutinin-stimulated human blood lymphocytes was purified as described elsewhere (25). This procedure includes a separation of DNA polymerases I and II on DEAE-cellulose prior to phosphocellulose chromatography and units gel filtration and final chromatography on DEAE-cellulose.

The extent of purification of DNA polymerase II from both RPMI 1640 and phytohemagglutinin-stimulated blood lymphocytes is comparable to that previously reported, i.e. 200- to 300-fold (5).

Viral DNA Polymerases—DNA-directed DNA polymerases from avian myeloblastosis virus, murine leukemia virus, gibbon ape lymphosarcoma virus, simian sarcoma virus, and Mason-Pfizer virus were purified according to published procedures (31), yielding approximately 1000-fold purifications.

Analysis of Enzyme Purity

DNA-cellulose Chromatography—DNA polymerase I (Fraction 5) was processed on DNA-cellulose prepared as previously described (25, 33). A column (0.9 × 4.8 cm) was successively washed with 5 ml of Buffer C + 1 mg per ml of bovine serum albumin and 30 ml of Buffer C + 1.0 M KCl. After equilibration with Buffer C, 1 ml of enzyme was applied to the column over about 1 hour. The column was washed with Buffer C + 0.025 M KCl, and the enzyme eluted with a linear gradient (15 × 15 ml) of KCl in Buffer C extending between 0.05 and 0.4 M. The flow was about 5 ml per hour. About 96% of the applied activity (measured in Assay System A) was recovered; all of the recovered activity was bound.

Polyacrylamide Gel Electrophoresis—Analysis of enzyme fractions on 7.5% polyacrylamide gels containing sodium dodecyl sulfate was performed according to the method of Weber and Osborn (35), yielding an upper limit of protein concentration for these enzymes; hence the values reported in Table II and the legend to Fig. 7.

Other Methods—Sedimentation of enzymes in sucrose gradients containing 0.33 M KCl was performed as previously described (5). Protein concentrations of solutions containing DNA polymerases were determined by the method of Lowry et al. (36). Because of scarcity of certain viral DNA polymerases, large volumes were not sacrificed to determine protein concentrations of these partially purified enzymes. However, based on the extent of concentration required to detect these proteins on polyacrylamide gels stained with Coomassie blue dye (31), we have estimated an upper limit of protein concentration for these enzymes; hence the values reported in Table II and the legend to Fig. 7.

Immunological Methods

Preparation and Purification of Antiserum—Solutions containing DNA polymerase I (Fraction 5, 0.33 to 0.47 mg of protein per ml) were divided into aliquots of 1 ml each. For the initial immunization, one of these aliquots was emulsified with an equal volume of complete Freund's adjuvant and injected into subcutaneous sites on a (W/Fu × BN)F1 young adult male rat. Subsequent immunizations, made at 3-week intervals, were similarly performed, except that incomplete adjuvant was used. A total of nine immunizations were achieved in the same rat. The animal was bled by tail amputation (37) at weekly or biweekly intervals following the third immunization. Six to eight milliliters of blood were taken at each bleeding. The serum was collected by centrifugation (20 × 1, 124 pg) was then added and the mixture incubated for an additional 12 hours at 4°C. This mixture was then centrifuged at 15,000 × g for 5 min at 4°C in a Brinkmann model 3300 centrifuge. Aliquots of 25 pg of the supernatant were withdrawn and assayed for DNA polymerase activity in 50-μl reaction volumes under optimal reaction conditions as described above. In control experiments, goat anti-rat IgG did not inhibit DNA polymerase activity.

In other experiments, a constant amount of DNA polymerase I (130 ng) was titrated against serial dilutions of immune IgG, keeping the total rat IgG input constant at 2.6 μg by diluting immune IgG in nonimmune IgG. After an incubation lasting 48 hours, 124 μg of goat anti-rat IgG was added. The mixture was then further incubated and centrifuged, and the supernatant was assayed for DNA polymerase activity as described above. Further control tests were made of the ability of the goat anti-rat IgG to precipitate the rat IgG which inactivates DNA polymerase I. Mixtures of immune or nonimmune rat IgG (2.2 μg) and Buffer C were prepared as described above and incubated for 48 hours at 4°C. Goat anti-rat IgG or nonimmune goat serum (20 μl, 124 μg) was then added and the mixture incubated for 12 hours at 4°C. After centrifugation, 20 μl of supernatant were aspirated and mixed with DNA polymerase I (130 ng, 10 μl). After 60 hours of additional incubation at 4°C, enzyme activity was measured in Assay System A in volumes of 80 μl. In the absence of precipitating goat antibody, enzyme activity was inhibited by 84%; in the presence of goat anti-rat IgG, enzyme activity was inhibited by 9%.

