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The Clinical Applications of Reverse Transcriptase for the Early Detection of Cancer

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THE CLINICAL APPLICATIONS OF REVERSE TRANSCRIPTASE FOR THE EARLY DETECTION OF CANCER

by

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INTRODUCTION

Viruses have been implicated as oncogenic agents as early as 1903 by Borel (1). Utilizing recently developed chemical purification techniques, numerous tumor filtrates have yielded virus consistently associated with oncogenicity (1). However, other animal cancers morphologically similiar to those produced by viruses have failed to yield virus. Experiments with the oncornaviruses have demonstrated (2) that in the proper host tumors could be induced. Little or no virus was recovered from these transformed cells and no cytopathic effect on the cell was observed. This transformed state remained in daughter cells with no virus production. However, the question of whether or not certain viruses are the etiologic agents of certain (or all) cancers remains unanswered.

Unlike other infectious agents, viruses contain either ribonucleic (RNA) or deoxyribonucleic acid (DNA) as their genomic materials. Tumorogenic viruses have been isolated from both the RNA- and DNA-containing groups. The RNA-containing viruses (oncornaviruses) have been implicated in leukemias in chickens, mice, and cats (1) and a mammary carcinoma in mice (2). The DNA-containing viruses have been established as the causative agent for Marek's disease (3) in turkeys and strong evidence has been advanced that these viruses are etiologic agents for certain types of cervical carcinomas in humans (4).

To date the viral etiology of any human cancer has not been proven conclusively. However, many recent studies (5,6,7,8,9) in vitro and in vivo on known animal tumor viruses indicate several possible mechanisms by which occult viruses or genetic viral information could reside within a cell for hundreds of cell generations before being expressed.

Other studies (10) aimed at developing antiviral chemotherapeutic agents showed that many drugs capable of blocking viral specific DNA metabolism also interfered with the tumorogenic activities of DNA-containing viruses. An adventitious finding was that some of these same drugs inhibited transformation by oncornaviruses (11,12). Since the oncornaviruses contained only RNA as their genomic material, these experiments suggested a DNA dependent step in transformation, a potential reverse transcription from the genomic RNA to a DNA intermediate. At this time, 1964, the concepts of nucleic acid metabolism (Figure 1) proposed by Watson and Crick (13) did not include transcription from RNA to a DNA product (reverse transcription).

In an effort to explain the above (or certain of the above) results Temin (14) proposed the provirus theory. This theory was based upon the well known phenomenon of bacteriophage lysogenicity together with observations on the effect of metabolic inhibitors on oncornavirus replication. Essentially, the theory proposed required the horizontal transmission of the intact viral RNA by transcription of the viral genome to a DNA copy. This would represent a reversal of the accepted scheme of transcription of DNA to RNA. The

resultant DNA copy would then be integrated into the cell's genome and remain latent until some future stimulating or derepressing event. It was not until a decade later that Temin (15) and Baltimore (16) established independently and conclusively the presence of an enzyme which could catalyze the reverse transcription. The enzyme, termed reverse transcriptase (RT), was isolated from two different oncornaviruses. Following these original observations all oncornaviruses examined have yielded RT and all non-tumorogenic RNA viruses tested, with the possible exceptions of Visna and foamy viruses, have lacked this enzyme activity (17).

Recently Temin has revised his provirus theory (now known as the protovirus theory) now stating that it is unnecessary to transmit an intact genome for tumor induction (18,19). Supportive experimental evidence for this model was the demonstration (20,21) of an endonuclease and a ligase in the virion. These enzymes were presumed to aid in the insertion of the viral DNA copy by uncoupling the cell's genome and coupling the viral DNA copy to this genomic material. Two general types of oncornaviruses were proposed by Temin - complete and incomplete viruses. Complete viruses were capable of the normal horizontal transmission of genetic information and ultimately complete oncogenic expression with virion production. Incomplete viruses integrated a partial DNA copy of viral genome and acted in conjunction with a normal cellular component for oncogenic expression. In recent studies Temin (22,23) has established that reverse transcription is a normal process during differentiation. He postulated that when the

incomplete viral gene is located near or adjacent to a host gene, which undergoes transcription during differentiation, then the viral gene may be altered to an oncogene expressing its oncogenic potential.

Alternate proposals have been made to explain the viral transformation of normal cells to the tumorogenic state and the inability to isolate virus from the induced tumor. Huebner (24,25) advanced the oncogene theory which states that all cells of the body possess foreign genetic information inserted during evolutionary development. Malignancy is expressed by derepression of the replication of this genetic material. The primary difference between Huebner's and Temin's hypotheses is that the former proposed the oncogene's presence in all cells (because of in ovo transfer) while the latter asserted that the oncogenic information was present only in transformed cells. Credence has been given to the oncogene theory through studies in many laboratories (5,7) in which diverse somatic cells, not known or demonstrated to have virus infections, were induced to produce viruses on exposure to the nucleosides 5-bro- or 5-iododeoxyuridine (IUDR or BUDR). These viruses were invariably C-type particles which have been frequently associated with oncornaviruses.

Since the original discovery of a RT several hundreds of publications have confirmed and extended these observations. There remains little doubt that all oncornaviruses of animals have a RT (2), that the levels of RT activity can be used to indicate the concentration of virus (19), and that RT activity in

animal tissues is often associated with the presence of oncornaviruses (19). Based upon these facts, several laboratories assayed the levels of RT activity in those human tumor tissues which closely resembled their virus-containing animal counterparts. These studies showed that the enzyme was associated with 70S RNA from patients with leukemias (26,27), sarcomas (28), lymphomas (29, 30), and breast cancer (31,32). The significance of these findings is that the 70S RNA is unique to RNA tumor viruses (2) and that the enzyme activity is frequently associated with the electron microscopic observations of C-type viral particles found in these tissues (19).

The purpose of the study to be presented was to develop an assay clinically applicable to detect tumorous tissue and to apply the test as a screen for the presence of unrecognized developing tumors. The RT assays previously employed had been primarily developed as experimental laboratory procedures and were not applicable for routine clinical screening. Several obstacles were obvious immediately. First, all human tumors were probably not of viral origin and certainly not exclusively induced by oncornaviruses. Second, the enzyme must be detectable in a small clinical sample. Third, the enzyme must be detectable in a biologically heterogeneous sample since exhaustive purification of 70S RNA seemed beyond most laboratory capabilities.

