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RAPID DETECTION OF CYTOMEGALOVIRUS IN HUMAN LUNG AND BONE MARROW TISSUE SECTIONS BY IN SITU HYBRIDIZATION TECHNIQUES WITH A COMMERCIALLY AVAILABLE DNA PROBE

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ABSTRACT

RAPID DETECTION OF CYTOMEGALOVIRUS IN HUMAN LUNG AND BONE MARROW TISSUE SECTIONS BY IN SITU HYBRIDIZATION TECHNIQUES WITH A COMMERCIALLY AVAILABLE DNA PROBE

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A c-DNA probe from ENZO BIOCHEM was used to elucidate Cytomegalovirus infection on formalin-fixed, paraffin-embedded human lung and bone marrow sectiions by in situ hybridization techniques. The kit protocol was modified to circumvent problems. The specificity of the probe was evaluated using tissue samples that were determined to be either positive or negative for CMV based on clinical and laboratory data. Generally speaking, the probe is fairly honest in terms of supporting the diagnosis of human Cytomegalovirus infection in lung tissue. A brick red inclusion indicates a positive reaction which is easily differentiated from negative cases. However in bone marrow samples, it shows nonspecific staining for white blood cells in relatively fresh-prepared tissue blocks.

This is dedicated to my parent for their love and care.

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. Douglas Estry, for the outstanding job he has done in providing me with encourgement and advice. I am also indebted to Dr. Estry and Dr. Joan Mattson for providing laboratory space, patient specimens and reagents to allow this project to be completed. I would like to thank my committee, Martha Thomas, for her assistance in accomplishing the requirements for my Master's degree.

Special thanks to Dr. Tom Bell at Animal Health Diagnositic Laboratory at Michigan State University for his technical assistance.

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LITERATURE REVIEW

History of Cytomegalovirus

Human Cytomegalovirus (HCMV) was first described in 1904 by Ribbert (Ribbert, 1904) as large "protozoan like" cells in the kidney of an alleged leutic stillborn. In 1907, Löwenstein (Löwenstein, 1907) described cytoplasmic as well as intranuclear inclusions. After submitting their findings to various zoologists for opinions, they concluded that the inclusions represented protozoa, i.e; coccidia, sporozoa (gregarines), or amoebas. This idea was refuted by Lange (Lange, 1922) and Mueller (Mueller, 1922), who reported seeing cytomegalic inclusions in the kidney cells of infants. They pointed out that since the inclusions could be observed in stillbirths, they were probably present before birth. As protozoa could not pass the placental barrier this possibility was ruled out. Later investigators continued to find similar inclusions that were constantly and characteristically associated with lesions produced by herpes virus. They referred to this as cytomegalic inclusion disease (C.I.D.).

Since the cytomegaloviruses are largely species

specific, and human CMV does not grow in experimental animals, the isolation of CMV was not possible until human cells could be routinely grown in cultures (Enders et al, 1949). This work was done by three different groups, Smith (Smith, 1956), Weller et al. (Weller, 1957), and Rowe et al. (Rowe, 1956).

Characteritics of Human Cytomegalovirus

According mainly to its viral morphology, HCMV (Betaherpesvirinae) is a member of the Herpesviridae family. This family includes two other subfamilies representing the herpes simplex virus (Alphaherpesvirinae) and the lymphoproliferative virus groups (Gammaherpesvirinae) (Ho, 1982). It replicates in the nucleus of its host cell where fibers of viral DNA are seen to penetrate into protein capsids thus forming nucleocapsids (Haguenau, 1975). These nucleocapsids of icosahedral symmetry 'bud' through the internal nuclear membrane from which they acquire an envelope. The virion has a density of 1.20 - 1.21 g/cm^3 (Kim, 1976). An amorphous protein material (the tegument) is found between the envelope and the nucleocapsid. The inclusion body may have the diameter of almost half of the nucleus. Ultrastructural studies have shown that the nuclear inclusions comprise condensed, structurally altered

chromatin, intimately associated with enveloping virions. Basophilic smaller cytoplasmic inclusions are also present, but these are of more variable distribution and conformation. They appear to be associated with assembly of the complete virion within the cytoplasm so range from particles that probably represent viral protein coat to naked nucleoids to complete viral units (Robbins and Cotran, 1974). Like all members of this family, HCMV is unique among DNA viruses in that its genome contains repeating sequences (Somogyi, 1986). The entire sequence of HCMV has been cloned in cosmids (Fleckenstein B, et al, 1982; Demarchi, 1981).

In light of its importance as a pathogen causing a wide spectrum of clinical disorders, this virus has received increasing attention in recent years. Infections with HCMV are the largest single cause of congenital malformation today. Six to 7 % of all infectious mononucleosis is due to HCMV (Lamberson, 1985). Infections with this virus may also constitute a serious problem for immunosuppressed individuals such as cancer patients, recipients of organ transplants and acquired immunodeficient patients (H0, 1975-1977; Lonnqvist, et at, 1984). Primary infection with CMV is usually not diagnosed because it is asyptomatic or associated with a mild influenza-like illness in the normal host. Symptomatic primary CMV may occur in surgical patients

who acquire CMV from blood transfusions and is one of the causes of the post cardiotomy syndrome after open heart surgery (Doerr, et al, 1985). Like other herpesviruses, CMV causes latent infection. The reactivation of latent CMV in immunocompromised patients is common but it is often difficult to be certain that the illness is due to CMV because of the nonspecific nature of the clinical manifestation of CMV infection. Under certain laboratory conditions, HCMV can transform or immortalize embryonic hamster and human fibroblast cells, a capacity it shares with two other human herpesviruses, herpes simplex and Epstein Barr viruses (Thapa, et al, 1985). In addition to this, HCMV plays an as yet undetermined role in transformed lymphocytes. Both of these have been implicated to a greater or lesser degree in human malignancies (Evans, 1976).

Diagnostic Methods

(1) Virus isolation :

The shedding of CMV from the throat, and especially in the urine, may continue for several weeks and even months after the acute illness has subsided. In contrast to urine and throat specimens, CMV can be recovered from blood only during the acute phase of infection (Colimon et al, 1984). After inoculation of the specimen into human diploid cell

cultures, up to four weeks may be required before the cytopathic effects of CMV are detected and this depends upon the initial amount of virus in the sample (Panjwani, et al, 1985).

(2) Serological tests :

Because of the fastidious growth characteristics of the virus, blood cultures may be negative despite CMV infection. Therefore, serological studies are recommended in addition to CMV cultures. A fourfold or greater increase in antibody level between acute and convalescent phase sera collected two to three weeks apart is evidence of CMV infection. Such an increase in antibody titer may occur even though blood and urine cultures are negative (Smith, 1981).

