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New Prospects for the Diagnosis of Viral Infections

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The diagnosis of viral infections is important for the accurate management of patients with infectious diseases and for the monitoring of the course of epidemics in susceptible populations. The utility of traditional viral diagnostic assays is limited by the time, expense, and expertise required for the performance of tissue culture techniques. Similarly, the application of immunoassay techniques has been inhibited by the limited degrees of sensitivity and specificity which can be attained by most immunoassay methods.

Recently, techniques for the identification of DNA and RNA have been applied to the detection of viral nucleic acids in clinical samples. Such assays have a number of potential advantages over corresponding immunoassays directed at the detection of viral antigens. In order to be generally applicable to clinical diagnosis, however, formats for the detection of viral nucleic acids have to be devised which allow for the reproducible quantitation of target DNA or RNA in human body fluids. Furthermore, formats need to be devised which allow enhanced assay sensitivity while maintaining high degrees of specificity and reproducibility. The use of non-isotopic labeling, liquid-phase hybridization, and target amplification techniques offers partial solutions to these problems. The development of practical assays for the detection of viral nucleic acids under a broad range of clinical and laboratory conditions would represent a major advance in the ability of physicians to care for patients with suspected infections.

Since 1980, we have witnessed an epidemic of a devastating new viral disease and a corresponding search for new methods for disease treatment and prevention. During the course of the acquired immunodeficiency syndrome (AIDS) epidemic, the expanding knowledge of the basic science of retroviruses has been applied rapidly to the development of diagnostic tests for the identification of infected individuals and for the monitoring of disease transmission. The knowledge gained from the application of these diagnostic tests has been invaluable in the understanding of the epidemic and in the development of strategies for disease prevention and treatment [1–6].

The most important recent development in the area of viral diagnosis has been the application of nucleic acid hybridization techniques to the direct detection of viral nucleic acid in human body fluids. Such assays utilize labeled deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) complementary to viral genomes extracted from virions in clinical samples. It should be noted that this technique is a direct extension of a great deal of research effort which has been applied to the understanding of the biochemistry and biophysics of nucleic acid interactions in aqueous solutions and on solid-phase surfaces. Furthermore, the vast body of information relating to the nucleotide sequences of pathogenic viruses can be directly applied to the construction of nucleotide probes and to their use for viral detection and characterization.

Abbreviations: AIDS: acquired immunodeficiency syndrome CMV: cytomegalovirus DNA: deoxyribonucleic acid HIV: human immunodeficiency virus RNA: ribonucleic acid

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Nucleic acid hybridization offers a number of advantages in terms of the accurate diagnosis and study of viral diseases [7–10]. First, the specificity of nucleotide base-pair interactions allows definitive identification of viral sequences in human body fluids. This specificity should permit the direct identification of genetic markers of viral pathogenesis. Such identification should result not only in the accurate diagnosis of viral infections but also in the accurate prediction of the disease process and in the efficient targeting of antiviral chemotherapy.

Another important advantage of nucleic acid hybridization techniques is that they are capable of detecting viral DNA sequences which are not expressing RNA and which are not being translated into measurable proteins. This property allows the detection of virus in a latent state before the complete clinical expression of the disease, which has proven to be valuable for the study of complicated disease processes such as those associated with human herpesviruses [11] and human immunodeficiency virus infections [12].

Another advantage of nucleic acid hybridization techniques is that they allow the detection of new viral pathogens before the antigenic nature of the viruses is elucidated. Since the sequencing of viral genomes can be accomplished fairly rapidly, this process permits the implementation of diagnostic assays soon after a viral species has been identified as a possible human pathogen. Finally, the fact that nucleic acids can be easily separated from proteins allows the detection of viruses which are bound to endogenous antibodies in the form of immune complexes or which are otherwise inaccessible to labeled antibodies [13]. It is thus particularly effective to utilize nucleic acid hybridization techniques late in the course of illness when the ability to detect infection by conventional techniques has diminished due to the generation of a host immune response to viral antigens [14]. Assays for the direct detection of viral nucleic acids have been developed for a number of medically important viruses, including herpes simplex virus [15], cytomegalovirus (CMV) [16], Epstein-Barr virus [17], varicella-zoster virus [18], rotaviruses [19], adenoviruses [20], papilloma viruses [21], rabies [22], and human immunodeficiency viruses.