MATERIALS

The artifical oligonucleotide, oligodeoxythmidine·polyribo-adenosine nucleic acid (oligo dT·poly rA) was obtained from Collaborative Research, Inc. Thymidine methyl-H^3 5'-triphosphate, tetrasodium salt was obtained from New England Nuclear. The deoxyribonucleotide triphosphates were purchased from Sigma Chemical Company. Highly polymerized calf thymus DNA, type V, was from Sigma Chemical Company. Purified murine Rauscher leukemia virus (RLV) was received from Electro-Nucleonics Laboratories, Inc. Nonidet (NP-40) was a gift from the Shell Oil Company. All routine chemicals used were purchased from Sigma Chemical Company. Heparin was purchased from The Upjohn Company. Ready-Solv scintillation fluid was from Beckman Chemicals and Scintisol Complete was purchased from Isolab, Inc.

METHODS

Assay Procedures for Reverse Transcriptase

Since the purpose of this study was to develop a clinically adaptable assay for RT, the known methods for RT assay were first tested and modified as necessary. For simplification the various procedures are presented in chronological order, with modifications given in the text.

Method I

The method of Kiessling (33) was examined first for the following reasons: 1) it appeared capable of detecting enzyme in small amounts of human plasma, 2) it did not require complicated concentration and purification procedures, and 3) it was not necessary to perform extensive purification procedures to obtain the enzyme and template. The unmodified reaction is detailed in the following description.

The reagents (Table 1) were dissolved in a total volume of 100 ml of water at pH 8.3. To this was added 10 µl of purified avian myeloblastosis virus enzyme. The mixture was incubated for a predetermined time and stopped by the addition of 100% trichloroacetic acid (w/v) (TCA). The precipitated material was collected immediately on a 0.2 millimicron (mµ) Millipore nitrocellulose filter (prior to use the filter was washed with 5 ml of washing mixture [WM] consisting of 8% TCA in 0.02M NaH_2PO_4 and 0.04M

$Na_4P_2O_7 \cdot 5H_2O$). The filter and TCA precipitate were first washed with 10 ml followed by 5 ml of WM, removed and placed in a scintillation vial. To this vial was added 0.5 ml of 0.5N perchloric acid and the vial was incubated at 70°C for 40 minutes. After incubation 10 ml of Ready-Solv scintillation fluid was added and the sample assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of approximately 30%.

Method II

This was the procedure used by Schlom and Spiegelman (31) for the detection of RT in human milk. Crude enzyme samples were suspended in 50 µl of 0.01M tris(hydroxymethyl)aminomethane hydrochloride (Tris)(pH 8.3) containing 0.03% NP-40 and 0.1M dithiothreitol (DTT) and incubated at 4°C for 10 minutes. To begin each reaction 10 µl of sample containing RT and the endogenous RNA template of the virion was added to a 100 µl volume of reaction mixture (Table 2). This was then incubated at 37°C for the specified time. The reaction was stopped by the addition of 10 µl of carrier DNA (1 mg/ml) and 0.3 ml of 100% TCA (w/v), modified from 10% TCA, and allowed to stand for 20 minutes. The terminated reaction mixture was filtered through a 0.3 mµ Millipore filter, modified from a ground glass filter, pretreated by immersion in WM followed by rinsing with 5 ml of WM. The reaction vessel was washed with an additional 5 ml of WM and finally treated as described previously in method I.

Method III

This procedure is that of Roy-Burman (34) designed for the detection of RT using the artificial template oligo dT·poly rA. The procedure, in our hands, yielded consistently reproducable data and, with only minor changes, was used throughout the remainder of the study. The sample was suspended in 50 µl of 0.01M Tris (pH 8.3) containing 0.03% NP-40 and 0.1M DTT and incubated at 4°C for 10 minutes. A 10 µl fraction of this preincubated sample was added to 100 µl of reaction mixture (Table 3) and incubated at 37°C for the indicated times. The reaction was stopped by the addition of 0.8 ml of WM (10% TCA [w/v] and 1% $Na_4P_2O_7 \cdot 5H_2O$) and 0.1 ml of carrier DNA (1 mg/ml). Roy-Burman had originally used a different buffer for the TCA, but this buffer proved to be better. It was then allowed to stand for 10 minutes and filtered through a 0.3 mµ Millipore nitrocellulose filter (prewashed with 3 ml of WM) using 18 ml of WM. The filter was then placed in the scintillation vial and 0.5 ml of 0.5N perchloric acid added. The vial was capped and incubated at 70°C for 30 minutes. Ten ml of Scintisol Complete was added and the sample assayed for radioactivity in a Packard Tri-Carb scintillation spectrometer.

Heparin Inhibition Study

Blood was collected in vacutainers containing heparin (28.6 USP/ml) or ethylenediaminetetraacetate (EDTA)(1.44 mg/ml), glass beads or with citrate-phosphate-dextrose solution (CPD). The

plasma collected with CPD was stored at -70°C prior to use; all other samples were collected on the day of the experiment. Plasma was separated from cells by centrifugation at 23°C for 10 minutes at 220 x g and clarified by centrifugation at 4°C for 10 minutes at 7800 x g. The particulate fraction was centrifuged at 4°C for 30 minutes at 215,000 x g, the supernatant fluid decanted and the pellet resuspended in 110 µl of virus buffer (0.1M NaCl, 0.01M Tris [pH 7.0], and 0.001M EDTA).

To detect RLV in the presence of the various anticoagulants, the virus was added subsequent to the clarification step. Studies on the effect of dilution of the plasma particulate fraction were carried out by first resuspending this fraction in 100 µl of virus buffer. Dilutions of 1:10 and 1:1000 were made with virus buffer. Fifty µl of RLV was added to a 50 µl portion of each dilution.

