Non-radioactive in situ hybridization for the detection of cytomegalovirus infections*

A.K. Raap1, J.L. Geelen2, J.W.M. van der Meer3, F.M. van de Rijke1, P. van den Boogaart4, and M. van der Ploeg2

1 Department of Cytochemistry and Cytometry, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands
2 Department of Medical Virology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
3 Department of Infectious Diseases, University of Leiden, P.O. Box 9600, 2300 RC Leiden, The Netherlands
4 Department of Microbiology, Organon International B.V., P.O. Box 20, 5340 BH Oss, The Netherlands

Accepted June 19, 1987

Summary. Acetylaminofluorene (AAF) modified cytomegalovirus (CMV) DNA probes have been applied for the rapid detection of CMV genomes by non-radioactive in situ hybridization in routinely obtained pathological material. To establish proper protocols, AAF modified mouse satellite DNA and mouse liver were used to investigate the procedural variables. Among these were type and time of fixation, glass slide coating for improved tissue adherence, protease permeabilization of sections, type and time of denaturation and hybridization, probe concentration, post-hybridization washing conditions and immunocytochemical detection. This research has led to a user-friendly procedure which, in addition to cells displaying a cytopathological effect typical for CMV infection, detects with high sensitivity CMV carrying cells that show no histo-pathological alterations. It can be readily applied in routine clinical-diagnostic laboratories.

Introduction

In recent years the interest for the application of in situ hybridization techniques for the diagnosis of viral infections has grown considerably, especially, since a number of non-radioactive DNA and RNA labeling procedures has been developed (Rudkin and Stollar 1977; Bauman et al. 1980; Van Prooijen-Knegt et al. 1982; Brigatti et al. 1983; Tchen et al. 1984; Landegent et al. 1984; Forster et al. 1985; Hopman et al. 1986; Viscidi et al. 1986). These methods are based on the fact that nucleic acid probes can be modified with compounds which do not, or only little, interfere with the hybridization reaction and which allow an immunocytochemical detection of in situ hybridized target sequences. The absence of radioactivity, the sharp localization that can be obtained, the long shelf-life of the modified probes and the speed with which results can be obtained make these methods important alternatives for existing methods of routine histo-pathological diagnosis of e.g. viral infections. An example of a viral infection for which rapid diagnosis by non-radioactive in situ hybridization may be of value is the cytomegalovirus (CMV) infection. CMV infections constitute a major problem in organ transplantation patients (Glenn 1981; Preiksitsis et al. 1983). The clinical phenomena associated with it, strongly mimic those of graft rejection. Thus, when such ambiguously explicable phenomena occur, it is necessary to run specific, sensitive and rapid laboratory tests in order to adequately adjust the immunosuppressive regime, or alternatively, supplement it with anti-viral therapy.

In this communication we report the use of acetylaminofluorene (AAF) labeling (Landegent et al. 1984) of CMV-DNA probes for the detection of CMV infections as an alternative for previously reported procedures with biotin-labeled probes (Myerson et al. 1984a, b; Unger et al. 1986; Loning et al. 1986). To establish proper protocols, practically important aspects of in situ hybridization to tissue sections have been investigated using mouse liver and mouse satellite DNA. The optimized protocols resulting from these investigations have been successfully applied to tissue sections of routinely formaldehyde fixed and frozen material of histopathologically proven cases of CMV infection.

Materials and methods

DNAs, antibodies and chemicals. Cosmid pCM-1050 was originally obtained from Dr. Fleckenstein FRG (Fleckenstein et al. 1982). Seven CMV-DNA fragments were cloned in the single stranded DNA phage M13 by one of us (PvdB). The CMV-DNA inserts of these M13 clones span 29-9 kb of the CMV genome (230 kb) and do not cross react with human DNA. Mouse satellite DNA was isolated according to Manuelidis (1977). Rabbit-anti mouse peroxidase and goat-anti rabbit peroxidase were obtained from Dakopatts (Denmark), goat-anti mouse alkaline phosphatase was obtained from Sigma (USA). Proteinase K was from Boehringer (FRG), Bio-11-DUTP was from Bethesda Research Labs (USA) and used in a standard nick-translation procedure. Acetoxy-acetylaminofluorene (acetoxy-AAF), rabbit anti-AAF and monoclonal mouse anti-AAF were generous gifts of Dr. R.A. Baan (Medical Biology Laboratory, Rijswijk, The Netherlands). Glutaraldehyde (25%) was from Aldrich (Belgium). All other chemicals used were of analytical grade.

Tissues. CMV and other virus positive autopsy materials were obtained from various pathology departments in Europe. They were routinely fixed in neutral 10% formalin for 8 to 24 h and embedded.
in paraffin. Mouse (Balb/c, male) livers were similarly processed with control of fixation time. Mouse liver nuclei isolation and methanol/acetic acid fixation were performed as described by Raup et al. (1986).