Differentiation of sera positive and negative for antibodies to CMV has historically been carried out by the complement fixation (CF) test. However, the CF test has several disadvantages: some subclasses of immunoglobulin G (IgG) do not fix complement (Haikin, 1980); certain sera show anticomplementary activity; and the test procedure itself takes two days. A second test, the indirect fluorescent antibody (IFA) technique, has been very useful for the detection of CMV IgG (Chiang, 1970). It is more sensitive and rapid than the CF test. The indirect peroxidase antibody (IPA) technique that has been developed

recently (Gerna, 1976) is as rapid and sensitive as IFA and requires a light microscope instead of a fluorescence microscope. However, IFA and IPA techniques both have the problem of nonspecific cytoplasmic staining attributed to the presence of Fc receptors induced by CMV infection (Sakuma, 1977). The enzyme-linked immunosorbent assay (ELISA) is a highly sensitive technique (Engvall, 1971) that is simple and permits use of stored antigen (Re, et al, 1980). The most sensitive antibody assay technique is generally acknowledged to be radioimmunoassay (RIA). The development of RIA for mass screening of HCMV infection (Kimmel, 1980) has allowed testing of large numbers of samples with small amounts of serum.

Primary infection may also be diagnosed by demonstration of CMV-specific IgM in a single serum sample. The indirect fluorescent method was the first technique used for detection of CMV IgM antibodies. However, rheumatoid factors in human sera often give rise to false-positive results. Elimination of this effect can be accomplished by separation techniques such as column chromatography and ultracentrifugation, although the handling of large numbers of specimens is laborious (Grint, et al, 1985). With respect to recurrent CMV infections, it appears that IgM is only detected in some of these cases and, therefore, its

appearance cannot be relied upon as an indication of reactivated or recurrent infection (Kangro, 1980). However, by the use of ELISA, RIA and immunoperoxidase assays for the detection of CMV-IgA antibodies, diagnosticians were able to detect recurrent infection in kidney transplant patients, in many cases, before the CF titer rise was apparent (Sarov, 1980 -1983).

(3) Microscopic methods :

In the past, cells in the urine sediment have been examined microscopically for CMV inclusions. However, because of the low sensitivity of this method, it has been discontinued. Two groups of investigators have recently used electron microscopy (EM) to rapidly detect the presence of CMV in infected infants (Lee, 1978; Henry, 1978; Martin, et al, 1984). Both groups detected virus in the urine, and Lee et al, were able to detect CMV particles from oral specimens as well.

Nucleic acid hybridization analysis --- New techniques

One aspect in clinical microbiology practice has not significantly changed in the elucidation of specific etiologic agents since the 1880s. Both in the 1880s and 1980s, the laboratory required agar-based media to yield the microorganism before analysis. Nucleic acid hybridization techniques and monoclonal antibody technology may potentially eliminate the need for growth of many microbes and thus reduce the time for identification (Engleberg, et al, 1984; Molden, 1986).

Steps for creating a probe

The five essential steps in the production of a nucleic acid hybridization probe test or monoclonal antibody analysis are listed in Table I (Edberg, 1986). The discovery of restriction endonucleases was the critical event that allowed scientists to fragment nucleic acid for specific analysis. Large numbers of microbial pathogens are grown and the nucleic acids are removed from them by the combination of physical (sonication) and chemical (detergent and alkali) means. After hydrolysis with restriction endonucleases, the small pieces of nucleic acid are separated from each other

Table I : Cloning Protocols Used in Constructing Nucleic Acid Hybridization Probes

Cloning Protocol Step	DNA Hybridization
1. Selection of template	Restriction Endonuclease and capture of DNA fragments
2. Insertion of template	Ligation to a plasmid-insertion in recipient Escherichia coli
3. Seletion of active template	Successful insertion shown by antibiotic sensitivity
4. Amplification of template product	Growth of vector in Escherichia coli
5. Selection of clones with desired properties	DNA hybidization

using electrophoresis on gel and column chromatography. Based on size distribution and other parameters (Grillner, et al, 1984), the investigators can choose fragments as probe to insert into a vector for amplification. The vector is grown inside a properly selected host cell in large numbers so that billions of copies of the original fragment(s) are made. Investigators have to examine each clone until one is found that has pathogen-specific nucleic acid processing the right properties for nucleic acid hybridization analysis. After a clone having the specific DNA fragment is selected, it must be grown in large quantities, removed from the host cell and labelled.

Traditionally, labelling occurred by a nick translation process. Single-stranded nicks are introduced into double-stranded DNA using DNase I. These nicks are subsequently repaired utilizing a DNA polymerase in the presence of radioisotope-labelled nucleotides (Mckeating, et al, 1985; Rigby, 1977). A procedure designed to replace radiolabelled nucleic acid was first sucessfully developed by Ward and associates in 1981 (Leary, 1983). They covalently linked biotin to the pyrimidine ring through an allylamine linker. It was found that the attachment of biotin to the nucleic acid did not affect the association properties of the hybridization probe. The assay itself uses the affinity of biotin for the egg white protein avidin. This protein has a dissociation constant for biotin of $Kd = 10^{-15}$, and four binding sites for biotin. Avidin can be labelled with an enzyme or fluorescent probe. When the enzyme-linked avidin attaches to biotin, a substrate for the enzyme can be added resulting in the development of color (Bayer, 1980; Langer, et al, 1981). A second strategy to label nucleic acid nonradiometrically is the introduction of a hapten directly onto a pyrimidine ring. Tchen and colleagues (Tchen 1984) found that guanine residues could be labelled with N-acetoxyl-N-2-acetylaminofluorene (AAF) and its 7-iodo (AAIF) derivatives. In practice, the probe consists of nucleic

acids to which hapten had been attached. When the probe interacts with its target, antibody to the hapten can then be added to the reaction mixture and the end point can be measured by any secondary antibody technique.

Basic assay techniques

After the nucleic acid has been captured from the microbial pathogen, inserted into a vector, amplified in a host, and labelled, the assay method itself is the last step in the diagnosis of an infectious disease using a nucleic acid hybridization technique. There are four basic assay techniques in use. The most extensively studied is the dot, or dot-blot, hybridization method, which employs a nitrocellulose filter paper support. Nucleic acid of the infectious microbe from the patient sample is released and then bound to a spot on the nitrocellulose filter by a combination of physical and chemical means (Bittner, et al, 1980). The labelled hybridization probe is added to the spot under a predetermined set of stringent conditions. If there is no complementary nucleic acid in the spot, the hybridization probe will not be bound and will be washed away during a rinsing step. If there is complementary nucleic acid from the patient sample, then the nucleic acid hybridization probe will be bound. A substrate to the label

