DNA Polymerase Activity in Human Serum: Studies with Australia Antigen (37356)

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Recently, Temin and Mizutani (1) and Baltimore (2) reported the presence of a DNA polymerase in RNA tumor viruses which could use viral RNA as a template for the synthesis of DNA. Immediately thereafter, we began to investigate the possible presence of such a polymerase ("reverse transcriptase") in normal human serum and in serum from patients with diseases of probable viral origin. During the course of this work a preliminary report from another laboratory suggested that a DNA polymerase might be a constituent of Australia antigen [Au(1)] (3). After we determined DNA polymerase activity in occasional samples of normal serum and in one preparation of purified Australia antigen, we then began a controlled study of patients with and without Au(1) in their serum. The results of these studies are reported herein.

Materials and Methods. Poly dAT was made by the *de novo* reaction catalyzed by *E. coli* DNA polymerase I (4). The latter was the most purified fraction, 7, by the method of Jovin *et al.* (5). Poly rA·dT, poly rA·rU, poly rI·rC were supplied by Miles Laboratories. "Activated" calf thymus DNA was made as previously described (6). Pancreatic ribonuclease and deoxyribonuclease were products of Worthington. *E. coli* RNase I was a gift of Dr. J. W. Abrell. Rauscher murine leukemia virus was obtained from Electro-Nucleonics Laboratories, Inc. ^{3}H- and [α-^{32}P]-labeled deoxynucleotides were products of New England Nuclear Corp. and unlabeled deoxynucleotides were products of California Foundation for Biochemical Research.

A study was designed to test the association of DNA polymerase activity with Australia antigen in serum. For this purpose, each of the 14 patients with Down's syndrome who had Australia antigen in their serum was matched for age, sex and institution with, (a) a Down's syndrome patient without Australia antigen and, (b) another mentally retarded patient with Down's syndrome or Australia antigen. Serum was obtained from these 14 triplets, and kept on ice for about 2 hr prior to the assay for DNA polymerase activity.

In another investigation sedimented fractions of serum were obtained after centrifugation at 300,000*g* for 18 hr. The pellets were dispersed in an amount of 0.02 *M* KCl corresponding to ¼ the initial volume of serum. This study included 5 matched pairs of mentally retarded patients with and without Australia antigen, 2 additional patients with acute hepatitis and Australia antigen, and 12 other individuals without Australia antigen.

Australia antigen was purified as previously described except that treatment with enzymes was omitted (7). In brief, human serum was centrifuged at 300,000*g* for 18 hr, the pellet was suspended in 0.15 *M* NaCl and then subjected to gel filtration through Sephadex G-200. The fractions containing Au(1) were combined, dialyzed against 0.01 *M* KCl and then centrifuged through sucrose density gradients. The fractions containing Au(1) were sedimented to approach equilibrium in cesium chloride density gradients. The fractions from this centrifugation which reacted with specific antibody at the highest dilution were used in these studies.

The assay for DNA polymerase activity measures the incorporation of an appropriately labeled deoxynucleotide into an acid-insoluble product. The reaction without add-

ed template (an endogenous reaction) contained in a volume of 0.05 ml; 1–10 μl of serum; 0.15 μl Triton-X; 2 μmole Tris-HCl, pH 8.0; 0.25 μmole magnesium chloride; 0.2 μmole dithiothreitol; 3 nmole each dATP, dCTP, dGTP; 0.5 nmole ^{3}H-dTTP or [α-^{32}P]-dTTP (about 1000 cpm/pmole). Unless indicated, all assays were incubated for 60 min at 37°. The reactions were terminated with 0.5 ml 1.0 *N* perchloric acid, 0.01 *M* potassium pyrophosphate and 300 nmole calf thymus DNA (Worthington) as carrier. The precipitate was collected by centrifugation at 7000*g* for 10 min, drained and dissolved in 0.2 ml of 0.2 *N* NaOH. After reprecipitation with 1.0 ml of 1 *N* perchloric acid the acid-insoluble precipitate was again dissolved in NaOH and again precipitated with acid. Radioactivity was determined by liquid scintillation spectroscopy after collecting the precipitate on glass fiber filters. With each set of determinations, Rauscher murine leukemia virus containing a known amount of polymerase activity (2) (reverse transcriptase) was used as a standard.