Despite these advantages, there are still some limitations to the widespread application of nucleic acid hybridization techniques to the clinical diagnosis of viral infections. One limitation is related to the fact that hybridization techniques are often not sufficiently sensitive for the detection of all viral nucleic acids in human body fluids. The level of sensitivity is a particular problem when non-isotopic probes are utilized [23–27]. Since it is difficult for clinical laboratories to utilize probes such as ^{32}P at high levels of specific activity, this limitation is particularly problematic for diagnostic testing. Another limitation of standard nucleic acid hybridization technology for practical viral diagnosis is related to the solid-phase Southern transfer or "dot-blot" detection formats which are widely used in research laboratories. While such formats can be quite effective for the detection of relatively pure preparations of nucleic acids, they are difficult to apply in situations in which the nucleic acids are not fully exposed but are contaminated with viral and host proteins. Such proteins can compete with the nucleic acids for binding sites on the solid phase and thus reduce the diagnostic efficiency of the hybridization systems for the diagnosis of viral infections.

There are a number of methods which can be utilized to improve the diagnostic utility of nucleic acid hybridization procedures for the detection of viral nucleic acids in crude clinical samples. One way of improving the kinetics and practicality of nucleic acid hybridization reactions is by the performance of hybridization reactions in a

fluid-phase environment. Since the kinetics of nucleic acid reassociation are more favorable in fluid phases as compared to the kinetics of reassociation on a solid-phase surface, hybridization reactions can be performed more rapidly in such systems. Also, such systems are not as limited by the presence of extraneous materials which can compete for binding sites on the solid-phase surface [29]. Formats which make use of fluid-phase reactions are thus more easily adaptable to the detection of viral nucleic acids in crude clinical samples.

One system which utilizes mixed solid-phase and liquid-phase reactions makes use of non-overlapping oligonucleotides in a "sandwich" hybridization format. In this system, one oligonucleotide which is complementary to a target sequence is bound to the solid phase, such as the well of a microtiter plate. A second oligonucleotide, which is complementary to a juxtaposed but non-overlapping sequence on the viral genome, is labeled with a detectable marker and reacted with nucleic acids extracted from the clinical specimen [9,20]. If the specimen contains the virus to be measured, the labeled marker is bound to the complementary viral nucleotides which are, in turn, bound to the sequences immobilized on the solid-phase surface. Following the removal of extraneous material by washing, the reaction is quantitated by the measurement of the appropriate label. This format has been utilized to detect a number of viral antigens in body fluids. While the system originally utilized radioactive labels, it can also be adapted to make use of enzymatic and high-energy fluorescent markers [30].

Another approach to the performance of liquid-phase hybridization involves the re-annealing of labeled probe and target sequences in liquid phase and the subsequent binding of the nucleic acid hybrids to a solid-phase surface. This binding can be accomplished by means of affinity columns or microtiter plates. In the latter case, one method of binding nucleic acid duplexes to a solid phase makes use of antibodies directed at DNA-RNA hybrids [31,32]. The format for such systems is presented in Fig. 1. In this system, biotin-labeled DNA is utilized for the detection of viral RNA and biotin-labeled RNA is utilized for the detection of viral DNA. Following completion of the hybridization reaction in liquid phase, the probe is bound to the solid phase by means of reaction to avidin or anti-biotin antibodies. The presence of DNA-RNA hybrids is quantitated by reaction with enzyme-labeled anti-DNA-RNA antibody and enzyme substrate using this assay system.