on the nucleic acid hybridization probe is added and the reaction is determined as positive or negative. The second major technique is the sandwich hybridization procedure. This method uses the production of two noncomplementary nucleic acid sequence reagents. These reagents are produced from adjacent sites on the gene of the microbial pathogen. The first nucleic acid sequence is immobilized onto the solid nitrocellulose support and serves as a target sequence to interact with nucleic acid sequences from the microbial pathogen. The second sequence is labelled and serves as the nucleic acid detector probe. Like dot hybridization, nucleic acid is first extracted from the clinical sample. The extracted nucleic acid is mixed with a probe nucleic acid and hybridized against a target sequence that has been bound to the nitrocellulose paper. If the microbial pathogen nucleic acid is complementary, a sandwich will be formed and the complex will be detected by the addtion of a substrate to the probe label (Virtnen, et al, 1982). A third method is the Southern blot technique. This method, described in 1975 (Southern, 1975; 1980), has become the standard by which the specificity of the nucleic acid sequence is established. After endonuclease digestion, the mixture is separated using agarose gel electrophoresis. The DNA fragments are transferred using either blotting or electroelution to nitrocellulose paper. The nucleic acid probes are added to

the nitrocellulose paper to establish identity. This technique is primarily utilized to establish identity and is not amenable to routine clinical testing because of the large amount of time and reagents required. A fourth technology, the in situ hybridization procedure, has the most applicability to clinical diagnostic pathology (Marlowe, et al, 1983; Wilkinson, et al, 1986). In this procedure, human tissue (ie, biopsy, cell culture, etc) is treated with a combination of diluted detergents, mild acids, and proteases. This treatment fixes the cells in their natural configuration while allowing the introduction of labelled nucleic acid hybridization probes. Analogous to fluorescent antibody detection of antigens in tissue, in situ hybridization allows the direct visualization of specific DNA sequences as they occur in vivo (Unger, et al, 1986). This method has proven to be particularly useful for the detection of viruses from tissue. Myerson and coworkers (Myerson, 1984) detected cytomegalovirus in open lung biopsy specimens within 24 hours. In 1982, Chou and Merrigan (Chou and Merrigan, 1982) reported the detection and quantitation of cytomegalovirus from human urine specimens utilizing DNA hybridization with ^{32}P. They were able to detect as few as 10^3 virus particles per milliliter of urine.

Based on the concepts of DNA hybridization assays,

variations can be made by changing the probe synthesis method, probe length, probe label, assay conditions, sample pretreatments and methods to immobilize DNA and choose from them the most suitable assay for each particular use.

METHOD AND MATERIAL

The kit being evaluated was purchased from Enzo Biochem, Inc., New York, N.Y. and contained a mixture of two cDNA fragments. The fragments were cloned into the BamHR site of the vector pBR322. One of them is 13.3 kilobase pairs, and the other 16.6 kilobase pairs. By nick translation with the thymidine analog 5-[N-(N-biotinyl-ε-aminocaproyl)-3-aminoally]-deoxyuridine triphosphate the probe was biotinylated. Strepavidin-biotinylated horseradish peroxidase was used as the detection complex with aminoethylcarbazole (AEC) as chromogen. The kit wash buffer salt consists of 1X PBS, 1% BSA, 0.5m NaCl and 5mM EDTA, pH 7.5.

Either cytomegalovirus infected human lung tissue embedded in paraffin or paraffin embedded CMV infected bone marrows were used. Five cases of paraffin embedded bone marrows and one case of lung tissue, negative for CMV based on clinical criteria were used as negative controls. One positive lung tissue, two positive bone marrow were used as positive controls. Tabe II shows a brief description of patient history, and the patients are assogned a letter for easy reference and spaces. Some modifications of the kit protocol have been made according to Forghani's (Forghani,

Table II: Brief Description of Patient History

	Patient #	Clinical History
A	H85-408	: 25 year-old white female with a 2 year history of chronic ulcerative colitis and a one week history of acute febrile illness; abnormal lymphocytes were seen on her admission CBC and the urine CMV titer was positive (1:128).
B	H87-875	: 65 year-old male with massive lymphadenopathy, renal shotdown and acute liver failure. CMV was recovered from the blood culture.
C	H87-759	: 17 year-old white male with Hodkin's disease, nodular sclerosis type, diagnosed by excision of mass on right side of neck.
D	H87-908	: 87 year-old female with multiple myeloma.
E	H87-912	: white female with mild chronic neutropenia.
F	H86-681	: 81 year-old man, extensive traveler, with fever of unknown origin. Liver enzymes were elevated with a non-diagnostic carcinoma in the past. Serum iron, TIBC and % saturation were decreased; transferrin was normaal. Patient died with lymphoma vs virus infection.
G	H68-53	: Unidentified patient with Erythroid Hyperplasia.
H	H68-140	: Unidentified patient with Infectious Mononucleosis.

et al 1985) modification.

To prepare tissue sections for hybridization, glass slides were first coated with poly-L-lysine to prevent loss of tissue during the test procedures. Specifically, one

milliliter of the poly-L-lysine solution (50ug / ml in deionized water) was evenly distributed on the surface of the slide. The slides were allowed to stand for thirty minutes at room temperature. The excess poly-L-lysine solution was rinsed off the slides by three successive washes with deionized water. Following air drying the slides were ready for use and could be stored at room temperature for one week. Paraffin embedded tissue was cut using standard histological techniques and then deparaffinized and rehydrated by sequential immersion into xylene, absolute ethanol, 95% ethanol, 80% ethanol and deionized water for approximately one minute each. The slides were then immersed into 3% H_2O_2 in water for five minutes followed by rinsing in deionized water. This was to remove endogenous peroxidase activity which might be present in some types of cells. Due to the large amount of red blood cells (hemoglobin has a proxidase-like activity), the bone marrow sections were treated twice with 3% H_2O_2. The tissue sections were digested with 100ug Proteinase K per ml in PBS by covering the tissue with solution and incubating for thirty minutes at 37 C. The reaction was stopped by washing the slide with 5uM EDTA in PBS (wash buffer) for ten to fifteen seconds. The excess liquid was wiped off the slide and then the slides were air dried.

In situ hybridization for detection of CMV genomic material was performed according to the kit protocol, with only minor modifications. After completing the pretreatment of the slides, one drop of BIOPROBE (SOLUTION 1, biotinylated DNA probe) was applied to each specimen and then covered with a glass coverslip just large enough to cover the surface of the tissue. Trapping air bubbles under the coverslip must be avoided. The slides were placed in a 92 C water bath for ten minutes, followed by incubation in a 37 C water bath for thirty minutes. Modification of this incubation procedure are described in the results. The coverslip was removed by gently lifting one corner and four drops of PROBE WASH SOLUTION (SOLUTION 2, 50% deionized formamide in 0.1X PBS) was applied to each specimen. The sample was then allowed to stand at room temperature for seven to ten minutes. The slides were rinsed with WASH BUFFER using an even stream of solution from a squeeze bottle for ten to fifteen seconds. The excess moisture was tapped off the slides and three drops of DETECTION COMPLEX (SOLUTION 3) were added to each specimen. The slides were then incubated for ten to fifteen minutes at room temperature, followed by rinsing with WASH BUFFER for five to ten seconds. The slides should be left wet while preparing the solution for the next step. The chromogen/substrate solution was prepared just before use by

removing the nipple from a vial of SOLUTION 4c (100 m mole acetate buffer, pH7.4) and adding, in the following order, one drop of SOLUTION 4a (AEC) and mixing gently and then adding one drop of SOLUTION 4b (H_2O_2) to the vial. This mixture was mixed well. Four drops of well mixed substrate/chromogen were added after the excess moisture was tapped off the slide and followed by incubation for ten to fifteen minutes at room temperature. Finally, the slides were rinsed with a soft stream of WASH BUFFER for five to ten seconds. Polyvinyl alcohol (Elvanol; Du Pont Co; Wilmington, Del.) can be used as the mounting medium for permanent preservation. The slides were evaluated by light microscopy and a brick red to brown deposit in the cells indicates a positive reaction.