RESULTS

Purification of DNA Polymerase I—A representative purification of DNA polymerase I from 100 × 10^6 RPMI 1758 cells is summarized in Table I. The over-all purification was about 152-fold; the yield was about 4%. One difficulty was the large loss of activity sustained during chromatography on phosphocellulose, which did not occur in previous work (5). This loss may be due in part to difficulties encountered in scaling up the procedures; e.g., the large volume of Fraction 2 required 3 days to process on phosphocellulose. The final step of purification, chromatography on DEAE-cellulose, is illustrated in Fig. 1A.

Chromatography of DNA polymerase I (Fraction 5) on DNA-cellulose is shown in Fig. 1B. The major peak of activity (using
Table I

Purification of DNA polymerase I from RPMI 1788 cells

Enzyme activity was assayed in System A (5) as described under "Experimental Procedure."

| Fraction | Volume (ml) | Protein (mg/ml) | Specific activity (units/µg) | Total activity (units X 10^-4) |
|----------|-------------|----------------|----------------------------|-------------------------------|
| 1. Crude extract | 3530 | 4.4 | 2.72 | 42.2 | |
| 2. DEAE-1 | 4665 | 3.5 | 1.64 | 26.8 | |
| 3. Phosphocellulose | 40 | 3.32 | 20.62 | 3.93 | |
| 4. Sephadex G-200 | 82 | 0.268 | 137.4 | 2.34 | |
| 5. DEAE-2 | 13 | 0.326 | 359.1 | 1.52 | |

* Activity of fractions prior to phosphocellulose chromatography represents the sum of activities of (at least) DNA polymerases I, II, and III.

![Figure 1](https://example.com/figure1.png)

Fig. 1. Chromatography of DNA polymerase I on DEAE-cellulose (A) and DNA-cellulose (B). A, Fraction 4 (82 ml) was applied to a column (1.8 X 16 cm) of DEAE-cellulose as described under "Experimental Procedure." DNA polymerase activity (O-O) was assayed in System B, and the optical absorbance at 280 nm was measured with a continuous recording spectrophotometer. B, Fraction 5 (1 ml) was applied to a column (0.9 X 4.8 cm) of DNA-cellulose as described under "Experimental Procedure." DNA polymerase activity was assayed in System B (O-O) or in the assay for DNA polymerase III (□-□). The concentration of KCl in fractions (△-△) was measured with a Lab-Line conductivity bridge.

![Figure 2](https://example.com/figure2.png)

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Fractions 1 through 5 of DNA polymerase I (see Table I), arranged in order from left to right, performed in 7.5% polyacrylamide gels ("Experimental Procedure"). Gels were 9 cm long; electrophoresis was for 7 hours at 7.8 ma per tube. The amounts of protein applied to each gel were 66, 52, 50, 31, and 33 µg, respectively, for Fractions 1 through 5.

![Properties of DNA Polymerases I and II](https://example.com/properties.png)

**Properties of DNA Polymerases I and II**—DNA polymerase I used for antigen possesses properties that clearly distinguish this enzyme from other cellular DNA polymerases. The enzyme sediments in sucrose gradients at $s_{20,w} = 6.5$, and elutes at 1.15 times the void volume on gel filtration through Sephadex G-200. The enzyme binds strongly to DEAE-cellulose at pH 7.0, suggesting that DNA polymerase I is an acidic protein (isoelectric point < 7.0). This enzyme is fully inhibited by 0.25 mM N-ethylmaleimide and has a $K_i$ of 2.7 µM for the dCTP analogue 1-β-d-arabinofuranosylcytosine 5'-triphosphate (32).

DNA polymerase II purified from RPMI 1788 cells sediments at $s_{20,w} = 3.6$ and elutes at about 2 times the void volume on Sephadex G-200. This enzyme binds strongly to phosphocellulose and does not bind to DEAE-cellulose at pH 7.0, suggesting that this enzyme is a basic protein, as is DNA polymerase II purified from blood lymphocytes (isoelectric point 9.4) (5). DNA polymerase II is not inhibited by 0.25 mM N-ethylmaleimide and has a $K_i$ of 12.8 µM for 1-β-d-arabinofuranosylcytosine 5'-triphosphate (32).