In assays with added poly rA·dT as a template (2.5 μg/assay) $MnCl_2$ (0.7 μmoles) was used in place of $MgCl_2$. By base pairing, the incorporation of the labeled TTP would be directed by the ribose (rA) strand of the hybrid. With each set of measurements, extracts of phytohemagglutinin-stimulated human lymphocytes was used as a standard (8). When the alternating polymer of deoxyadenosine and deoxythymidine nucleotides, poly d(A-T) (4.0 μg/assay), was used as a template, a known amount of homogeneous *E. coli* DNA polymerase I (5) was used as a standard.

Results. In our initial survey, we assayed preparations of human serum and of purified Au(1) for the presence of DNA polymerase activity. These assays were carried out with a variety of added natural and synthetic polynucleotide templates, with Mg^{2+} or Mn^{2+} as metal activators, and with the complementary deoxynucleoside triphosphate substrates. DNA polymerase activity was detected in one of the preparations of purified Au(1) which had been obtained from the serum of a patient (E.H.) with Down's syndrome (Table I, column I). Surprisingly, this activity was not increased by added polynucleotide templates. The decreased activity with the added polynucleotides (rA·dT, rA·rU and rI·rC) apparently reflects the requirement for all four deoxynucleoside triphosphates in the reaction mixture (*vide infra*). Using the added templates only the complementary nucleotides were present in each reaction mixture. Activity was not detected in unfractionated serum from the same patient, in two fractions obtained early in the purification, as well as in another preparation of purified Au(1) from a different patient. Yet, a DNA polymerase activity was found in a normal patient's serum from which we were unable to detect Au(1) by either immunodiffusion or radioimmunoassay (9).

Requirements for activity. Prior to studying the association of DNA polymerase activity with Au(1), we investigated the requirements of the reaction using the purified preparation of Au(1) from patient (E.H). Table II shows that the requirements for activity are typical of DNA polymerase systems; Mg^{2+} and all four deoxynucleoside triphosphates. In the presence of only one of the deoxynucleotides (dTTP), incorporation was 24% of that obtained with all four deoxynucleotides (Table II). When the four corresponding ribonucleoside triphosphates were used in place of the deoxynucleotides, RNA synthesis was not detected. DNA synthesis was linear for about 90 min; activity was proportional to the amount of Au(1) tested over a range of 0.5–10.0 μg of protein. In the absence of added Mg^{2+} or Mn^{2+} there was no detectable incorporation; the optimal concentration of Mg^{2+} and Mn^{2+} was about 5 m*M* and 0.5 m*M*, respectively.

The lack of stimulation by added templates was further investigated. The activity in this preparation of Au(1) was not increased by any of the following polynucleotides: poly d(A-T), "activated DNA," poly rA·dT or poly rI·rC using varying concentrations of Mg^{2+} and Mn^{2+}. The former two acted to stimulate human lymphocyte enzyme (10) and the latter two stimulated the enzyme found in RNA tumor viruses (11). The most direct explanation for this lack of stimula-

TABLE I. DNA Polymerase Activity Found in Serum and Purified Australia Antigen.[a]

Template	Divalent ion + deoxynucleotides	DNA polymerase activity (pmoles labeled nucleotide/hr)				
		Purified Au(1) E.H.	Serum E.H. Au(1)	Serum J.B. Au(1)	Purified Au(1) D.M.	Serum Au(0)
None added	(5 m*M* Mg^{2+}, all four)	6.5	—	—	—	2.6
Yeast RNA	(1.4 m*M* Mn^{2+}, all four)	1.2	<0.1	<0.1	<0.1	0.4
Yeast RNA	(8 m*M* Mg^{2+}, all four)	5.1	<0.1	0.1	<0.1	2.9
poly rA • dT	(1.4 m*M* Mn^{2+}, TTP-^{3}H)	0.6	<0.1	0.1	<0.1	0.1
poly rA • rU	(1.4 m*M* Mn^{2+}, dATP, TTP-^{3}H)	0.3	<0.1	<0.1	<0.1	<0.1
poly rI • rC	(1.4 m*M* Mn^{2+}, dCTP, dGTP-^{3}H)	0.2	—	—	<0.1	0.1
Activated DNA	(7 m*M* Mg^{2+}, all four)	4.2	—	0.2	—	2.4