Pretreatments. To promote binding of the sections to glass, gelatin chrome-alum (GCA) coated glass slides were activated by immersion in 2.5% glutaraldehyde in PBS (15 min), washed with demineralized water and air-dried. The glass slides were stored dry for 1 to 7 days at 4°C. Alternatively, a polylysine coating (1 mg/ml) was used. Paraffin sections (5 μm) were brought on glutaraldehyde or poly-lysine coated glass slides, baked for 1 to 16 h at 52°C, deparaffinized in xylene and air-dried. When peroxidase was used as the final label, endogenous peroxidase was inactivated with 1% H2O2 in methanol for 30 min; thereafter the sections were washed with methanol and air-dried. To inactivate aldehyde groups, sections were immersed for 15 min in 1% hydroxylammoniumchloride in PBS, pH 7.4. After dehydration in a graded series of ethanol, the sections were treated with (predigested) proteinase K in 20 mM Tris-HCl, 2 mM CaCl2, pH 7.4 at 37°C for 30 min, dehydrated and air-dried. 5-μm-thick cryostat sections from frozen material of a known CMV positive lung were fixed in either 4% paraformaldehyde in PBS or in Bouin’s fixative for 20 min at room temperature.

Denaturation, hybridization and washings. The following denaturation/hybridization protocols were used. Sections were denatured in 0.15 M NaOH in 70% ethanol for 4 min or by immersion in 50% deionized formamide/2× SSC at 70°C or 100°C for 10 min and after ethanol dehydration, air-dried. The denatured probe was applied under a coverslip and hybridization was allowed to proceed at 37°C in a moist chamber equilibrated with 50% formamide/2× SSC. Alternatively, probe and section were denatured simultaneously. For this purpose, probe was applied on the slide under a coverslip and sealed with rubber cement. Next, the sections were incubated for 10 min in an oven of 80°C after which they were brought to 37°C for the indicated time of hybridization. For cryostat sections the denaturation time was reduced to 2 min. The probe mixture consisted of 5 mg/μl AAF-DNA in 50% formamide, 4× SSC, 40 mM NaH2PO4, 10% dextran sulphate, 0.1% BSA, 0.1% polyvinlypyrrolidone, 0.1% Ficoll and 250 μg/ml sonicated salmon sperm DNA, pH 6.8.

Two post-hybridization washing protocols were used. Hybridized sections were either washed 3 times for 20 min in 50% formamide/2× SSC at room temperature, or 2 times 5 min in 2× SSC RT, 0.1% SDS, 10 min 0.1× SSC, 0.1% SDS at 50°C and finally briefly in 2× SSC.

Immunocytochemistry. After post-hybridization washings, sections were briefly washed with PBS and incubated for 20 min at 37°C in 2% BSA in PBS to block specific binding of anti-sera. Then a 400-fold dilution of monoclonal anti-AAF (IgG2a) in 2% BSA, 0.05% Tween-20 in PBS was applied for 45 min at 37°C. After washing 3× with PBS containing 0.05% Tween-20, anti-mouse peroxidase (1:50) or anti-mouse alkaline phosphatase (1:50) in PBS, BSA/Tween was allowed to react for 45 min at 37°C. Finally, sections were washed for 3 times with PBS/Tween. Peroxidase activity was visualized by staining for 5 min in 0.5 mg/ml DAB, 0.05% H2O2 in 50 mM Tris-HCl containing 0.01 M imidazole. Alkaline phosphatase activity was visualized by staining for 10 to 20 min in 0.167 mg/ml 5-bromo-3-chloroindolylphosphosphate (BCIP), 0.33 mg/ml nitroblue tetrazolium salt (NBT) in 200 mM Tris-HCl, 10 mM MgCl2, pH 9.6. DAB/H2O2 stained sections were counterstained with Mayer’s haematoxylin or with methylene blue, and BCIP/NBT stained sections with Nuclear Fast Red.

Results

Coating of glass slides

When gelatin-chrome alum coated object slides were used, severe problems arose due to loss of tissue sections during the in situ hybridization procedure. Therefore various coating procedures were tested for their ability to keep the tissue sections fixed to the glass slides. Good results were obtained with GCA glass which was activated with glutaraldehyde or with poly-lysine coated glass. Without the use of such coatings, the tissue sections are completely lost from the glass slides during processing. With such coatings there is hardly any detachment of tissue sections even after considerable proteinase K digestion.

Effect of protease treatment and formaldehyde fixation time

Figure 1 shows the effect of proteinase K treatments on the detectability of satellite sequences in formalin fixed mouse liver. The more intense the protease treatment is, the more satellite DNA sequences become available to in situ hybridization. Use of stronger protease treatments leads ultimately to deterioration of the tissue morphology.