RESULTS

Pretreatment of the Sections

(1) Proteinase K digestion study :

A series of sections were digested for varying incubation times and with varying Proteinase K concentrations to determine the optimal treatment for human lung tissue. According to guidelines provided by the kit, we choose 0.01, 0.1, and 1mg/ml as test concentrations and 10, 20 and 30 minutes as incubation periods. Results, shown in Table III, suggested that incubation of the sections in 0.1 mg/ml proteinase K solution for at least 30 minutes was the minimum. If proteinase K digestion was not sufficient (Fig. 1), we could not find brick red inclusions as we did with the appropriate treatment, 0.1mg/ml Proteinase K for 30 minutes at 37 C (Fig. 2-3).

As for bone marrow sections, 0.01, 0.1 mg/ml and 20, 30 minutes were employed as test concentrations and incubation periods. A positive bone marrow control showed negative results when the Proteinase K concentration was less than 0.1 mg/ml or the incubation time was less than 30 minutes. Result showed the false negative reaction on a positive case when the Proteinase K treatment was not

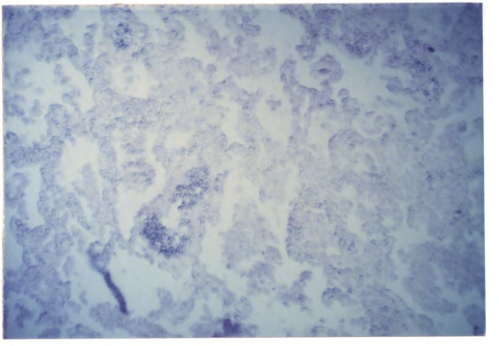

Figure 1: Insufficient Proteinase K digestion of lung

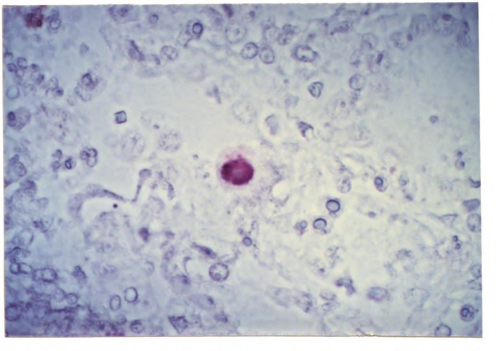

Figure 2: Adequate Proteinase K and H_2O_2 treated lung

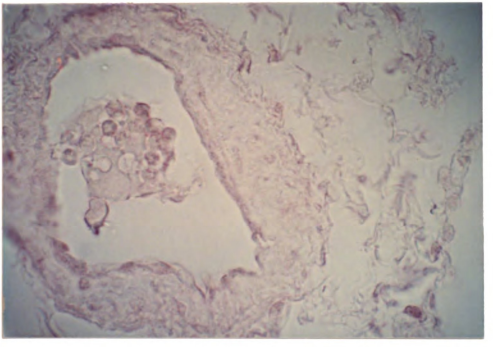

Figure 3: Adequate Proteinase K digestion of negative human lung

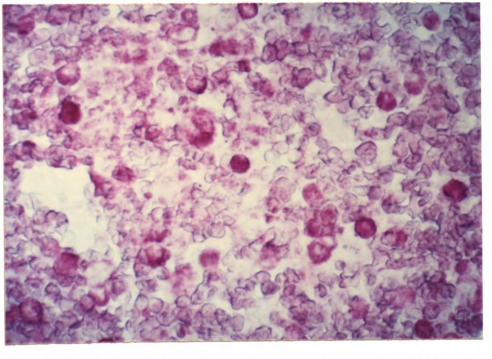

Figure 4: Endogenous peroxidase-like activity of bone marrow due to insufficient H_2O_2 incubation

optimun. Therefore, we decided to use 0.1 mg/ml Proteinase for 30 minutes at 37 C as the test condition.

Table III: Varying Durations of time and concentration of Proteinase K treatment for lung tissue. +: red inclusions were seen in positive control (Fig.2); 0: red inclusions could not be found in positive control (Fig.1).

	0.01 mg/ml	0.10 mg/ml	1.0 mg/ml
10 min	0	0	+
20 min	0	0	+
30 min	0	+	+

(2) Hydrogen Peroxide Treatment :

Immersion of the specimens into 3% H_2O_2 in water for 5 minutes was enough to inhibit the endogenous peroxidase activity in lung tissue (Fig. 2). But for bone marrow sections the five minute incubation still resulted in a red color in white as well as red blood cells (Fig. 4). This indicated nonspecific peroxidase-like acitivity in red cells. An additional five-minute incubation with 3% H_2O_2 was added to determine if this eliminated the endogenous peroxidase activity from a negative control. Red cell peroxidase-like activity was successfully removed (shown in Fig. 5) by this modification. As for the nonspecific

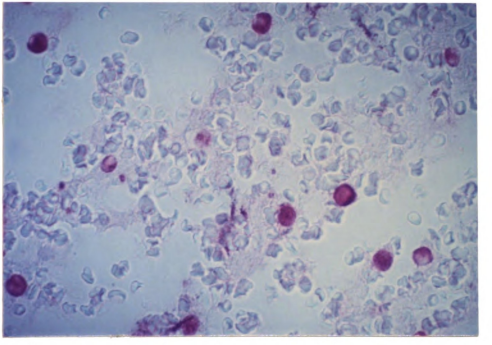

Figure 5: Bone marrow incubated twice with 3% H_2O_2

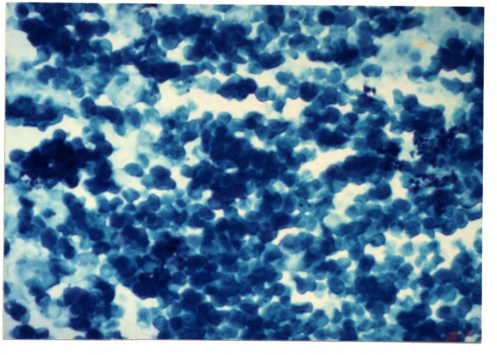

Figure 6: Bone marrow counterstained with Fast Green

staining seen in the white cells, further experiments were needed to determine whether it was caused by remaining white cell peroxidase activity or nonspecific binding of avidin to white cells.

