

[54] **DIAGNOSTIC METHOD UTILIZING
SYNTHETIC DEOXYRILIONUCLEOTIDE
OLIGOMER TEMPLATE**

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[56] **References Cited**

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[57] **ABSTRACT**

A diagnostic method for the detection of virus-related neoplastic disease states is described. This method involves employing synthetic nucleotide oligomers hybridized with RNA-type polymers as a template for assaying RNA-dependent DNA polymerase activity. RNA-dependent DNA polymerase activity has been found to be specifically characteristic of several neoplastic disease states including human leukemia. In a preferred embodiment the instant method employs synthetic thymidylic acid oligomers (d-pT) hybridized with polymeric ribonucleotide rA.

11 Claims, No Drawings

DIAGNOSTIC METHOD UTILIZING SYNTHETIC DEOXYRILIONUCLEOTIDE OLIGOMER TEMPLATE

BACKGROUND OF THE INVENTION

It has recently been demonstrated that an RNA-dependent DNA polymerase is present in the virions of Rauscher mouse leukemia virus and Rous sarcoma virus, both viruses being RNA tumor viruses (Baltimore, *Nature*, 226, 1209-11 [1970]). The template for the RNA-dependent DNA polymerase was shown to be the viral RNA. Activity of this polymerase was determined by employing a standard DNA polymerase assay utilizing the incorporation of radioactivity from ^3H -TTP (thymidine triphosphate) into an acid-insoluble (polymeric) product as the mode of measurement. It was postulated that all RNA tumor viruses tested have such RNA-dependent DNA polymerase activity.

In a contemporaneous publication, Temin and Mizutani (*Nature*, 226, 1211-13 [1970]) confirmed Baltimore's finding with respect to Rous sarcoma virus and extended this discovery to avian myeloblastosis virus (AMV). Furthermore, they reported that RNA-dependent DNA polymerase activity was not present in supernatant of normal cells even if treated with detergent which served to increase polymerase activity tenfold in infected cell supernatants.

Most recently, Gallo, Yang and Ting reported that an RNA-dependent DNA polymerase analogous to that found in RNA tumor viruses above had been found in lymphoblasts obtained from humans suffering from acute leukemia whereas lymphoblasts produced from lymphocytes obtained from normal subjects were shown to be devoid of such activity (*Nature*, 228, 927-29 [1970]). Additionally, Gallo et al. discovered that this RNA-dependent DNA polymerase did not have specificity for viral RNA. They are able to employ not only mammalian RNA, but also the synthetic ribopolynucleotide poly rA:poly rU as template for this activity. The use of synthetic polymeric DNA-RNA hybrids and RNA-RNA duplexes as templates for oncogenic DNA polymerase assay was reported by Spiegelman et al. (*Nature*, 228, 430-32 [1970]).

These findings enhance the possibility of an eventual discovery of a preventive and/or therapeutic treatment for disease states whose etiology involves RNA-dependent DNA polymerase activity. However, of more immediate practicality is the use of these discoveries as a basis for a diagnostic method to screen populations for the presence of such diseases, to monitor the effectiveness of present treatments for these diseases in patients known to be afflicted or to monitor patients who have obtained remissions of their diseases so as to alert the treating physicians of the initiation of a relapse so that treatment can be reinstated prior to the exhibition of clinical symptoms. The basic problem preventing the utilization of such an assay on a practical clinical level, however, is the fact that the template materials used in assaying for the RNA-dependent DNA polymerase activity, i.e., viral RNA, mammalian (rat liver) RNA, and synthetic ribonucleotides (poly rA:poly rU) are obtainable only in small quantities in the laboratory and thus are prohibitively expensive. It is evident that a low-cost source of template material is necessary in order to make effective use of any diagnostic technique directed to the assay of RNA-dependent DNA polymerase activity.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to an improved diagnostic method for the detection of those virus-related neoplastic disease states in whose etiology RNA-dependent DNA polymerase activity is likely to be involved. Exemplary of such disease states are Rous sarcoma, Rauscher mouse Leukemia, avian myeloblastosis and human leukemia. In the diagnostic method of this invention known procedures for the assay of RNA-dependent DNA polymerase activity using modified standard reactive mixtures are utilized wherein the incorporation of a labeled nucleotide, i.e., ^3H -TTP into an acid insoluble fraction is monitored. However, the selective template for the RNA-dependent DNA polymerase activity in the instant method is a synthetic nucleotide oligomer hybridized with an RNA-type polymer. Since both components of this template are readily available and are relatively inexpensive, the diagnostic method of this invention may be readily employed in the clinic to screen subjects suspected of having the aforesaid diseases or to monitor treatment in patients known to be suffering from such diseases.