RLV was added to either virus buffer or virus buffer with heparin (57.2 USP/ml) to make a 1:1 (v/v) dilution for the kinetic study. A 10 µl fraction of either buffer A (0.05 µmoles DTT, 0.002 µl NP-40, 0.05 µmoles $MgCl_2 \cdot 6H_2O$, 5.0 µmoles Tris [pH 7.9]) or buffer A with heparin (28.6 USP/ml) was added to the reaction mixture during incubation where indicated. Assay method III was used for the quantitation of RT in all samples prepared.

Clinical Sample Preparation

Two problems were encountered in preparing clinical samples for assay. First, the anticoagulants used for the collection of plasma samples interfered with the assay by differing amounts.

Secondly, when the particulate fraction was pelleted by centrifugation, resuspension of the pellet was difficult. Two methods were evaluated for preparation of these samples before arriving at a satisfactory procedure.

Method I

Venous blood was collected in vacutainers containing EDTA (1.44 mg/ml). Plasma was collected by centrifugation at 1000 x g for 10 minutes at 23°C and clarified by centrifugation at 7800 x g for 10 minutes at 4°C. The clarified plasma was centrifuged at 215,000 x g for 30 minutes at 4°C and the pellet was resuspended in 110 µl of virus buffer with the aid of a rotating glass probe. A 100 µl portion of this suspension was removed and preincubated in 0.25% NP-40 for 10 minutes at 4°C. The RT activity was measured according to enzyme method III.

Method II

A plasma sample was centrifuged at 31,000 x g for 10 minutes at 4°C and the resultant pellet was resuspended in 100 µl of incubation mixture (1.5% NP-40 and 0.048M DTT) plus 100 µl of virus buffer followed by incubation for 10 minutes at 23°C. The RT activity was determined with the enzyme method III.

Method III

A plasma sample was placed in a nitrocellulose centrifuge tube containing a fitted flat-topped teflon boss with a circular 12 mm

coverslip on top of the boss. This centrifuge tube with plasma was then centrifuged at 31,000 x g (maximum radius) for 10 minutes at 4°C in a SW65 rotor. The pellet on the coverslip was resuspended with 250 µl of virus buffer and 100 µl of incubation mixture and incubated for 10 minutes at 23°C. The RT activity was determined using the enzyme assay method III.

RESULTS AND DISCUSSION

Kiessling's studies (33) indicated a quantitative difference in plasma polymerase activity between normal and chronic lymphocytic leukemia (CLL) patients. It should be emphasized however that his system, according to recent data (19,36) would not differentiate RT from other DNA polymerase activity present. The assay system was unique in that human plasma was used directly without gradient purification or collection of large quantities of leucocytes. The relative simplicity of the procedure indicated that it was a logical choice to monitor RT activity in cancer patients. The decision to determine the RT activity in human plasma was further supported by Gallo (17). Gallo reported an increased polymerase activity in acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), and chronic myelocytic leukemia (CML) when using plasma samples. The data in Figure 2 illustrate incorporation of thymidine monophosphate (TMP) over a 40 minute incubation at 37°C when Kiessling's procedure was repeated. This demonstrated that this procedure could be repeated in the laboratory using partially purified RT. The labelled thymidine triphosphate (H^3-TTP) used in these early experiments had a low specific activity (5 Ci/mM) when compared to that used in later procedures. Actually in the reaction mixture the specific activity was much lower (2 μCi/mM) because unlabeled TTP was also added to the reaction. This addition was made because the low concentration of TTP in the reaction with only

H^3-TTP was rate limiting for the polymerase assay. During 30 minutes a total of 800 counts per minute (cpm) of TMP was incorporated using method I.

Although easily performed and yielding satisfactory results, two important factors negated further use of this procedure. First, the template was nicked DNA which is not thought specific for RT (25,42,43). Second, the quantitative differences (33) between normal and CLL patients was only 500 cpm after a 90 minute incubation.

Shortly after the appearance of Kiessling's report Schlom and Spiegelman (31), studying RT activity in human colostrum, presented an RT assay for the endogenous RNA template of oncornaviruses. Figure 3 is a kinetic curve of a RT assay using method II and shows a linear incorporation, demonstrating that enzyme activity can be detected by using the RNA from the virion as the only template in the reaction. During 30 minutes only 400 cpm of TMP was incorporated using method II. Although only 400 cpm was detected, this incorporation was more representative of RT activity than was the incorporation observed using the nicked DNA template in method I since the endogenous RNA was utilized. This procedure gave valid experimental results, yet it was not readily adaptable to routine clinical screening because of the time required for isolation and purification of virus. It was obvious that a simpler, rapid and more specific assay was required.

In 1972, investigating DNA polymerase, Roy-Burman (34) reported experiments which showed an increase in TMP incorporation

which did not require complicated purification procedures. Furthermore, high specific activity (40 Ci/mM) for the TTP now became commercially available; the high specific activity would measurably increase assay sensitivity since more H^3-TTP would be available for incorporation into the DNA product. To increase the product formation the artificial template oligo dT·poly rA was added to the reaction mixture. This is a specific template for RT as evidenced by the work of Temin and Baltimore (19) and Wells (36). Results by Verma (35) demonstrated two separate enzyme activities within the virion, a RNA template dependent and a DNA template dependent activity, both forming a DNA product. The RNA-dependent activity was established and completed within the initial 10 minutes of incubation while the DNA-dependent activity was not established until a sufficient concentration of DNA was present in the reaction mixture. The best sample of a DNA product formed only because of RT activity should then be taken during the initial 10 minutes of incubation. Because the amount of DNA formed during this initial period was small, it was suggested by Verma that the second enzyme activity be utilized to amplify this initial RT product. It was further recommended that an incubation period of 30 minutes or less be used for amplification.

The results in Table 4 show a rate of TMP incorporation of 0.046 picomoles per minute with a backround of less than 0.01 picomoles. To demonstrate that this incorporation was virus dependent, the original concentration of virus particles was diluted and qualitatively correlated to TMP incorporation (Table 4).

Comparing the actual value to the expected value for 6.6×10^7 virions per ml, it was seen that assay method III was only able to detect a difference of one-half a power of ten of virions per ml. The undiluted sample was used as a reference point to compute the expected virus concentration when the TMP incorporation was 0.06 picomoles. It was determined that 3×10^6 virions per ml was expected. Also, the data presented in Table 4 show that the lowest level of detection by the RT assay method was at a concentration of 6×10^6 RLV virions per ml of virus buffer.