Modifications of the Kit Protocol

(1) Heating Process :

According to the protocol, after application of the BIOPROBE, the section is to be covered with a coverslip and placed on a 92 C heating block for 2 to 3 minutes. The slides usually dried out during this heating process so the sections fell off while removing the coverslip. In order to prevent drying of the slide we reduced the heating time to less than one minute. The results indicated no red inclusions in a positive CMV specimen. Two additional modifications were made to solve this problem. One was to heat the slide by steaming. In this modification, slides with probe were placed in a petri dish and put on the top of a beaker containing boiling water. The other method was to use a 92 C water bath to reduce the water loss during heating. Either way gave satifactory results. Incubation time was also modified and the results showed no difference between a 2-3 minute incubation at 92 C followed by 10-15

minute incubation at RT and a 10 minute incubation at 92 C followed by 30 minute incubation at 37 C.

(2) Counterstaining :

Fast green, which stains cytoplasm, was used as a counterstain to highlight the red inclusions in CMV positive specimens (Fig. 6-7). Specimens without counterstaining (Fig. 8) show even more clearly where the inclusions are. By comparing these two cases of the same area of lung, we found that counter staining tends to mask the positive reaction and specimens without counter staining still showed tissue details. So we decide to leave out this step for better results and less time and reagent needed.

(3) Specimen Mounting :

Water is suggested by the kit protocol to yield fine results. But the slides can not be preserved more than one week as the slides will dry out. A more permanent mounting media, polyvinyl alcohol, was used to replace water. Figure 1-22 shows a satisfactory result by using polyvinyl alcohol and the color was not changed after two weeks.

Reaction Patterns and Specificity

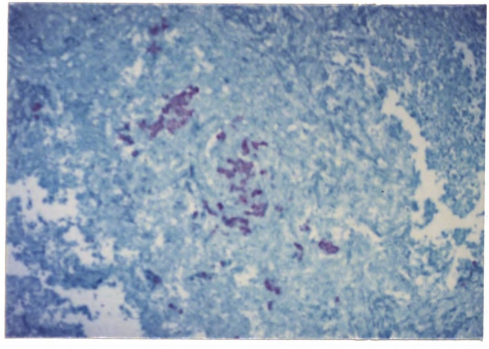

Figure 7: Human lung counterstained with Fast Green

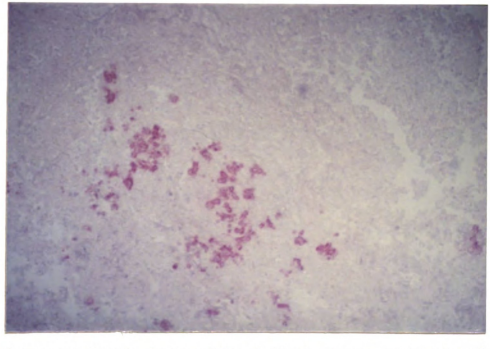

Figure 8: Human lung without Fast Green counterstaining

(1) Staining Patterns :

CMV replicates in the nucleus of the host cell and enters the cytoplasm by budding through the nuclear membrane. Therefore, as we can see in the routine microscopic examination, the staining pattern of this virus is expected to be cytoplasmic as well as intranuclear, punctate rather than diffuse. This characteristic staining pattern can be shown (Fig. 9-10) in the lung tissue sections tested. As for most bone marrow cases processed by this protocol, the staining pattern is predominently cytosolic and diffuse with only an occasional nuclear or punctate appearance (Fig. 11-14). This might due to the nonspecific staining problem we observed for relatively freshly-prepared bone marrow tissue blocks.

(2) Nonspecific Staining :

The cytoplasmic and diffuse staining pattern of white blood cells in bone marrow sections which could be shown even in a negative control case (Fig. 11) indicated the occurrance of the false-positive nonspecific staining. Two experiments were performed to rule out the possiblities of nonspecific avidin binding and remaining nonspecific peroxidase activity. At first, we left out the probe from the procedure. No color development indicated there was no nonspecific avidin from the detection complex bound to the

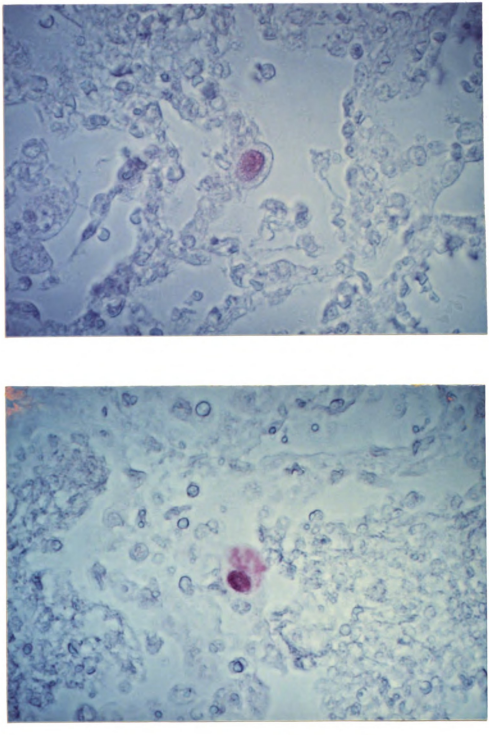

Figure 9-10: Chracteristic Staining of CMV in lung

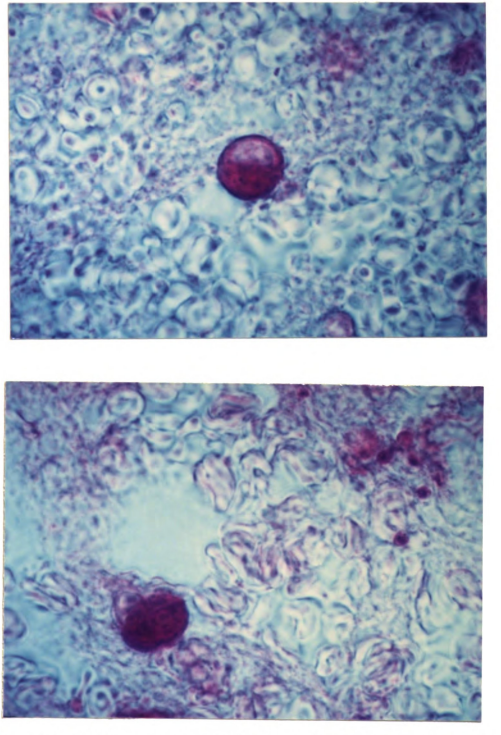

Figure 11-12: Predominantly cytoplasmic and diffuse staining in bone marrow specimens