The synthetic nucleotide oligomers useful in the preparation of a RNA-dependent DNA polymerase selective template are preferably linear thymidine polynucleotides containing from 2 to 24 thymidine nucleotide units per molecule, i.e., d-pT₂ to d-pT₂₄, most preferably d-pT₃ to d-pT₉. These linear nucleotide oligomers are known compounds and methods for their preparation, separation and purification and described in detail in the papers by Khorana and Vizsolyi, *J. Am. Chem. Soc.*, 83, 675-85 (1961) and Narang et al., *J. Chem. Soc.*, 90, 2702 (1968). For the purpose of the diagnostic method of this invention tri-, hexa-, octa- and nona-nucleotides d-pT₃, d-pT₆, d-pT₈ and d-pT₉ are preferred. While the higher nucleotide oligomers are preferred because of their greater activity in the assay this is balanced by the fact that the lower oligomers are more readily available and thus less expensive.

The second component of the template useful in the present invention are the RNA-type polymers, such as, for example, rA, rU, rG, rC and rI, a most particularly preferred polymer is rA, RNA type polymers are articles of commerce and thus readily available. Hybridization of the aforesaid two components to form the desired template is conveniently carried out by the addition of approximately equimolar amounts (on a monomer basis) of the nucleotide oligomer and the RNA-type polymer (100 $\mu\text{g}/\text{ml}$ in 0.01 M Tris-HCl, pH 7.4), making the solution 0.2 M with respect to NaCl and allowing the mixture to stand for 15 minutes at room temperature.

The assay method of the present invention can employ purified extracts of neoplastic cellular material or leukemic plasma for which the RNA-dependent DNA polymerase activity is to be determined. For purposes of illustration standard assay procedures for determining DNA polymerase activity in accordance with the method of the present invention is given utilizing alternative sample sources.

A suitable assay procedure for neoplastic cellular material, i.e., human leukemic cells derived from whole blood is as follows:

Heparinized blood samples (5-10 ml. whole blood) are kept at 4°C., and after separation the buffy coat is removed and transferred into a mortar, pre-cooled in

dry ice. The material is thoroughly ground with a precooled pestle and the contents transferred and weighed. It is then taken up (2ml, per gram) in TM buffer (0.1 M Tris, pH 8.3, 0.01 M $MgCl_2$, 0.002 M dithiothreitol [DTT]). The homogenized suspension is spun down in an SS-34 rotor in a Sorvall centrifuge for 30 min. at 305,000 xg. The supernatant is removed and the pellet is taken up in phosphate buffer (0.01 M potassium phosphate, pH 8). A convenient volume is 1 ml. of buffer per gram of original buffy coat.

The suspension obtained in this manner is further purified by running it through a glycerol gradient. The purification is done by layering 17 ml. of the buffy coat suspension in a 37 ml. polyallomer centrifuge tube on top of a 10 to 30 percent glycerol gradient (12 ml.) containing 0.01 M potassium phosphate and 0.003 M DTT, and running it for 3 h. at 25,000 xg. in a Spinco centrifuge with a SW 27 rotor. The glycerol gradient is made over a 100 percent glycerol pad (8 ml.). The material that collects on top of the pad, after centrifugation, is removed (usually about 2 ml. of suspension per tube). The suspension is then thoroughly homogenized with the help of a syringe and canula. If the suspension contains a large amount of protein and is very viscous, it is diluted with 0.01 M Tris pH 8.3. An aliquot of this material is used for a "Lowry" protein determination.

If the buffy coat suspension is small in quantity, the glycerol gradient purification can be done either in a SW 41 or SW 501 rotor. For example, a 0.3 ml. buffy coat suspension can be purified using 4.4 ml. of a 10 to 30 percent glycerol gradient with 0.3 ml. 100 percent glycerol pad in a 501 rotor at 50,000 xg. for 1½ hours.

In a standard DNA polymerase enzyme assay, 100–200 µg. of protein prepared above contained in 80 µl. with 0.01 M Tris (pH 8.3) is employed. The solution is made 1 percent with respect to Nonidet P-40, a non-ionic detergent, and incubated for 30 minutes at 0°C. The reaction is then carried out in a total volume of 125 µl. The incubation mixture contains 1–5 µg. of the synthetic template and is 5×10^{-2} M in Tris (pH 8.3), 4×10^{-2} M with respect to K^+ and 6×10^{-3} M with respect to Mg^{++} . The mixture also contains the two deoxyribonucleoside triphosphates complementary to each member of the synthetic template. At least one of the two nucleoside triphosphates is isotopically labeled. Thus, if the template were poly d-pT:rA, then cold dATP and labeled dTTP would be added. Cold deoxyribonucleoside triphosphate corresponding to the labeled one, i.e., TTP, is added until the concentration of this species equals that of the complementary triphosphate, i.e., both species are present at a concentration of 800 mµ moles/ml. The labeled triphosphate may be ^3H -TTP, α - ^{32}P -TTP or ^{14}C -TTP. Preferably ^3H -TTP is employed and is used at a specific activity of 100–500 cpm/pmol. For routine assays, a 20 minute reaction is employed.