[a] The assay for DNA polymerase activity is given in "Methods" with the indicated templates, metal activators, and complement of deoxynucleotide substrates. Either 25 μl of serum or 10 μl of purified Au(1) containing about 1 μg of protein was used as a possible source of activity. The amounts of RNA, synthetic polynucleotides and DNA when present in each assay was 1.0, 2.0, and 5.0 μg, respectively. In this study, 12 coded specimens were tested. These included 4 sera-containing Australia antigen from 4 patients with Down's syndrome (including EH), 2 specimens of purified Au(1) (including 1 from EH), 3 specimens from EH at various stages of purification, one whole serum from an individual without Australia antigen (Au(0)), one specimen of DNA polymerase from stimulated human lymphocytes (13), and one specimen containing Rauscher murine sarcoma virus. The last two were included as standard preparations with known amounts of polymerase activity.

tion is that the enzyme in this preparation of Au(1) is not exposed to these added templates. Alternatively, its template requirements could be more restricted than most other investigated DNA polymerases.

Even though Au(1) has been reported to contain 5% RNA (12) we have no evidence that this is indeed the template which is being copied *in vitro*; activity is not abolished by prior treatment of Au(1) with pancreatic ribonuclease (Table II), with *E. coli* RNase I or with ribonuclease T1. Yet the RNA could be deeply embedded within Au(1) and not accessible to hydrolysis by these added ribonucleases. Attempts at disrupting Au(1) by varying the concentration of the detergent Triton-X from 0.1 to 5% in the reaction mixture and preincubating for various times did not lead to subsequent inhibition by ribonuclease. It would be ambiguous to use deoxyribonuclease as a probe for determining the structure of a putative DNA template since this enzyme would easily hydrolyze the product of the reaction (Table II).

Having established the requirements for DNA synthetic activity in one preparation of purified Au(1) we then asked whether a similar activity could be found in other preparations of Au(1) and whether there was an association between the amount of activity present in human serum and the presence of Au(1).

Analysis of purified Australia antigen for DNA polymerase. We have performed DNA polymerase assays on four preparations of Au(1) purified by the method of Millman *et al.* (7). These reactions were carried out using different concentrations of Mg^{2+} and Mn^{2+} with a variety of added polynucleotide templates. In only one of these preparations

TABLE II. Requirements for DNA Polymerase Activity.[a]

Reaction mixture	Incorporation of ^{3}H-TMP (%)
Complete	100
Minus $MgCl_2$	5
Minus dGTP, dATP, dCTP	24
Minus Australia antigen (E.H.)	5
Preincubated with RNase	88
Product incubated with DNase	8

[a] The complete system is detailed in "Methods" using 1.2 μg of purified Au(1) from E.H. as an enzyme source. Treatment with RNase consisted of preincubated Au(1) with 10 μl of pancreatic ribonuclease (0.2 mg/ml) for 15 min at 37° prior to assaying for polymerase. Treatment with DNase consisted of adding 10 μl of deoxyribonuclease (0.2 mg/ml) immediately prior to stopping the reaction, then incubating an additional 15 min. All reactions were carried out in triplicate. Incorporation with the complete system was 3.0 pmole dTTP-^{3}H (2400 cpm) and incorporation without incubation was 0.12 pmole.

(E.H.), was polymerase activity found to be present. In addition, we were unable to detect DNA polymerase activity in a sample of purified Au(1) obtained from Electro-Nucleonics Laboratories. Preincubation of poly d(A-T) or poly rA·dT with purified Au(1) did not diminish the ability of these polynucleotides to serve as templates for *E. coli* DNA polymerase I. Thus, if a nuclease was present in purified Au(1) it was not responsible for the lack of stimulation by these polynucleotides.

The assay for DNA polymerase used in these experiments is sensitive enough to detect 0.002 μg of Rauscher leukemia virus protein (13). Thus, if only a small amount of Au(1) constitutes a particle with a DNA polymerase of similar specific activity it would be easily detected.

Analysis of serum. The finding of a DNA polymerizing activity in one sample of normal serum (Table I) prompted us to determine its occurrence in normal sera. First we investigated the requirements for activity using the most active serum from a 21-year-old married female, a healthy volunteer. The activity was proportional to the amount of serum in the assay, incorporation was linear for up to 90 min, and the Mg^{2+} optimum was 5 m*M*. The addition of RNA templates such as yeast RNA poly rA·dT or DNA templates such as "activated" calf thymus DNA did not stimulate the reaction. Thus, the requirements for activity determined in this serum appears similar, if not identical, to that required for the enzyme studied in the preparation of isolated Australia antigen.