To further demonstrate that the assay was dependent upon a RNA template, RNase was incorporated into the reaction. By demonstrating that only 0.01 picomoles of TMP (Table 5) was incorporated when RNase was included into the reaction mixture, it was concluded that less than 5% of the DNA product formed was not dependent on RNA sensitive to RNase.

It has been reported (37) that various divalent cations have an effect on the sensitivity of the assay for RT activity, e.g., the presence of manganese cation increased TMP incorporation three-fold over that observed with the magnesium cation. However, the authors did not offer an explanation for the difference. The specificity (for a RNA template opposed to a DNA template) of the reaction was lowered when the manganese cation was used.

Using the procedure as described by Roy-Burman (34) for the detection of RT activity it was established that: 1) there was an increase in H^3-TMP incorporated into the DNA product over a 30 minute incubation period, 2) the product formed was dependent on

the amount of virus present, and 3) the reaction was RNase sensitive. With this evidence and support that the template in the reaction mixture was specific for RT activity (19,36), this procedure was used to detect RT activity in plasma samples.

Preliminary experiments were performed using a positive control consisting of human plasma to which known amounts of RLV were added. Attempts to detect RT in positive plasma control samples were negative unless the manganese cation was added to the reaction mixture. Table 6 presents the results of RT assays performed to determine the optimal concentration of NP-40. The optimum concentration range found was 0.10-0.25%. These results confirm a previous report (19) that the reaction was dependent on NP-40. It has been shown (19) that there is a direct relationship between protein concentration and NP-40 concentrations for this assay, i.e., less NP-40 is required at low protein concentration and vice versa. It was determined in this laboratory and other laboratories (19,31) that much lower concentrations of NP-40 were optimum for reactions not involving protein. This optimum concentration was 0.03%.

A report by Mueller (38) had suggested that heparin inhibited RT activity. The inhibitory effect on RT activity of various anticoagulants are presented in Table 7. As compared to virus RT activity in virus buffer, all reagents showed some inhibition. Clearly heparin showed complete inhibition while EDTA and glass beads ranked below CPD. Since glass beads were essentially inert the observed inhibition might have been due to plasma inhibitors

and/or to heparin which might have been released from disrupted white blood cells. To determine whether the inhibition noted may be a combination of the factors in the plasma and the method of collection, an experiment with virus added to virus buffer or to virus buffer with EDTA (1.44 mg/ml) was carried out. As shown in Table 8 EDTA caused about 13% inhibition while heparin was completely inhibitory. From Table 7 it can be seen that when the RT activity in the plasma collected with EDTA was compared to virus in virus buffer there was about 42% inhibition.

The potency of heparin as an inhibitor could be a factor in the assay of RT activity from certain tissues which might contain naturally occuring heparin. Table 9 presents data illustrating that, even with a 1000-fold dilution of plasma particulate fraction collected from heparinized plasma, there was significant inhibition of RT activity when compared to EDTA plasma. This demonstrated that at least 66% of the available RT activity was masked by heparin inhibition. A 10-fold dilution of plasma particulate fraction prepared from EDTA treated plasma abolished RT inhibition entirely. Although the plasma was collected with a known amount of heparin, the concentration of heparin in the plasma particulate fraction was unknown but was assumed extremely low.

It was of interest, since heparin might be used as a metabolic inhibitor, to determine whether the reverse transcription step or the normal DNA to DNA transcription step or both was inhibited by heparin. As shown in Figure 4, heparin added to the RT reaction at 5 minutes or 15 minutes caused immediate inhibition of the incorpor-

ation of H^3-TTP. Since the RNA template specific activity of RT was completed during the initial 10 minutes (35), the results in Figure 4 demonstrated that heparin inhibited both the RNA-dependent and DNA-dependent enzyme activities. It should be noted that although the reaction was stopped at these times, the amount of product remained constant demonstrating that the DNA product was stable over the incubation period.

The results of these experiments showed EDTA was the anti-coagulant of choice because it caused least inhibition of the assay. Plasmas were collected in EDTA-Vacutainers from 22 patients with various malignancies such as leukemias, lymphomas and breast cancers. One case each of a colon cancer and a melanoma were included in the assay. Of the plasmas assayed none showed TMP incorporation over backround. Since other workers (26,27,28,29,30, 31,32,33) had demonstrated RT activity with the same type of malignancies it was obvious that a reevaluation of the assay method was necessary.

When a known amount of RT, in the form of virus, was added to plasma the recovery of the RT activity was only 10-15% of the activity obtained when the RT was assayed without plasma presence or centrifugation. The lowered recovery of virus particles was apparently due to the formation of a pellet which could not be completely resuspended. Next a procedure used by Mathews and Buthala (39) to form an even layer of virus particles instead of a clumped pellet was attempted. This newer method used a flat-topped teflon boss in the bottom of the centrifuge tube, supporting a flat

collecting surface for collecting virus particles. Plasma, contaminated with RLV particles, when sedimented at 31,000 x g for 10 minutes at 4°C consistently had a recovery rate of 50% when calculated as the previous recovery rate.

Theoretically a 100 mμ particle, the approximate size of oncornaviruses, will take 180 minutes at 31,000 x g to be pelleted. Although little RT activity would be expected using method III, the pellet from contaminated plasma incorporated 2.41 picomoles of TMP. Even at 38,000 x g, a 100 mμ particle would be expected to require 20 minutes to sediment 99.9% of the particles. To establish that this unusual result was not dependent only on the presence of plasma, the same amount of virus, 3×10^9 virions, was added to virus buffer and treated identically. An incorporation of 3.64 picomoles of TMP was found with pelleted material. When the supernatant was further centrifuged at 215,000 x g for 30 minutes at 4°C, sufficient to pellet 99.9% of the remaining virus particles, only 0.15 picomoles of TMP incorporation due to RT activity was detected. These results indicated that the RLV particles formed aggregates when in human plasma or virus buffer, and that 96% of the virus particles (in virus buffer) could be pelleted by centrifugation at 31,000 x g for 10 minutes at 4°C. Only 64% of the available particles in plasma were pelleted. The question remains as to whether the plasma particulate fraction interfers with the RT assay or whether the plasma itself impedes sedimentation. Using this procedure it was calculated (Table 10) that approximately 10^7 virions per ml of human plasma (collected

with CPD) was the minimum concentration detectable.