A second purification procedure utilizing leukemic plasma as a sample is as follows:

Heparinized whole blood is centrifuged to obtain a clear plasma. The following steps were then conducted at a temperature of 0°–4°C. A total of 200 ml. of the plasma is mixed with 2 gm. of Kieseluhr and centrifuged at 1,800 xg. for 10 minutes. The supernatant is filtered through a Buchner funnel containing a layer of Kieseluhr over filter paper. To remove the virus from the supernatant, the plasma is centrifuged against a 10 ml. glycerol pad in a Spinco SW-25.2 rotor at 75,000

xg. for 1 hour. The virus is removed from top of the glycerol pad by pipet and diluted with 40 ml. of 0.15 M NaCl–0.01 M Tris pH 8.8. The above glycerol purification is repeated three times.

A standard general assay system for the sample prepared by the second purification method utilizes the following procedure. About 25 µg. of the purified sample is initially incubated in 50 µl. of 1–2 percent Nonidet P-40, 0.003 M DTT and 0.01 M Tris-HCl (pH 8.3) for 10–30 minutes at 0°C. There is then added the following components (assuming a poly d-pT:rA template is being used) in an amount to achieve the respective concentration or quantities:

15	Tris-HCl (pH 7.5–9.0, i.e., 8.3)	.05M
	$MgCl_2$	.006M
	DTT	.002M
	dATP	.0008M
	^3H -TTP	.00016M
	KCl	.04M
	poly d-pT:rA template 1 µg.	

The total final volume of the above mixture is 125 µl. While the above procedure is particularly useful in assay of AMV, it is within the skill of the art to adapt the second purification method to utilize other sample materials. For example, Rauscher murine leukemia virus, Rous sarcoma virus and Mouse mammary tumor virus can be assayed by modifying the second purification procedure. A particulate-free fluid sample from a host infected with any of the foregoing viruses is treated at 0°–4°C. in the following procedure.

The fluid sample is layered over a 100 percent glycerol pad in a centrifuge tube and centrifuged at 95,000 xg. for 70 minutes. The material layered on the glycerol pad is transferred to the top of a 25–50 percent sucrose gradient in a centrifuge tube and centrifuged for 3 hours at 95,000 xg. The virus band is removed and diluted in 0.01 M Tris-HCl (pH 8.3), 0.1 M NaCl and 0.002 M EDTA (mixture identified as TNE). This mixture is centrifuged at 95,000 xg. for 2 hours to pellet the virus. The virus pellet is suspended in TNE and utilized as a sample in the second method assay procedure as described previously.

In both general methods described above the DNA polymerase activity in the respective reaction mixtures is determined by measuring labeled nucleotide uptake into an acid-insoluble polymer in a manner known per se. For example, the reaction mixture is incubated for about 20–30 minutes at from 30°–45°C., i.e., at 37°C. and the reaction is then terminated with 0.5 ml. of cold water and 0.3 ml. of a trichloroacetic acid solution comprising equal volumes of 100 percent trichloroacetic acid and saturated solution of sodium orthophosphate-sodium pyrophosphate. The precipitate is collected by filtration or centrifugation, washed with water and then counted by appropriate known methods employing liquid scintillation techniques. It is also possible to isolate polymeric product by passing the incubation mixture over an appropriate gel column, recovering the polymeric product in the exclusion volume and measuring the isotope content by known techniques.

While several specific general assay methods have been described, it is within the skill of the art to modify general assay procedures known in the art to utilize the present synthetic templates of the instant invention.

The assay method of the present invention is further illustrated by the following examples.

Example 1

This example demonstrates the template activity of various synthetic templates in an assay utilizing AMV as the source of RNA-dependent DNA polymerase. The templates employed were the following linear thymidylic acid oligomers: d-pT₂, d-pT₃, d-pT₄, d-pT₅, d-pT₆, d-pT₇, d-pT₈ and d-pT₉; each respectively hybridized with poly rA by the procedure described previously. Also tested were poly dT:rA, poly dT, poly rA, d-pT₉ and d-pT₃ for the sake of comparison of template activity.