* Unpublished observations.
The wide separation of DNA polymerase I from polymerase II achieved during each of the last three steps of purification should ensure that polymerase I (Fraction 5) is free of polymerase II. The final step should remove any remaining traces of polymerase II, since, under the conditions used, this enzyme appears quantitatively in the unbound fraction. Chromatography of DNA polymerase I (Fraction 5) on DNA-cellulose was performed to determine the extent of contamination by DNA polymerase II, since, under the conditions used, this enzyme appears quantitatively in the unbound fraction. Chromatography of DNA polymerase I (Fraction 5) on DNA-cellulose was performed to determine the extent of contamination by DNA polymerase III, since this procedure resolves these two enzymes (25, 33). Although the recovery of DNA polymerase I was low, DNA polymerase III was undetectable at a sensitivity of assay adequate to detect 1% of the activity of DNA polymerase I (Fig. 1B). In direct assays of DNA polymerase I (Fraction 5), under conditions optimized to measure DNA polymerase III (8), a specific activity of 1 unit per μg was detected. This is 0.27% of the specific activity measured in Assay System A. DNA polymerase I (Fraction 5) contains no detectable terminal deoxynucleotidyltransferase activity.

Preparation of Antiserum—A prolonged course of immunization was necessary before enzyme neutralizing activity was obtained; five immunizations of 470 μg protein per injection were required. Subsequently, the titer of neutralizing antibodies was maintained at an approximately constant level by triweekly immunizations with DNA polymerase I for an additional four immunizations. A total of approximately 30 ml of immune serum was recovered from the animal.

Properties of Neutralization Reaction—The kinetic data of neutralization of DNA polymerase I are shown in Fig. 3. In this experiment, small quantities of IgG (1.0 μg) and enzyme (65 μg) were used. It is noteworthy that under these conditions, a prolonged preincubation (48 to 72 hours) of polymerase and immune IgG was required to achieve maximal inhibition of enzyme activity. At this low concentration, nonimmune IgG did not consistently influence enzyme activity; amounts greater than 5 μg per reaction mixture stimulated enzyme activity. For this reason, in comparative neutralization assays (Figs. 6 and 7), total rat IgG was kept constant, whereas immune IgG was varied. Incubation for longer than 12 hours caused a partial loss of enzyme activity even in the absence of immune IgG (Fig. 3). This loss was minimized by including purified bovine serum albumin in the reaction mixture. The albumin also minimized but did not completely abolish the stimulation of enzyme activity by nonimmune IgG.

If larger amounts of immune IgG (>25 μg) were used, partial inhibition of DNA polymerase I occurred without any preliminary incubation of antigen and IgG. Under these conditions inhibition was independent of the time of incubation up to 1 hour, or of the temperature of incubation up to 37°C. Assessment of the effect of longer incubation periods at 25°C or 37°C was impossible because of thermolability of the polymerase. Therefore, in the routine neutralization reaction, a period of 48 to 72 hours of incubation of antigen and IgG at 4°C was adopted as the best compromise between completion of immune inhibition and avoidance of thermal loss of enzyme activity.

A titration of increasing amounts of immune IgG against two fixed amounts of DNA polymerase I is shown in Fig. 4. The enzyme was incubated with IgG for 72 hours prior to determination of residual enzyme activity, which is expressed as a percent of the activity in the presence of the same amount of nonimmune IgG. The activity of 16.3 ng of enzyme was reduced to 40% of control by 1.2 μg of immune IgG; approximately 16 times as much immune IgG was required to reduce the activity of 1630 ng of enzyme to this level. By increasing the immune IgG to 19 μg, more than 95% of the activity of the smaller amount of enzyme was inhibited.

A titration of increasing amounts of DNA polymerase I against a fixed amount of immune IgG (3.8 μg) is shown in Fig. 5. Above enzyme inputs of 100 μg, progressively less inhibition was observed, until at 1630 ng of enzyme only 15% inhibition occurred. This experiment clearly defines a region of “antigen excess” in the titration.

In experiments not shown, immunological inhibition of DNA polymerase I was about the same whether the template-primer was activated DNA, (dA)n·(dT)n-18, or (dC)n·(dG)n-14. Inhibition was also independent of concentration of template-primer over a range of 10 to 40 μg per ml for activated DNA and from 2.5 to 20 μg per ml for (dA)n·(dT)n-18. These ranges extend from limiting to saturating concentrations of template-primer under Assay Conditions A and B, respectively.