There is a minimal amount of enzyme activity observed in serum of most normal volunteers (Fig. 1). A detailed analysis could not be carried out with samples containing such low amounts of activity. However, the activity measured in normal serum is determined under the same conditions used to measure the activity in the serum having high activity as well in the preparation of purified Au(1).

Patients with Down's syndrome. In order to determine whether the presence of Au(1) in serum was correlated with the amount of DNA polymerase activity, we measured the activity in serum with and without detectable Au(1). In a study analyzed by Wilcoxon

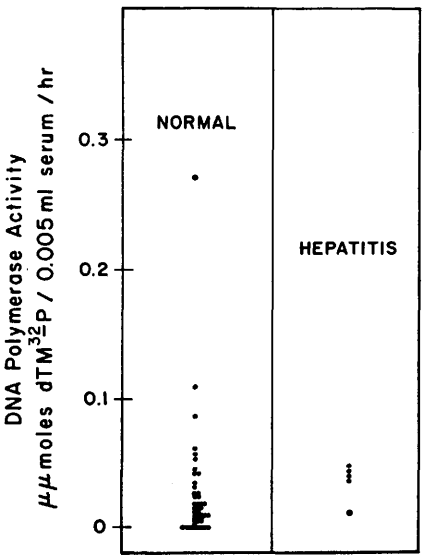

FIG. 1. DNA polymerase activity in serum from patients with hepatitis and individuals without apparent disease. Assays were carried out with 5 μl of serum and without added template as given in "Methods." All reactions were carried out in triplicate with and without incubation.

(14), matched pair signed rank test consisting of five matched pairs of mentally retarded individuals with and without Au(1) we found a marginal positive association between the presence of Au(1) in the serum and the amount of DNA synthetic activity only without an added template (Table III). A more detailed study on 14 triplets [each consisting of age- and sex-matched patients with Down's syndrome with and without Au(1) and a mentally retarded patient from the same institution without Au(1)] revealed no association (unpublished results). This lack of association was apparent when activity was measured without added template as well as with either poly d(A-T) ("DNA-dependent") or poly rA·dT ("RNA-dependent"). When the pellets from the sera of the five pairs of matched subjects with and without Australia antigen were analyzed by the Wilcoxon test (Table III), there was no evidence of association of DNA polymerizing activity with Au(1) in any of the reactions. Our results differ from those of Hirschman, *et al.* (3); for with poly d(A-T) we find that the Au(1) pellet had significantly less activity than did the Au(0) pellet. The use of the Mann–Whitney test (15) to analyze a total of seven pellets from sera with Au(1) and seventeen pellets from Au(1) negative sera revealed similar findings: no increased activity in the Au(1) pellet, and actually less activity in Au(1) pellet with poly d(A-T) primer.

Patients with acute hepatitis. Also presented in Fig. 1 is a comparison of the polymerase activity between 46 patients without apparent disease and 5 patients with acute

TABLE III. Polymerase Activity in Serum and Particulate Fractions.[a]

	(a) Sera			(b) Pellets		
Serum	poly rA • dT	poly d(A-T)	Endogenous	poly rA • dT	poly d(A-T)	Endogenous
1. Au(1)	281	277	128	233	325	55
Au(0)	229	225	134	350	484	86
2. Au(1)	332	268	209	304	298	59
Au(0)	202	290	142	446	610	115
3. Au(1)	286	281	160	515	261	53
Au(0)	257	243	115	335	493	67
4. Au(1)	274	271	182	414	567	108
Au(0)	293	295	141	270	354	63
5. Au(1)	238	225	168	254	365	60
Au(0)	244	243	107	450	565	90
p [Au(1) vs Au(0)] =	.112	.343	.040	.446	.112	.173