As little as 10 pg/ml of target RNA can be detected, using monoclonal hybridization formats. This level of sensitivity can be obtained following 20 minutes of substrate incubation, provided that substrates generating fluorescent products are utilized to quantitate the enzymatic reaction (Fig. 2). When the colorigenic substrate nitrophenyl phosphate is utilized, a reaction period of more than two hours is required to attain this degree of sensitivity (Fig. 3). Thus convenient colorimetric reactions with alkaline phosphatase-labeled antibody can be used for this reaction system, provided that longer incubation periods are utilized. The sensitivity of these assays compares favorably with those which utilize radiolabeled probes for the detection of viral RNA [9,22].

Non-isotopic hybridization reactions allow the practical measurement of nucleic acids, provided that the target nucleic acids are present in sufficient quantities. The levels of sensitivity achieved are, however, somewhat less than those required for the diagnosis of all viral agents in human disease states.

A number of techniques have, however, recently been developed which can greatly amplify the quantity of nucleic acid in a clinical sample prior to measurement by

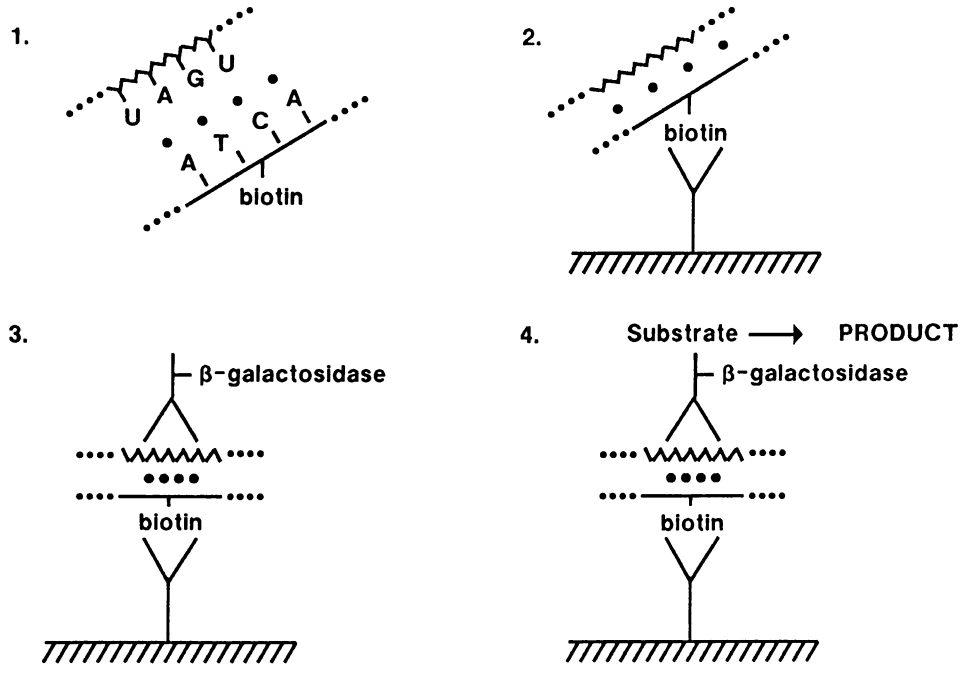

FIG. 1. Monoclonal hybridization assay for the detection of viral nucleic acids. In the first step of the assay, microbial RNA extracted from a clinical sample is hybridized to a biotinylated microbe-specific DNA probe. Following completion of the hybridization reaction, the test mixture is transferred to the wells of a microtiter plate coated with a goat antibiotin antibody (step 2). Biotinylated DNA-RNA hybrids bound to the solid phase are detected in a reaction with β-d galactosidase-labeled monoclonal antibody directed at DNA-RNA duplexes (step 3). The assay is completed by adding a fluorescent substrate to each well and by measuring the enzymatic reaction in microplate fluorometer (step 4).