To attempt further dispersion of the virus particles the mucilaginous material deposited on the coverslip was treated with hyaluronidase with the results presented in Table 10. Although the mucilaginous material dissolved, as noticed by visual observation after 10 minutes preincubation at room temperature, the use of the hyaluronidase during the preincubation did not affect the percent recovery (see Table 10). The incubation of the coverslip and virus with hyaluronidase over a 24 hour period, to ensure complete hyaluronidase treatment, resulted in a decreased TMP incorporation. In view of these results, it was decided that use of hyaluronidase was of no benefit as a solubilizing agent.

Using clinical preparation method II, four different subjects were examined for presence of RT activity in plasma collected with EDTA. One patient had clinically evident acute granulocytic leukemia (AGL) and the other three subjects were normal healthy individuals. These results are presented in Table 11. Plasma samples were taken from the cancer patient on three different dates. The levels of TMP incorporation in all subjects was low, and no significant difference was seen between the normal subjects and the cancer patient. It should be remembered that this method had an enzyme recovery rate of only 10-15%.

Later, the improved preparation method III was used (Table 12) to analyze the RT activity in plasmas of 12 additional patients. One normal subject, K and the AGL patient, W, were included within the sample. Even though the percent recovery of enzyme activity

was 50% using this method, low levels of TMP incorporation were again found in all subjects. No significant difference between normal and cancer subjects, using the magnesium cation, was observed. Since, as stated earlier, the manganese cation increases the sensitivity of the enzyme assay, replacement of the magnesium cation with manganese was attempted in a normal and an AGL subject. As reported in Table 12, a greater sensitivity was observed using this cation. Although more TMP incorporation was found, less RT activity was observed in the AGL patient than in the normal subject. Because of the apparent lack of specificity, as predicted (37), use of the manganese cation was discontinued.

The RT assay was developed to yield presumptive evidence predicting malignancy. Although no oncornavirus has yet been demonstrated as an etiologic agent for a human malignancy, evidence (26,27,28,29,30,31,32) indicates that some malignant diseases increase the RT activity in human tissues. No significant difference was observed between the enzyme activities of normal and cancer subjects. Evidence of others predicted that some leukemias and lymphomas (26,27,29,30) would have elevated enzyme activities. Therefore, it must be concluded that the enzyme assay was not successfully adapted as a rapid screening procedure for any malignancy tested.

An attempt was made to determine whether RT activity could be correlated with virus titer in tissue culture fluid. This would provide an investigator with a simple and rapid assay procedure. A RLV sample obtained from the laboratories of The Upjohn Company

having a titer with 2.6×10^5 infectious units per ml of tissue culture fluid was assayed for RT activity two different times. The virus had been stored frozen and the titer was established only before storage at -90°C. The preliminary assay showed a titer of 0.23 picomoles per ml and after one month, using another sample, a titer of 0.07 picomoles per ml was found. The first sample volume was only 0.3 ml compared to a much larger 1.75 ml in the second RLV sample. Because the results with the enzyme assay showed a three-fold difference and because this assay system has been shown (page 16) to detect only one-half a power of ten difference in virus concentration, use of the RT assay system to quantitate viral presence is limited.

SUMMARY

This study was originally designed to examine rapid, clinically suitable methods to detect cancer using the criteria of RT activity. RT had been demonstrated by others (26,27,28,29,30,31,32,33) to be a potential predictive signal of some forms of oncogenesis. Three assay methods were tested, with the selection of the procedure described by Roy-Burman (34). The procedure was dependent on: 1) virus presence, 2) RNA template presence, 3) specific cations, and 4) detergent (NP-40) concentration. Although the enzyme assay was dependent on the specific cation used, others (37) had reported that the manganese cation was less specific for the RNA template than was the magnesium cation. Perhaps the manganese cation acts to shift the equilibrium of the reaction toward DNA replication and the early enzyme activity dependent on a RNA template is shortened.

Heparin and other anticoagulants were shown to cause inhibition of the RT activity in biological samples (Tables 7 and 8). Inhibition by heparin was especially significant because of its possible presence in biological samples. The heparin inhibition affected both early (RNA template) and late transcription (DNA template) suggesting that it was not a selective inhibitor of RT.

As the methods for adapting Roy-Burman's assay method to a clinical system evolved, attempts were made to increase the recovery of virus from the collected plasma samples. Originally the

procedure was capable of recovering 10-15% of the available RT activity, but after modifying the procedure to avoid a pellet, recovery of 50% was achieved. Also, during the evolution of the clinical assay method, it was shown that the virions aggregated while in solution. The final procedure for use in clinical systems was able to detect approximately 10^7 virions per ml of human plasma or greater.

Plasma particulates from normals and patients with malignancies were assayed for RT. No significant difference was found between normal subjects and subjects with malignancies. The lack or significant difference was probably due to the inability of the assay to detect the levels of RT found in human plasma. Still, the possibility of RT not being present at any level within the samples tested cannot be discounted. Because of these results it was concluded that the clinical assay system developed was not suitable for detecting differences in RT activity between subjects.

Although the clinical assay system was not able to detect RT activity in the subjects tested, an attempt to demonstrate the utility of this procedure to detect oncornaviruses in tissue culture was made. Because this system was biologically less complex, some utility was shown in preliminary experiments. More work will need to be done to further establish the effectiveness of the assay in tissue culture.