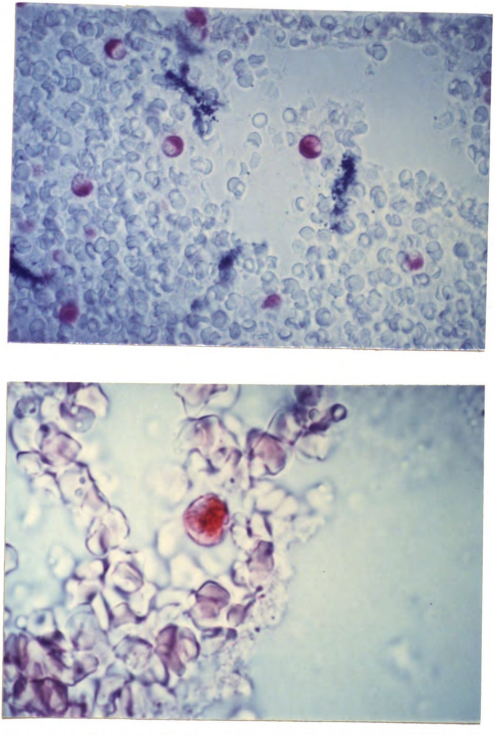

Figure 13-14: Predominantly cytoplsmic and diffuse staining in bone marrow specimens

sample (Fig.15). Streptavidin from another source was also tested and gave the same results with the detection complex provided in the kit. Both the probe and the detection complex were left out to see whether remaining nonspecific peroxidase alone could develop a color reaction. Figure 16 shows that there was no brick red color development in the whilte blood cells thus no endogenous nonspecific peroxidase activity was left in the tissue.

Four more cases of negative control were also tested against the CMV probe and a probe for Epstain-Barr virus (EBV) infections. The EBV probe was used as a control. The staining procedure was similar to that for CMV and it was felt that if probe specificity was a problem, the EBV probe would not give the same nonspecific staining seen with CMV probe. Alternatively, if the nonspecific reaction was due to interactions of cellular RNA or DNA with the probe vector (pBR322 in both cases), then both probes would give the same nonspecific positive result. For case G and H, which are fairly old bone marrow tissue blocks, we did not get nonspecific reaction either with CMV or EBV probe. The case H, diagnosed as Infectious mononucleosis, stained positively with the EBV probe (Fig. 17) and negative with the CMV probe (Fig. 18). While case G, diagnosed as Erythroid hyperplasia, stained negatively with both CMV and EBV probes (Fig. 19-20). However, two other relatively freshly-prepared bone

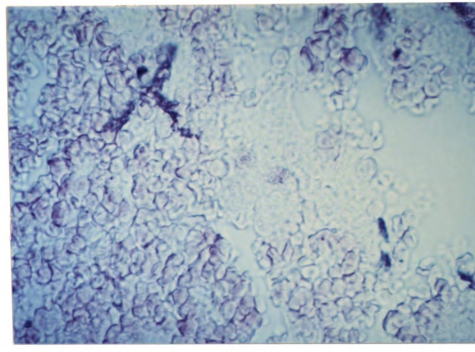

Figure 15: Bone Marrow tested without probe

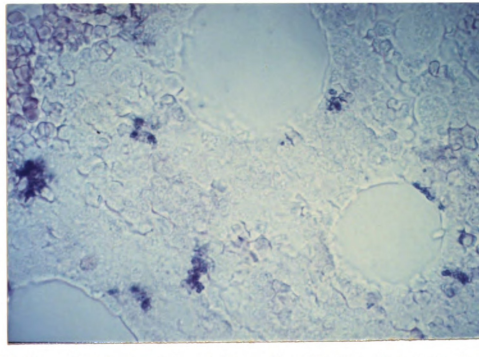

Figure 16: Bone marrow tested without either probe or detection complex

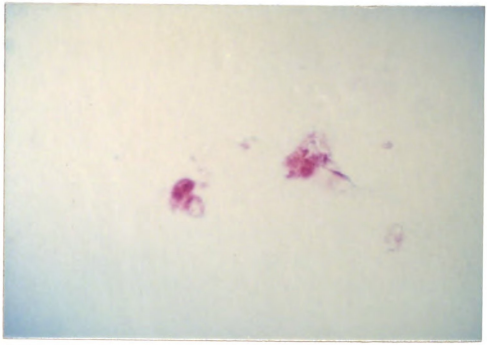

Figure 17: Old Infectious Mononucleosis specimen tested with EBV probe

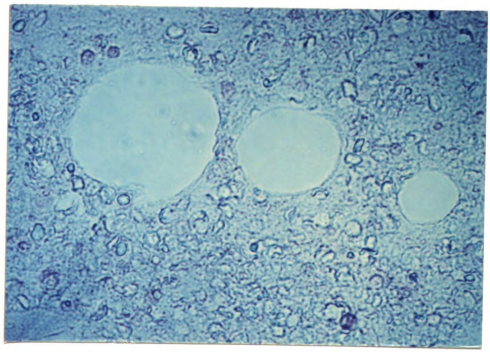

Figure 18: Old Infectious Mononucleosis specimen tested with CMV probe

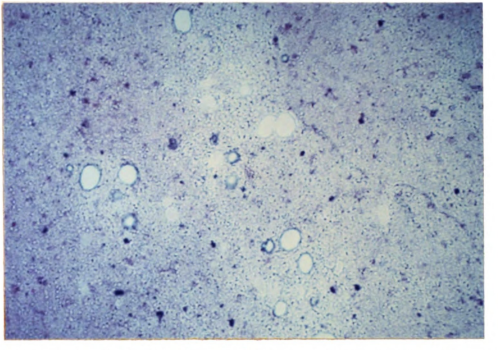

Figure 19: Old Erythroid Hyperplasia specimen tested with CMV probe

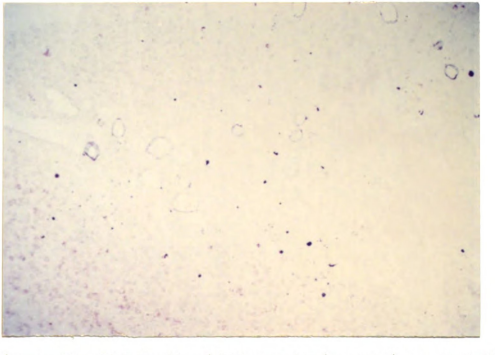

Figure 20: Old Erythroid Hyperplasia specimen tested with EBV probe

marrow blocks, I and J, all stained positively with both EBV and CMV rpobes indicating a nonspecific reaction with both probes (Fig.21-22).