[a] This study includes 5 matched pairs of mentally retarded patients with and without Au(1) in their serum. Measurements of polymerase activity is detailed in "Methods." Each reaction was carried out in triplicate with 5 μl of serum or 10 μl of the dispersed particulate fractions. All four deoxynucleotides were present in reaction mixtures with alternating poly d(A-T) and with no added template. With poly rA • dT, ^{3}H-dTTP (2000 cpm/pmole) was the only nucleotide in the reaction mixture. With the endogenous reaction α-^{32}P-dTTP (400 cpm/pmole) was used while with poly d(A-T), ^{3}H-dTTP (2000 cpm/pmole) was the labeled nucleotide. Results are given in cpm and represent the average of triplicate determination after subtracting the cpm of nonincubated controls for each enzyme source. The effect of sera and pellets on the ability of polynucleotides to serve as templates for homogeneous *E. coli* DNA polymerase I was determined in separate experiments. With poly rA • dT, poly d(A-T) and "activated" DNA as templates, incorporation using 0.04 μg of *E. coli* polymerase plus 5 μl of sera was 121, 76 and 96% of that obtained with *E. coli* polymerase alone, respectively. In similar experiments, 10 μl of the particulate fraction did not significantly alter synthesis catalyzed by the *E. coli* polymerase.

hepatitis. The serum enzyme activities from the 5 patients with acute hepatitis having Au(1) were all low (Fig. 1), but there was a marginally significant association of Au(1) hepatitis serum with the higher levels of enzyme activity ($p = 0.063$) as analyzed by the Mann–Whitney test.

Discussion. The small amounts of incorporation of deoxynucleotides into acid-precipitable material by most sera may not represent a DNA polymerase activity. This apparent activity we find in normal serum could be derived from breakdown of host cells or represent ubiquitous virions. Yet, the inability of the enzyme to use added templates is at variance with the properties of these sources. An attractive possible explanation is that the DNA polymerase in normal serum is associated with a template and functions in normal processes including the exchange of information between cells. Thus, it will be of interest to measure polymerase activity in serum from patients having diseases associated with altered immune functions. The presence of this activity in normal serum must be considered in evaluating serum polymerase activity as an index of viral infection (3, 16).

The evidence presented indicates that DNA polymerase is not a constituent of Australia antigen. We were able to detect DNA polymerase activity in only one of five preparations of purified Au(1). This activity was not abolished by treating the Au(1) with ribonuclease, suggesting that RNA was not the template in this reaction. Since we find evidence for a similar activity in normal serum, the most direct explanation for the presence of DNA polymerase activity in one of five preparations of purified Au(1) is that it is a component of serum which copurified with Au(1).

Analysis of human sera and particulate fractions from these sera in three series of patients with Down's syndrome and hepatitis revealed that in two of the three series, there was a statistical correlation between the amount of polymerase activity without an added template and the presence of Au(1). But in the most extensive series consisting of 14 triplets matched as to age and sex we were unable to detect this correlation. Thus, the marginal statistical correlation between the amount of polymerase activity and the presence of Au(1) in sera is probably of an indirect nature and only suggests that individuals with Au(1) are more likely to have higher serum "DNA polymerase activity" than individuals without Au(1). Furthermore, the amount of polymerase activity present in pelleted fractions was not related to the presence of Au(1) in the serum from which these fractions were derived. Our findings do not support those of Hirschman *et al.* (3) who found polymerase activity in three pelleted fractions from serum containing Au(1), and who were unable to detect the activity in sera without Au(1).

Summary. The presence of DNA polymerase in normal human serum and its possible association with Australia antigen was investigated. DNA polymerase activity was determined in 135 samples of normal serum. Only 3 samples revealed appreciable amounts of activity. The addition of a variety of polynucleotide templates did not stimulate polymerase activity.

Australia antigen is a particle associated with hepatitis and is reported to contain RNA. A DNA polymerase, "reverse transcriptase" might be required for replication of this RNA. We found DNA polymerase activity in only one of five preparations of purified Australia antigen. A similar activity was also found to be present in serum from certain normal volunteers. In either case, polymerase activity does not require nor is it stimulated by added polynucleotides suggesting that the enzyme may already be associated with a template such as RNA or DNA. A comparison between the amount of DNA polymerase activity in serum and the presence of Australia antigen in three series of patients with Down's syndrome and hepatitis revealed at most a marginal positive association. Thus, DNA polymerase is probably not a part of Australia antigen and the association observed is probably of an indirect nature. Its presence in occasional normal sera must be considered when using this activity as an index for detection of oncogenic viruses.

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