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FIGURE 1. - Molecular-Genetics Model
Known in 1964

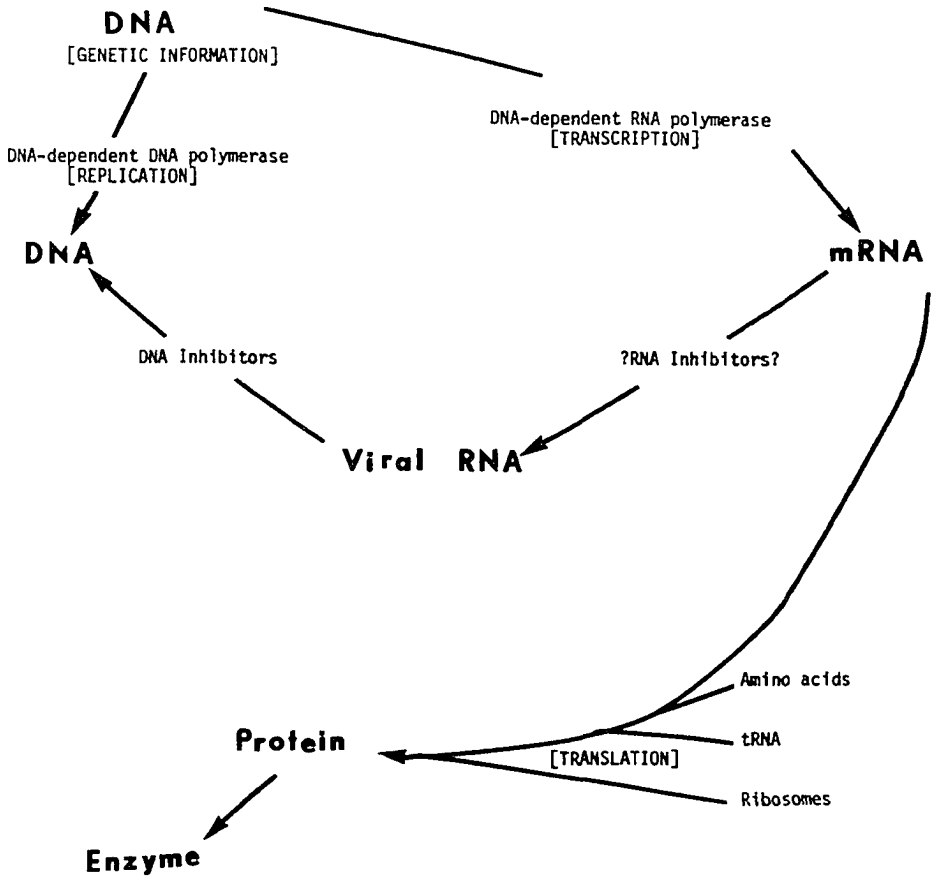
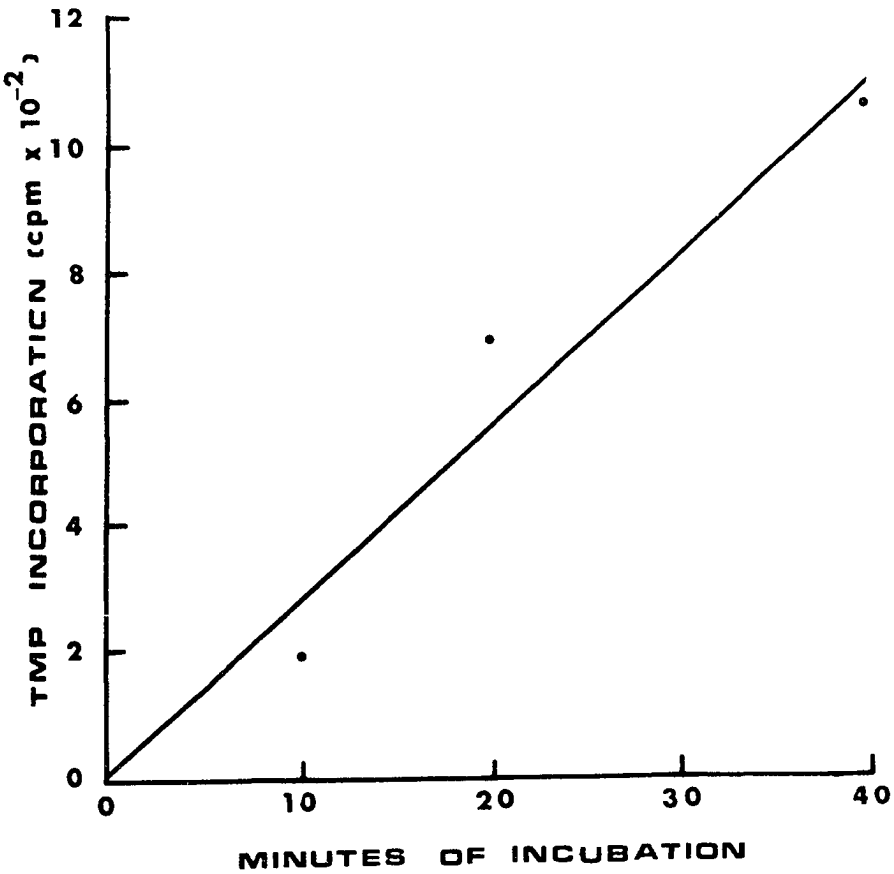
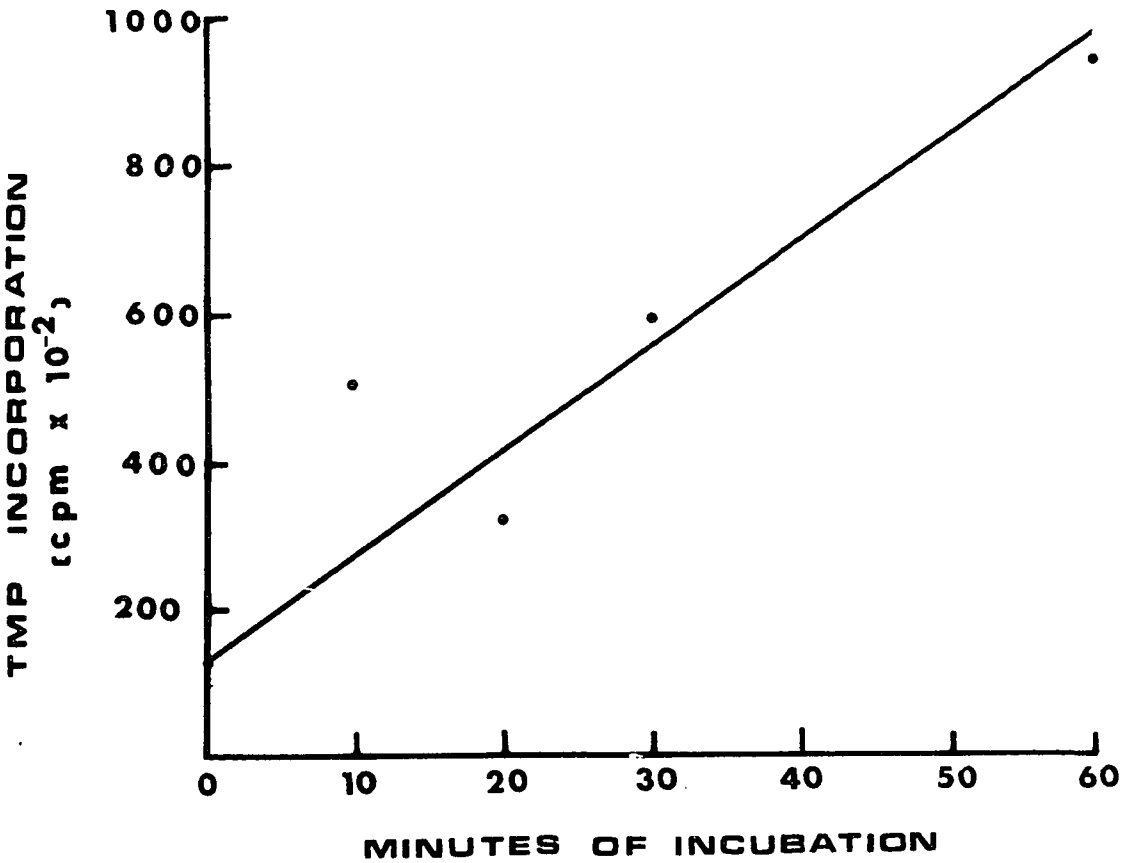