means of nucleic acid hybridization protocols. One of the most widely used of these techniques makes use of the enzyme DNA polymerase and flanking oligonucleotide primers to generate amplified copies of a defined region. The steps of primer-target annealing and enzymatic generation of DNA can be repeated in a cyclical manner in a format which has been termed the polymerase chain reaction [33]. While DNA polymerase enzymes will only efficiently catalyze the amplifications of deoxyribonucleotides, the polymerase chain technique can be utilized to detect viral RNA, provided that the RNA is converted to complementary DNA by means of reaction with the primers and the enzyme reverse transcriptase [34]. The recent availability of a thermostable enzyme from the organism *T. aquaticus* has markedly improved the utility of this technique, since the use of this enzyme allows the performance of cycles of DNA generation, heat-induced strand separation, and re-annealing without the need to add additional enzyme at each cycle [35].

The polymerase chain reaction offers a number of important advantages for the detection of retroviral nucleic acids in clinical samples. In fact, polymerase chain reaction has been applied to the detection of human immunodeficiency virus (HIV) DNA and RNA in small numbers of samples. The technique has also been utilized for the detection of HIV CMV DNA in the blood and urine of patients with CMV infection [36,37] and HIV RNA and c-DNA in small numbers of patients with HIV infection [34,35,38–40]. In the latter case, evidence of retroviral c-DNA has been