Double Antibody Immunoprecipitation Assay—In these experiments, nonimmune or anti-DNA polymerase I IgG was mixed...
ENZYME PROTEIN (ng)

FIG. 5. Titration of a fixed amount of immune IgG against increasing concentrations of DNA polymerase I. Neutralization mixtures were established as described under "Experimental Procedure," containing 3.8 µg of nonimmune or immune IgG (obtained following the ninth immunization of the rat) and serial dilutions of DNA polymerase I (Fraction 5) in Buffer C. After incubation at 4° for 66 hours, polymerase activity was assayed in System A. Enzyme activity is expressed as a percentage of the activity in the presence of nonimmune IgG.

TABLE II
DNA polymerase activity remaining soluble after double antibody immunoprecipitation

The assay is described under "Experimental Procedure." The units refer to the supernatant activity in the presence of nonimmune rat IgG; the activity remaining soluble is the percentage of this activity in the supernatant in the presence of immune IgG. The immune IgG used in this experiment (2.2 µg) was obtained following the seventh immunization.

| Enzyme                          | Enzyme protein | Units ± Activity remaining soluble |
|---------------------------------|----------------|-----------------------------------|
| DNA polymerase I (Fraction 5)   | 33             | 0.41 ± 0%                         |
| DNA polymerase II               | 0.45           | 0.64 ± 158%                      |
| RPMI 1788 cells                 | 685            | 0.48 ± 76.9%                     |
| Blood lymphocytes               |                | 0.43 ± 117%                      |
| RNA-directed DNA polymerase     |                |                                   |
| Avian myeloblastosis virus      | <2.5           | 0.37 ± 68.2%                     |
| Murine leukemia virus (Rauscher)| <5             | 0.70 ± 110%                      |
| Mason-Pfizer virus              | <12.5          | 0.80 ± 90.8%                     |
| Simian sarcoma virus            | <20            | 1.35 ± 61.7%                     |
| Gibbon ape lymphosarcoma virus  | 9              | 0.43 ± 117%                      |

and incubated with various DNA polymerases as shown in Table II. Then precipitating antiserum against rat IgG was added and the mixture was further incubated. After centrifugation, the residual DNA polymerase activity in the supernatant was determined in the assay systems optimized for the particular enzyme tested. In some experiments a constant amount of DNA polymerase I (130 ng) was titrated against increasing concentrations of immune IgG. In these experiments, 2.6 µg of immune IgG were found to bind 95% of the enzyme activity; 0.5 µg bound 50% of the activity. In other experiments, an amount of rat IgG (2.5 µg) was chosen that inhibited about 82% of the activity of DNA polymerase I in the neutralization assay (Figs. 6 and 7). All of the activity of this polymerase is precipitable in the double antibody assay (Table II). Therefore, under these conditions, 2.2 µg of immune IgG quantitatively bound 33 ng of DNA polymerase I.

Specifity of Antiserum—In the neutralization assay (Figs. 6 and 7), 1.0 µg of immune IgG neutralized 66% of the activity of DNA polymerase I. DNA polymerase II from RPMI 1788 cells or from blood lymphocytes was not inhibited by this quantity of immune IgG (Fig. 6), nor were the DNA polymerases from five RNA tumor viruses (Fig. 7). Therefore, at low concentra-
tions of immune IgG, this antiserum distinguishes DNA polymerase I from these other polymerases. At much higher inputs of immune IgG, some of the other polymerases were partially inhibited. DNA polymerase II from RPMI 1788 cells was not inhibited by immune IgG at 64 μg per reaction mixture; in a control reaction, this amount of IgG inhibited 99% of the activity of DNA polymerase I (Fig. 6). In the same experiment, 64 μg of immune IgG inhibited 33% of the activity of DNA polymerase II from blood lymphocytes (Fig. 6). This quantity of immune IgG inhibited none of the activity of the DNA polymerase from the Mason-Pfizer virus and a minority of the activities of the polymerases from murine leukemia virus, simian sarcoma virus, and gibbon ape lymphosarcoma virus (Fig. 7). Only the polymerase from avian myeloblastosis virus was inhibited more than 50% (Fig. 7).

In the double antibody immunoprecipitation assay, none of the activity of DNA polymerase II from RPMI 1788 cells or of the RNA-directed DNA polymerases from murine leukemia virus or gibbon ape lymphosarcoma virus was precipitable. About one-fourth of the activity of DNA polymerase II from blood lymphocytes and one-third of the activities of the RNA-directed DNA polymerases from avian myeloblastosis and simian sarcoma viruses were precipitable (Table II).