FIGURE 2. - Thymidine Incorporation due to RT using Method I[a]



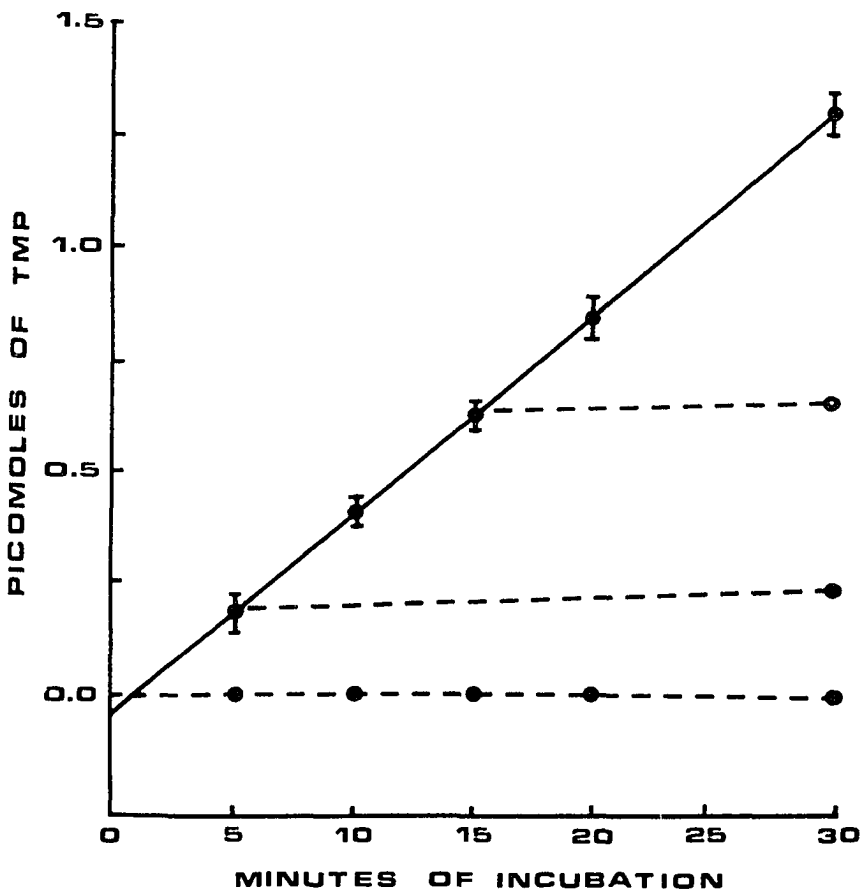
[a] In virus buffer

FIGURE 3. - Thymidine Incorporation due to RT using Method II[a]



[a]In virus buffer

FIGURE 4. - Heparin as a Metabolic Inhibitor[a]



[a]Heparin added at 0, 5, or 15 minutes (-----) or virus buffer with no heparin added (——) at 0, 5, or 15 minutes. All samples were incubated at 37°C and assayed with method III.

TABLE 1. - Reagents for the Reaction Mixture or Assay Method I

Reagent	Amount [a]
Tris (hydroxmethyl) aminomethane hydrochloride (Tris)	8.0 µmoles
NaCl	12.0 µmoles
$MgCl_2 \cdot 6H_2O$	1.2 µmoles
Reduced glutathione	0.74 µmoles
DNase nicked myeloblastic DNA	0.01 ml
H^3-thymidine 5'-triphosphate (H^3TTP)	0.02 µCi
Thymidine 5'-triphosphate (TTP)	4.0 pmoles
Deoxycytidine 5'-triphosphate (dCTP)	0.4 nmoles
Deoxyadenosine 5'-triphosphate (dATP)	0.4 nmoles
Deoxyguanosine 5'-triphosphate (dGTP)	0.4 nmoles

[a] Abbreviations are: micromoles = µmoles, curies per millimolar = Ci/mM, picomoles = pmoles, and nanamoles = nmoles, microcuries = µCi

TABLE 2. - Reagents for the Reaction Mixture of Assay Method II

Reagents	Amounts
Tris (pH 8.3)	5.0 µmoles
$MgCl_2 \cdot 6H_2O$	0.37 µmoles
NaCl	0.21 µmoles
dATP	0.16 µmoles
dCTP	0.16 µmoles
dGTP	0.16 µmoles
TTP	0.87 nmoles
H^3-TTP	0.02 µCi