The BAI strain A myeloblastosis virus plasma was obtained by tissue culture propagation and was purified according to the second purification method in this specification. A total of 26.7 μ g. of protein was employed for each template tested. The labeled ³H-TTP used had a specific activity of 100 cpm/pmole. The incubation time was 20 minutes at 37°C. The polymerization product was precipitated, filtered through nitrocellulose filters, the filters washed with water and dried. The filter was counted in a BBOT scintillation fluid and the results obtained are summarized in the following table.

TABLE I

Template	Cpm
poly dT:rA	62,679
d-pT ₉ :rA	177,312
d-pT ₈ :rA	147,706
d-pT ₇ :rA	156,225
d-pT ₆ :rA	178,397
d-pT ₅ :rA	75,788
d-pT ₄ :rA	40,311
d-pT ₃ :rA	80,076
d-pT ₂ :rA	9,974
poly dT	2,499
poly rA	471
d-pT ₉	8,702
d-pT ₃	3,383

It is seen from the above that synthetic templates utilizing a synthetic thymidylic acid oligomer can be employed successfully in an assay for the measurement of RNA-dependent DNA polymerase activity. A substantial number of such templates unexpectedly exhibit superior template activity compared to the polymeric dT:rA. Control runs with polymeric or synthetic unhybridized oligomeric materials are also shown and are seen to provide generally negligible activity levels when used as templates in this assay.

Example 2

This example demonstrates the template activity of various synthetic templates in an assay employing RNA-dependent DNA polymerase obtained from human leukemic cells by means of the first purification method described in the specification. A total of 100 μ g. of protein was employed using a 20 minute incubation period at 37°C. Labeled ³H-TTP having a specific activity of 500 cpm/pmole was used. The polymerization product was prepared for counting by the same procedure as in Example 1. Results of these experiments are summarized in Table 2 below.

TABLE 2

Template	Cpm
poly dT:rA	794
p-dT ₉ :rA	688
p-dT ₈ :rA	543
p-dT ₇ :rA	478
p-dT ₆ :rA	450
p-dT ₅ :rA	730

p-dT₄:rA
p-dT₃:rA
p-dT₂

472
387
383

This example demonstrates the utility of the synthetic templates of the present invention in an assay employing cellular RNA-dependent DNA polymerase as sample. The templates of the present invention can thus be used to determine or monitor the presence of RNA-dependent DNA polymerase in human cells.

I claim:

1. A method for detecting RNA-dependent DNA polymerase activity in a purified mammalian or avian cellular extract or plasma sample which method comprises adding said purified sample to a DNA polymerase assay reaction mixture, said reaction mixture being particularly distinguished in containing a template consisting of a synthetic deoxyribonucleotide oligomer containing 2 to 24 nucleotide units per oligomer molecule hybridized with a RNA-type polymer selected from the group consisting of rA, rU, rG, rC and rI, and deoxyribonucleoside triphosphates complementary to said RNA-type polymer, at least one of which is isotopically labeled; incubating said mixture whereby polymeric product is formed under the direction of said template; terminating reaction in said mixture; and measuring the incorporation of labeled nucleotide into said polymeric product wherein the amount of said incorporation is proportional to the presence of RNA-dependent DNA polymerase activity.

2. The method of claim 1 wherein said synthetic nucleotide oligomer is a linear thymidine polynucleotide.

3. The method of claim 2 wherein said synthetic nucleotide oligomer is d-pT₉.

4. The method of claim 2 wherein said synthetic nucleotide oligomer is d-pT₈.

5. The method of claim 2 wherein said synthetic nucleotide oligomer is d-pT₇.

6. The method of claim 2 wherein said synthetic nucleotide oligomer is d-pT₆.

7. The method of claim 1 wherein said RNA-type polymer is rA.

8. The method of claim 1 wherein said sample is obtained from human leukemia cells.

9. The method of claim 1 wherein said sample is a mammalian cellular extract obtained from whole blood which has been purified by running it through a glycerol gradient.

10. The method of claim 9 wherein said reaction mixture comprises 100-200 μ g. of purified sample, 1-5 μ g. of said template; and is 4×10^{-3} M with respect to K⁺ and 6×10^{-3} M with respect to Mg⁺⁺; and contains 800 m μ moles/ml. of two deoxyribonucleotides complementary to said template, at least one of which is isotopically labeled having a specific activity of about 500 cpm/pmole.

11. The method of claim 1 wherein said sample is a purified plasma obtained from whole blood purified by treatment with Kieselguhr and said reaction mixture comprises 25 μ g. of said purified sample; is 0.006 M in Mg⁺⁺ is 0.04 M in K⁺, is 0.008 M in a first complementary nucleoside triphosphate and is 0.00016 M in an isotopically labeled second complementary nucleoside triphosphate having a specific activity of about 100 cpm/pmole, and contains about 1 μ g. of said synthetic template.

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