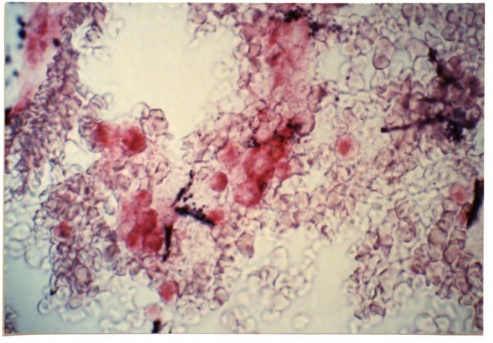

Figure 21: Negative bone marrow tested with EBV probe

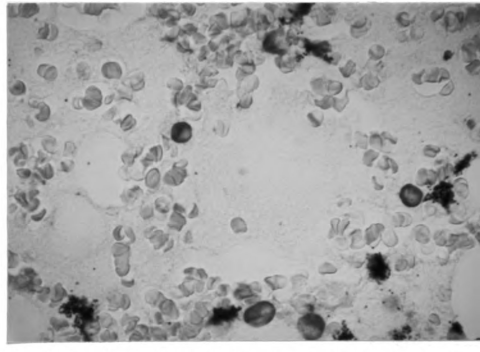

Figure 22: Negative bone marrow tested with CMV probe

DISCUSSION

Because of the specificity and potentially high sensitivity of the in situ hybridization technology, there has been increasing attention to the application of this technique in the detection of viral infection. This methodology recognizes the specific viral genome in a clinical specimen. In the above study we evaluated a commercially available biotinylated cytomegalovirus DNA probe for detection of CMV genomic material in human lung and bone marrow tissue sections. There are several things that need to be discussed in this set of experiments. The first of these is the Proteinase K digestion study. The Proteinase K digestion procedure is essential for formalin fixed tissue sections because it exposes the DNA inside the cells to make the hybridization possible. The exact concentration and incubation time depends largely on both the type of tissue being processed and the fixation condition of the tissue. A series of digestions were performed to determine the optimal conditions for both human lung and bone marrow tissue sections. According to the positive control reactions, 0.1 mg/ml proteinase K incubation at 37 C for 30 minutes was chosen for both tissue types without wasting the reagent or time, both critical

parameters to be evaluated when applying a test to clinical diagnostic usage.

The heating process which allows the unwinding of the biotinylated probe DNA as well as target DNA is a second important topic to be discussed. The denaturation curve of double-stranded DNA (Fig. 23) indicates that it well become single-stranded DNA at about 100 C. Prior to this temperature the amount of single-stranded DNA decreases rapidly as the temperature decreases. In order to maximize the percentage of single-stranded DNA for hybridization, high temperature is required. Formamide (already in the probe) is added to this reaction to reduce the Tm (melting temperature) at which DNA unwinds.It is suggested by the kit protocol that, in order to reach this requirement, direct heating on a 92 C heating block for 2-3 minutes is required. Forghani et al. (Forghani et al. 1985) modified this procedure and used an 80 C water bath and a 10-minute incubation period. This modification was used to detect Herpes Simplex Virus DNA in human brain tissue with a probe purchased from the ENZO BIOCHEM company. Since direct heating resulted in the tissue sections drying and falling off the poly-L-lysine coated slides, a shortened incubation time (less than one minute) was tried to prevent drying of the slides. This, however resulted in a false negative reaction. The protocol was then modified by steaming,

incubation in a 92 C water bath for 2-3 minutes or 92 C water bath for 10 minutes. All three incubation protocols gave the same satisfactory results. Higher temperatures are not required since they do not cause a significant increase in the amount of single-stranded DNA but only result in the slides drying.

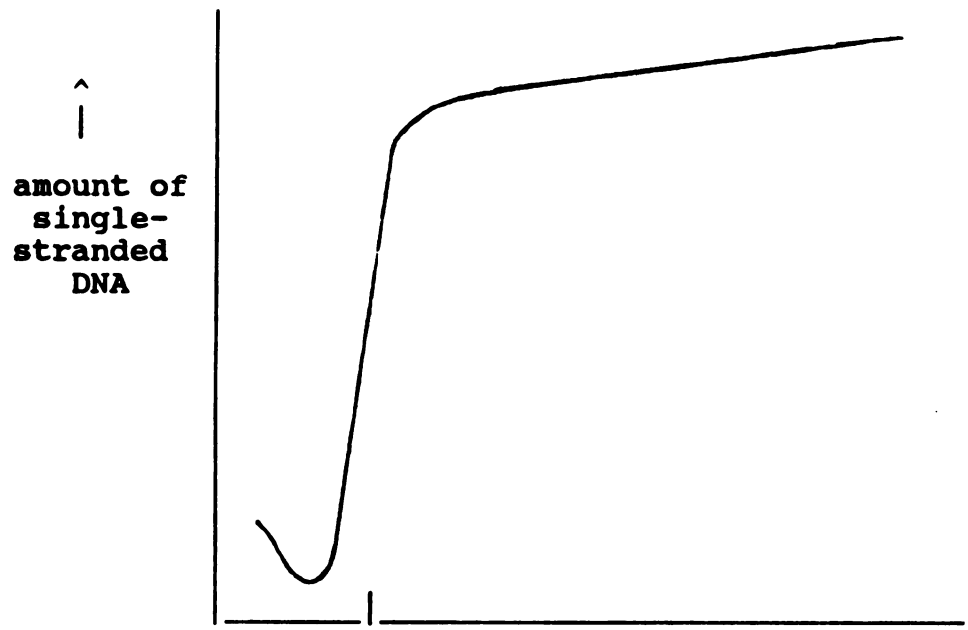

Figure 23 : DNA Denaturation Curve. Tm, melting point of duplex DNA, related to formamide concentration, G+C content of DNA and ionic strength of solvent.

As mentioned in the results, specimen counterstaining and mounting were also modified. Counterstaining with fast green, provided in the kit masked the brick red inclusions that could be seen clearly without counterstaining. This procedure was therefore omitted in our modification. As for mounting fluid, water is very good except for preservation. Polyvinyl alcohol gave excellent results and provide a much more permanent mounting media than water. For clinical use in laboratories that wish to preserve their specimens for a longer period of time it is suggested that polyvinyl alcohol be used. Otherwise, use of water is fine and can save money for reagents.

This probe is fairly honest in terms of differentiating positive and negative controls in the control slides provided in the kit as well as in human lung tissues. The control slides with infected and uninfected cells placed in different wells were tested against CMV infection along with other tissue sections. A positive brick red staining could always be seen in the positive well and no color reaction developed in the negative well. Positive and negative lung tissues based on clinical and laboratory criteria were also tested. Satisfactory results were obtained. Neither the control slide nor the human lung tissue has the problem of nonspecific staining. The positive staining patterns for both of them were intranuclear as well

as cytoplasmic.