TABLE 3. - Reagents for the Reaction Mixture of Assay Method III

Reagents	Amounts [a]
Tris (pH 7.9	5.0 µmoles
$MgCl_2 \cdot 6H_2O$	0.5 µmoles
NaCl	2.0 µmoles
DTT	0.5 µmoles
NP-40	0.02 µl
Oligo dT·poly rA	2.0 µg
H^3-TTP	2.0 µCi

[a]Abbreviations are the same as in Table 1 with the addition of: micrograms = µg

TABLE 4. - Picomoles of TMP Incorporated at Various RLV Dilutions[a]

Virions per milliliter	TMP Incorporation[b,c] (picomoles) Actual	Expected
7.2×10^8	1.39	----
6.6×10^7	0.06	0.13
6.0×10^6	0.01	0.01
5.5×10^5	0.00	0.00
5.0×10^4	0.00	0.00

a
The samples were preincubated in 0.03% NP-40 for 45 minutes at 4°C and then incubated for 30 minutes at 37°C with method III (all samples were in virus buffer)

b
Calculated from the dilution factor and the TMP incorporation of the undiluted sample (backround subtracted from all values)

c
Thymidine monophosphate = TMP

TABLE 5. - Sensitivity of the Artificial Template to RNase[a]

Incubation Time (minutes)	RNase	TMP Incorporation (picomoles)
0	absent	0.00
0	present	0.00
30	absent	0.22
30	present	0.01

a
The samples were preincubated in 0.03% NP-40 with RNase (0.13 milligrams per ml) or without for 45 minutes at 4°C

TABLE 6. - Effect on NP-40 Concentration on RT Assay[a]

Final Concentration of NP-40 (percent)	TMP Incorporation (picomoles)
0.05	0.04
0.10	0.05
0.25	0.05
0.50	0.02

[a] After adding 10^8 virus particles to human plasma collected with the anticoagulant, heparin, the virus particles were separated from the plasma by centrifugation at 215,000 x g for 30 minutes at 4°C. This pellet was resuspended in various concentrations of NP-40 in 0.1M DTT and examined for RT activity with enzyme method III.

TABLE 7. - The Effect of Various Anticoagulants on RT Activity in Plasma

Anticoagulant	ul of RLV	TMP Incorporation[a] (picomoles)
EDTA (1.44 mg/ml)	100	0.72 ± 0.03
Glass beads	100	0.66 ± 0.03
Heparin (28.6 USP/ml)	50	0.00
CPD[b]	100	0.83 ± 0.03
CPD[b]	50	0.41 ± 0.02
Virus buffer (no plasma)	100	1.24 ± 0.05

a
All reactions were incubated for 30 minutes at 37°C

b
These values were not obtained at the same time as other values presented. The value for CPD with 50 µl of RLV was the average of two different days, each run in triplicate.

TABLE 8. - The Effect of EDTA and Heparin on RT Activity in Virus Buffer[a]

Anticoagulant	TMP Incorporation (picomoles)	% Inhibition[b]
None	0.78 ± 0.03	0
EDTA (1.44 mg/ml)	0.68 ± 0.03	13
Heparin (2.40 USP/ml	0.00	100

a
All reactions were incubated for 30 minutes at 37°C with 1.5×10^{6} virus particles per reaction mixture

b
Percent inhibition calculated by comparison to no anticoagulant

TABLE 9. - Effect of Diluting the Anticoagulant on the RT Activity[a]

Dilution	Anticoagulant[b]	TMP Incorporation (picomoles)
No Dilution	EDTA	0.46
1:10	EDTA	0.98
1:1000	EDTA	0.88
No Dilution	Heparin	0.00
1:10	Heparin	0.01
1:1000	Heparin	0.66

a
All reactions were carried out at 37°C for 30 minutes with a virus concentration of 3×10^8 particles per reaction

b
The concentration of EDTA was 2.44 mg/ml and the concentration of heparin was 28.6 USP/ml

TABLE 10. - Use of Coverslip and Hyaluronidase to Increase the Percent Recovery of RT Using the Clinical Sample Preparation Methods II and III

Clinical Methods	Hyaluronidase[a]	TMP Incorporation (picomoles)	% Recovery[b]
II	none present	0.14	32
III	none present	0.44	100
III	present during preincubation	0.43	98
III	present 24 hours prior to preincubation	0.27	61

a
The hyaluronidase (Wydase) concentration was 37.5 NF units per preincubation volume. The visible mucoid substance was absent after incubation with hyaluronidase.

b
% Recovery was calculated by comparison to method III with no hyaluronidase present

TABLE 11. - RT Levels in Human Plasma
Using the Method II

Date	Patient	Diagnosis[a]	TMP Incorporation (picomoles)
5-4	W	AGL	0.01
5-7	W	AGL	0.00
5-10	K	Normal	0.01
5-10	H	Normal	0.02
5-10	Wa	Normal	0.02
5-14	W	AGL	0.01

[a] Abbreviations: AGL = acute granulocytic leukemia

TABLE 12. - Levels of RT in Various Cancer Patients Using the Clinical Sample Preparation Method III

Date	Patient	Diagnosis[a]	TMP Incorporation (picomoles)	
			Mg	Mn
5-24	B	AGL	0.02	-
5-24	P	CGL	0.02	-
6-6	Pe	ALL	0.01	-
6-6	W	AGL	0.01	-
6-6	B	AGL	0.01	-
6-9	C	AGL	0.01	-
6-21	V		0.02	-
6-21	G		0.01	-
6-21	I		0.01	-
6-29	Br	AGL	0.00	-
6-29	Pl	AGL	0.01	-
7-1	Pl	AGL	0.01	-
7-3	Pl	AGL	0.02	0.05
7-3	K	Normal	0.02	0.11
7-20	Va		0.03	-
7-20	O	Lymphoma	0.00	-
7-20	A		0.01	-

[a] Abbreviations: AGL = acute granulocytic leukemia, CGL = chronic granulocytic leukemia, and ALL = acute lymphoblastic leukemia