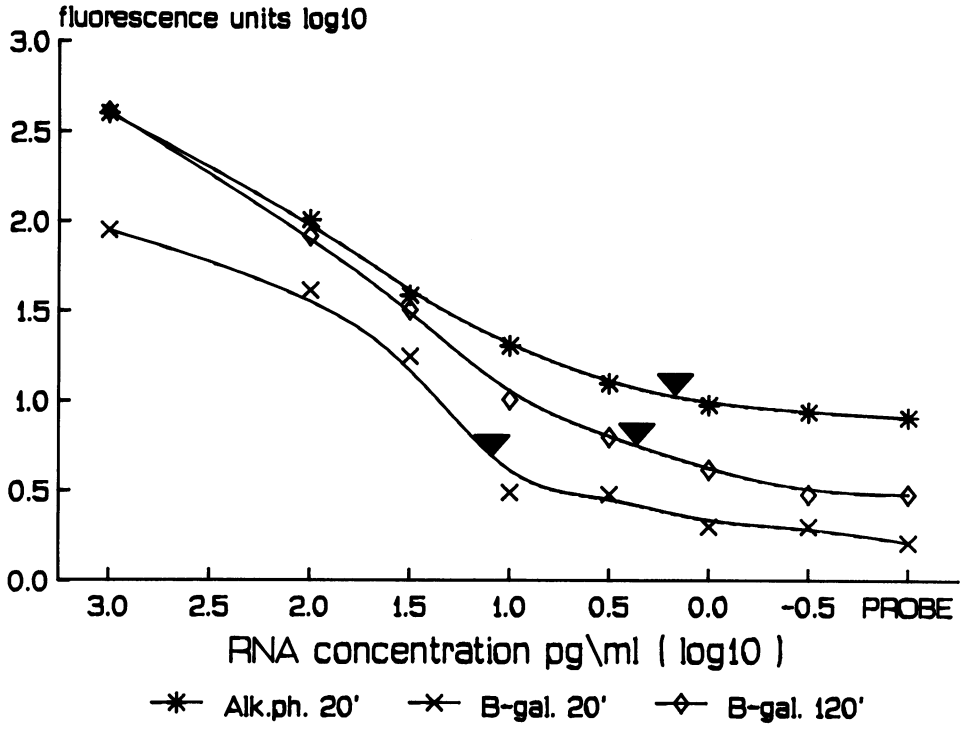

FIG. 2. Detection of RNA by monoclonal hybridization assay-fluorescent substrates. Wells of a black microtiter plate were coated overnight at 4°C with 50 μl of goat antibiotin in carbonate buffer, pH 9.6, at a concentration of 1 μg/ml (0.25 μg/well). Biotinylated pSP 65 plasmid DNA (0.4 μg/ml) was hybridized to serial dilutions of complementary pSP 65 RNA transcripts for 16 hours in a hybridization buffer (2 × SSC, 20 mM HEPES, 2 mM EDTA). After the addition of Triton-X, 50 μl of each of the nucleic acid mixtures were distributed on the plate coated with antibiotin Ab. After an incubation of one hour, the plates were washed and β-galactosidase- or alkaline phosphatase-labeled anti DNA-RNA monoclonal antibody was added. After another washing step, the presence of DNA-RNA hybrids was quantitated with the addition of a fluorescent substrate. The antibody labeled with alkaline phosphatase (alk. ph.) was detected with 10^{-4} M methylumbelliferyl phosphate in diethanolamine buffer, pH 9.6, with an incubation time of 20 minutes. Antibody labeled with β-d-galactosidase (β-gal.) was detected with 10^{-4} methylumbelliferyl-β-d-galactoside in PBS buffer, 1 mM $MgCl_2$, 50 μg/ml BSA with incubation times of 20 and 60 minutes. The data are means of three values from two experiments. Detection cut-off (▼): fluorescent activity of the probe +3 SD.

found in the lymphocytes of high-risk individuals in the absence of serological evidence of infection. This finding suggests that the detection of HIV nucleic acids may be a more sensitive indicator of infection than the development of a measurable serological response to HIV proteins [40,41]. The exact relationship between the detection of HIV nucleic acids by the polymerase chain reaction and the course of HIV-related diseases has, however, not been conclusively established.

Despite the advantages associated with these techniques, there are a number of problems which currently impede the general application of polymerase chain-reaction techniques for the general diagnosis of viral infections in patient-care settings [38,42]. Most important, the extreme sensitivity of polymerase chain-reaction techniques requires that extreme caution be utilized in the performance of the reactions and that the results of the reactions should be interpreted with caution unless extensive control reactions are performed. Problems which can occur include contamination with small