**DISCUSSION**

The purification of antigen was designed to prepare DNA polymerase I as free as possible from contamination by other human DNA polymerases, specifically DNA polymerase II (1-7) and DNA polymerase III (8-10, 25, 33). Therefore, chromatography on DEAE-cellulose at pH 7.0 was added to the purification scheme that we have previously described (5). This step effectively removed remaining traces of DNA polymerase II. Moreover, previous reports suggested that DEAE-cellulose chromatography separates DNA polymerase III from DNA polymerase I (8, 9). This procedure produced DNA polymerase I (Fraction 5) which was contaminated by less than 0.3% DNA polymerase III5. The purified enzyme (Fraction 5) was also free of terminal transferase activity. Thus, although Fraction 5 was not purified to homogeneity (Fig. 2), it was substantially free of other DNA polymerase activities found in human cells. This degree of purity is required for the production of analytically useful antiserum, i.e. useful in distinguishing or relating various human DNA polymerases and determining any possible relationships to the DNA polymerases of RNA tumor viruses.

The properties of DNA polymerase I are similar to those previously described by a number of workers (1-6, 25, 32).

An unusual feature of the neutralization reaction is the requirement for prolonged incubation of antigen and immune IgG in order to reach maximal inhibition (Fig. 3). This feature differs from the kinetics of immune inhibition of RNA-directed DNA polymerases described by others (21, 23). Chang and Bollum (39) obtained an antiserum in rabbits to calf thymus DNA polymerase I produced in rabbits (39). At approximately the same titers, this rabbit antiserum equally inhibited DNA polymerases I and II from several mammalian species (39). This rabbit antiserum conceivably recognizes antigenic site(s) on the enzymes that are different from the site(s) which our rat antiserum recognizes. DNA polymerase I and II may contain polypeptide regions in common which our antiserum does not recognize. Neutralization and immunoprecipitation tests in the reciprocal systems (anti-DNA polymerase I with use of both the neutralization and immunoprecipitation assays, we tested the specificity of anti-DNA polymerase I IgG. In neither of these assays did this IgG inhibit or bind to DNA polymerase II from RPMI 1788 cells, even at concentrations sufficient to inactivate or bind all of DNA polymerase I (Fig. 6 and Table II). On the other hand, a small fraction of DNA polymerase II from phytohemagglutinin-stimulated blood lymphocytes was bound (Table II) and inactivated (Fig. 6). It is possible that this enzyme was contaminated with a small amount of DNA polymerase I, since the technique of purification of DNA polymerase II from blood lymphocytes was less rigorous than that used with RPMI 1788 cells. At least 60 times as much immune IgG was required to inhibit DNA polymerase II to the same extent as DNA polymerase I (Fig. 6). This is a much greater difference than reported by Chang and Bollum for an antiserum to calf thymus DNA polymerase I produced in rabbits (39). A similar antiserum did not inhibit human DNA polymerase III (33). In those experiments, the incubation of antigens and IgG was brief (10 min). Further studies using more prolonged conditions of incubation are now in progress in order to define a possible serologic relationship between DNA polymerases I and III.

Several DNA polymerases isolated from RNA tumor viruses...
were inhibited or bound to a small extent by rat anti-DNA polymerase I (Fig. 7 and Table II). Surprisingly, the RNA-directed DNA polymerase of avian myeloblastosis virus was inhibited (Fig. 7) or bound (Table II) more than any other viral polymerase tested. In general, however, there were large differences in the concentration of immune IgG required to produce inhibitions of the viral enzymes equal to that of the homologous enzyme; for example, 54 times more immune IgG in the case of the polymerase from avian myeloblastosis virus (Fig. 7). These titrations are essentially the reciprocal of the experiments performed by others (20-22), and confirm in general that the antigenic relationships are distant between cellular DNA polymerase I and viral RNA-directed DNA polymerases. This specificity is a useful property of our rat anti-DNA polymerase I IgG, which should distinguish other polymerases from DNA polymerase I and detect contamination of RNA-directed DNA polymerases by DNA polymerase I when such enzymes are isolated from human tumor cells.

We have reported the isolation of RNA-directed DNA polymerases from the leukemia blood cells of several patients (15-17). These enzymes were inactivated by antisera to the DNA polymerase of simian sarcoma and gibbon ape lymphosarcoma viruses (16, 17). Two such enzymes were not inhibited by the rat anti-DNA polymerase I IgG. Neither did this IgG inhibit an RNA-directed DNA polymerase isolated from the placenta of rhesus monkeys (42). The activities of both the human leukemia and rhesus placental reverse transcriptases declined during prolonged incubations at 4°C to such an extent that incubations of less than 1 hour were necessary. However, under these circumstances, large amounts of immune IgG (up to 34 μg) were used so that up to 76% of the activity of DNA polymerase I was neutralized in control assays.

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