On the contrary, relatively fresh-prepared bone marrow samples were stained positively whether they had been infected with CMV or not. The staining patterns in these cases were predominently cytoplasmic and diffuse rather than intranuclear and punctate. Several possibilities were suggested as causes for this nonspecific reaction. Nonspecific avidin binding and remaining nonspecific peroxidase activity were two of them. They were subsequently ruled out by performing the experiments without probe or without both the probe and detection complex. If there were nonspecific avidin binding from the detection complex to the tissue, the color reaction should develop even with no probe added. For a similar reason, if there still was nonspecific peroxidase activity left in the tissue after 3% hydrogen peroxide treatment, chromogen/substrate solution should be sufficient for positive color development. We did not get positive staining in either case which suggested that these two possibilities were ruled out.

The literature suggested that CMV infection could be asymptomatic and hence not diagnosed. We suspected our negative controls were not truly negative. But after performing two positive, three negative and one suspicious cases of the relatively fresh bone marrow blocks, it is

unlikely that all of them have been infected with CMV. Two of these negative bone marrow blocks were also tested with EBV probe and had the same nonspecific staining problem although not as strong as in the CMV detection. The staining pattern in these cases were also predominantly cytosolic and diffuse.

Two relatively old bone marrow blocks, an Infectious mononucleosis and an Erythroid hyperplasia case, were used in the CMV hybridization test as negative controls and they were stained negatively. The same tissues were tested with EBV DNA probe for the detection of Epstein-Barr virus infection. The Infectious mononucleosis case stained positively and the Erythroid hyperplasia case negatively. This raises at least three questions. 1) Why is the nonspecific staining only found in freshly prepared bone marrow blocks and not in relatively old blocks? 2) Does the predominantly cytoplasmic staining pattern correlate with the nonspecific reaction? 3) Dose the viral DNA probe or the bacteria plasmid vector pBR322 have sequence homology with either native cellular DNA or cytoplsmic RNA? Further investigations may include trying to find a relatively old CMV positive bone marrow block and test it against this CMV probe from ENZO company to see whether there are some nonspecific factors present in white blood cells that will disappear during aging as is indicated by the EBV probe.

Perhaps old bone marrow blocks are totally nonreactive to this CMV probe. By comparing the staining patterns between lung and bone marrow sections, we find that predominent cytoplasmic staining is only found in relatively fresh bone marrow blocks. Cytoplasmic RNA is a possiblility for nonspecific hybridization with the probe. Because of the unstability of RNA, it would also explain why we did not get nonspecific staining in relatively old bone marrow blocks. Pretreatment of the bone marrow tissue sections with RNAse to remove RNA before hybridization may provide a way to eliminate this problem. If RNAse digested bone marrow sections loose the nonspecific cytoplsmic staining reaction, it would be concluded that RNA accounts for the nonspecific reaction. Both the CMV and EBV probe from ENZO are cloned into a pBR322 vector. Ambinder's group (Ambinder, et al, 1986) has suggested that this vector is the cause of their vector homology problem in diagnositic nucleic acid hybridization of various types of clinical specimens. They also emphasize that it is essential that all hybridization detection systems use a control probe of the vector alone in order to demonstrate the absence of material with vector homology in the specimen tested. In addition to this, several approaches to determine this problem may include the use of isolated insert probes, alternative cloning vectors,

and competitor pBR322 DNA in prehybridization and hybridization mixes.

APPENDIX

I. INSTRUCTIONS FOR HYBRIDIZATION/DETECTION OF CMV

1. Immerse slides sequentially into xylene, absolute alcohol, 95% alcohol, 80% alcohol and water.
2. Incubate in 3% hydrogen peroxide for 5 minutes. (once for lung; twice for bone marrow)
3. Stop the incubation with water.
4. Apply 1ml of 0.1 mg/ml Proteinase K solution.
5. Incubate at 37 C for 30 minutes.
6. Stop the reaction with PBS-EDTA (5 umole EDTA in PBS).
7. Apply one drop of probe solution to slide. (VIAL 1).
8. Cover with coverslip.
9. Heat on 92 C water bath for 10 minutes or 2-3 minutes.
10. Hybridize at 37 C for 30 minutes or RT for 10-15 minutes.
11. Remove coverslip (Rinse with wash to loosen coverslip).
12. Tap off excess liquid.
13. Apply 4 drops of probe wash solution. (VIAL 2)
14. Let sit at room temperature for 7-10 minutes.
15. Rinse with wash buffer for 5-10 seconds.
16. Tap excess moisture off the slide.
17. Apply 3 drops of detection complex. (VIAL 3)
18. Let sit at room temperature for 10-15 minutes.
19. Prepare substrate/chromogen solution.
    To one vial of acetate buffer (VIAL 4c) add one drop of chromogen (VIAL 4a) and one drop of substrate (VIAL 4b)

20. Rinse slide with wash buffer for 5-10 seconds.
21. Tap off excess moisture from the slide.
22. Apply substrate/chromogen solution to slide.
23. Incubate at room temperature for 10-15 minutes.
24. Rinse gently with water.
25. Mount coverslip with polyvinyl alcohol.
26. View under microscope.

II. INSTRUCTIONS FOR HYBRIDIZATION/DETECTION OF EBV

1. Immerse slides sequentially into xylene, absolute alcohol, 95% alcohol, 80% alcohol and water.
2. Incubate slide with 3% hydrogen peroxide for 5 minutes. (one change for lung; two changes for bone marrow)
3. Stop the incubation with water.
4. Apply 1 ml of 0.1 mg/ml Proteinase K solution to the slide.
5. Incubate at 37 C for 30 minutes.
6. Stop the reaction by rinsing the slide with 5 umole EDTA in PBS.
7. Mix 4ul of carrier DNA with 20ul probe.
8. Mix following for the hybridization mixture
   - 50 ul deionized formamide (hybridization solvent) pH6.8 to 7.2
   - 20 ul dextran sulfate (hybridization reaction accelerator)
   - 10 ul 20X SSC
   - 24 ul carrier-probe mixture
9. Apply 20 ul of hybridization mixture to the slide.
10. Cover with coverslip.

11. Incubate in 92 C water bath for 2-3 minutes (10 min).

12. Continue incubation at room temperature for 60 minutes.

13. Remove the coverslip by immersing the slide into 50% formamide in 0.1X PBS for 5-10 minutes at room temp.

14. Rinse in 1X PBS-EDTA at room temperature for 5 minutes.

15. Apply 3 drops of detection complex.

16. Let sit at room temperature for 10-15 minutes.

17. Prepare substrate/chromogen solution as follows : To one vial of acetate buffer add one drop of chromogen and one drop of substrate. Mix.

18. Rinse the slide with wash buffer.

19. Tap excess moisture off the slide.

20. Apply substrate/chromogen solution to slide.

21. Incubate at room temperature for 10-15 minutes.

22. Rinse gently with water.

23. Mount the coverslip with polyvinyl alcohol.

24. View under microscope.

NOTE : The hybridization buffer contains formamide. Each increase of 1% in the formamide concentration lowers the Tm of a DNA duplex by 0.7 C.
Tm, melting temperature of duplex DNA, related to formamide concentration, G+C content of DNA and ionic strength of solvent.

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