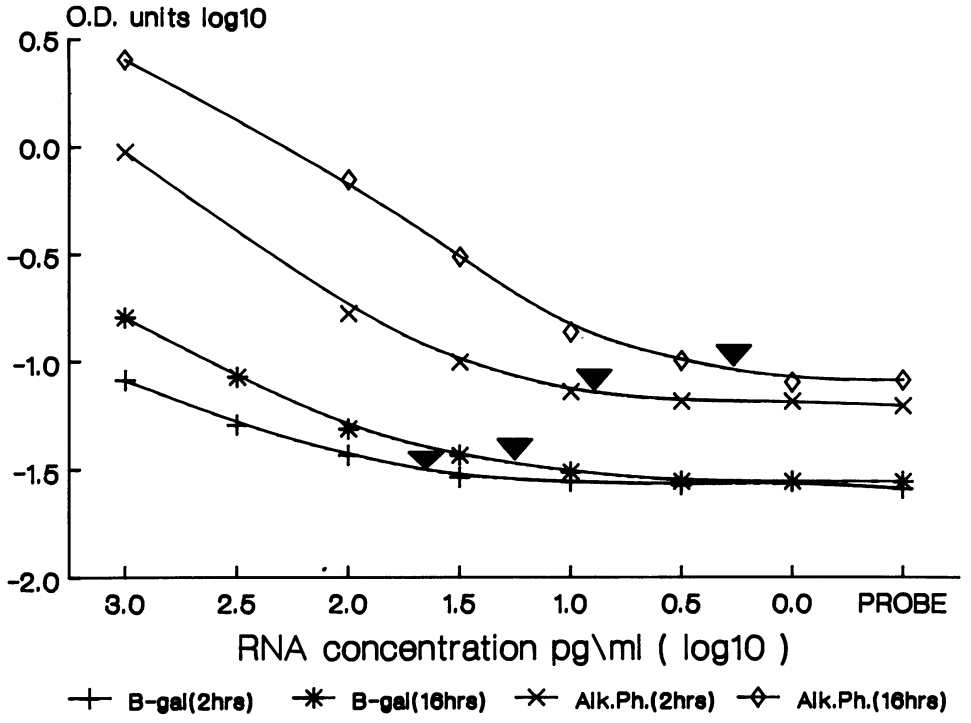

FIG. 3. Detection of RNA by monoclonal hybridization assay-colorimetric substrates. The reactivity of the monoclonal antibody against DNA-RNA was assessed with varying substrates and incubation times. The assay was performed as described in Fig. 2. Antibody labeled with β-d-galactosidase (β-gal.) was detected with 10^{-3} M nitrophenyl β-d-galactopyranoside in 50 mM PB buffer, pH 7.2, 1.5 mM $MgCl_2$, with incubation times of 2 and 16 hours. Antibody labeled with alkaline phosphatase (alk. ph.) was detected with 10^{-3} M nitrophenyl phosphate in diethanolamine buffer, pH 9.6, with incubation times of 2 and 16 hours. The data are means of three values from two experiments. Detection cut-off (▼): fluorescent activity of the probe +3 SD.

amounts of nucleic acid introduced during laboratory procedures and homologies with non-viral DNA, which generate amplified sequences similar in length to the HIV target. The problem of contamination is compounded by the extreme sensitivity of the polymerase chain-reaction procedure, in that the presence of very small amounts of extraneous nucleic acid can lead to the generation of a measurable signal. Contamination can occur from a number of sources, including plasmids, which are utilized as positive controls, and amplified pieces of DNA, which are generated as part of polymerase chain-reaction reactions in other tests.

The problem of contamination is best addressed by the use of rigorous isolation techniques in the preparation of the samples and in generation of the amplified targets and by the performance of large numbers of control reactions in which known negative samples are processed and analyzed in a manner identical to the test sample. The problem of homologies with non-viral nucleic acids which generate amplified segments can be partially overcome by the performance of a second hybridization reaction, using a probe sequence homologous to a viral sequence internal to that represented by the primer oligonucleotide. This technique, however, might not be capable of distinguishing the probe-virus interactions from reactions between viral probes and highly

homologous regions of the human genome. Careful studies are needed in order to determine the extent of these homologies and their potential effect on the specificity of sensitive techniques for the detection of viral nucleic acids in human body fluids.

An additional potential limitation of polymerase chain reaction is that the target nucleic acids must be highly conserved in order to react efficiently with the primer. This requirement can be a particular difficulty with such viruses as HIV, since the virus has the ability to undergo a great deal of genetic variation due to the low fidelity of the HIV reverse transcriptase reaction. This problem can be partially overcome by attempting to identify highly conserved sequences and by the use of multiple probes [38]; however, the use of multiple probes can compound the problem of non-specific reactions, since homology of only one of the probes with contaminating nucleic acids will result in false-positive reactions.

In all of the above cases, the performance of control reactions would be markedly simplified by the inclusion of a quantitative, non-isotopic method for the measurement of the DNA amplified by the polymerase chain-reaction procedures. It is likely that the quantitative techniques described above can be applied to the measurement of the amplified products of polymerase chain reactions. The application of these quantitative control reactions might markedly increase the specificity of amplification reactions and increase the likelihood that these procedures can be directly applied to the diagnosis and management of patients with suspected viral infections. In addition, the development of strategies for the optimization of assay specificity is particularly important, as the results of polymerase chain reactions are being utilized to support linkages between viral infections and diseases of idiopathic origin such as multiple sclerosis [43].

It is clear from the above discussion that nucleic acid hybridization will play an important role in the discovery, evaluation, and diagnosis of new viral pathogens of humans and other animals. It is still too early in the course of assay development to state with certainty the exact role of hybridization techniques in the clinical diagnostic laboratory. It can be predicted with certainty, however, that advances in the basic understanding of viral structure, genetics, and replication will continue to be applied to the diagnosis of human infections. Furthermore, it is inevitable that the development of practical techniques for the diagnosis of viral infections will lead not only to the improved care of patients with infectious diseases, but also to the eventual control of epidemic viral infections.

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