As it was anticipated that retrospective analysis of material from formalin archives will be important, the influence of formalin fixation time was investigated with mouse liver and mouse satellite DNA-AAF as probe. The results are given in Table 1. For the employed denaturation and hybridization protocol it was shown that longer formalin fixation periods have an adverse effect on the in situ hybridization signal. By increasing the proteinase concentration, however, some improvement can be obtained. The period of time that the tissue is in paraffin is of no influence. During the course of our investigations with human virus infected material it became apparent that for the detection of viral nucleic acid sequences the fixation time is less critical, which may be due to the fact that the viral DNA, in contrast to genomic human DNA, is not wrapped in the nucleosome and higher order structures. For instance, we have performed successful in situ hybridizations to tissues with a known formalin fixation time of 10 days. On the other hand, with CMV positive tissue that had been fixed for at least three weeks, no positive hybridization results were obtained. For cryostate sections a number of fixatives were tested i.e. 4% formaldehyde in PBS, freshly prepared paraformaldehyde (4%) in PBS, Bouin’s and Carnoy’s fixatives. Paraformaldehyde and Bouin’s fixative gave...
Fig. 1A and B. Effect of proteinase K treatment on the detectability of mouse satellite DNA sequences in 5 μm formalin fixed mouse liver sections. Denaturation was done at 70° C in 70% formamide/2SSC. A This section was incubated with 10 μg/ml proteinase K and scored as +/−; B This section was incubated with 50 μg/ml proteinase K and was scored as ++. No counter-staining. Bars represent 20 μm

the best signal to noise ratios after optimization of proteinase treatment and time of heat denaturation.

Kinetics of in situ hybridization and effect of probe concentration

To get an impression of the kinetics of in situ hybridization of double stranded DNA probes, we recorded microfluorometrically the immunofluorescent signal of methanol/acetic acid fixed mouse liver nuclei hybridized with mouse satellite DNA-AAF according to Raap et al. (1986). It can be seen in Fig. 2 that already after 5 min, measurable hybridization signals are obtained. Maximum signals are obtained after an overnight hybridization (16 h).

Figure 3 gives the effect of probe concentration on the in situ hybridization signal of isolated methanol/acetic acid fixed mouse liver nuclei. It can be seen that at 35 ng/μl a maximum signal is obtained. The use of this concentration would, however, imply the consumption of rather large amounts of probe DNA.

Denaturations and post-hybridization washings

Several denaturation protocols were investigated. The ethanolic alkaline denaturation, which works well with metaphase chromosomes and isolated methanol/acetic acid fixed

mouse liver nuclei (Landegent et al. 1984; Raap et al. 1986), resulted in less intense in situ hybridization signals than thermal denaturations. No gross differences in intensity or in number of CMV-DNA positive cell were observed between denaturation of the sections with 70% formamide/2SSC at 70° or 100° C and with 50% formamide/2SSC at 80° C with probe present, but the morphology obtained with the latter denaturation was by far superior. From the practical point of view, the simultaneous denaturation of
probe and target sequences has also the advantage that it prevents use of hot formamide solutions.

Simultaneous denaturation of probe and tissue has furthermore the theoretical advantage that in situ renaturation has a stronger competition with in situ hybridization (Unger et al. 1986). However, the speed with which in situ renaturation occurs (Raap et al. 1986) is such that the in situ hybridization has no chance to interfere with it.

The stringency of the post-hybridization washings determines to a large extent the specificity of the hybridization result. With both the 50% formamide/2 x SSC room temperature and the 0.1 x SSC/0.1% SDS, 50°C washings, no cross-hybridization reaction was observed with tissues known to be infected with Herpes Simplex virus type 1, the polyoma virus JC or parvo virus B19. On the other hand these control tissues reacted strongly when probed respectively with AAF labeled HSV-1, JC and parvo-B19 DNA (Boerman et al. in preparation; Salimans et al. in preparation).

Immunocytochemical detection

With the DAB/H2O2 imidazole peroxidase protocol it was sometimes difficult to distinguish the golden-brown DAB product from pigments present in the tissue. Therefore we have also used the alkaline phosphatase BCIP/NBT protocol which results in a blue precipitate. Previously we have shown that both immunoenzyme cytochemical protocols are of equal sensitivity (de Jong et al. 1985).

**CMV-DNA detection in tissue sections**

Initially we have used the cosmid clone pCM-1050 for detection CMV-DNA in infected tissues. When the preparations are screened microscopically, cells showing the CMV characteristic cytopathological effect are very easily detected with the 10 x objective. In addition hybridization positive non-cytomegalic cells are readily discerned. We have also used biotin-labeled pCM 1050 DNA according to Unger et al. (1986), but no differences in sensitivity were noticed.

In the latter part of our study we used CMV-DNA sequences cloned in the single-stranded DNA bacteriophage M13, comprising 29.9 kb of the CMV genome. Such single-stranded DNA probes have the advantage over double-stranded DNA probes that probe self-annealing does not occur. The formation of large, partially single-stranded probe DNA catenates, which may penetrate the sections slowly or not, is thus prevented, leading to enhanced in situ hybridization signals in comparison with double-stranded probes (Landegent, personal communication). Figure 4 shows some representative micrographs of results that can be obtained on a routine basis with these probes following the described DAB staining protocol. Furthermore, the M13-CMV-DNA-AAF mix allowed us to decrease the hybridization time to 2 h without loss in sensitivity.

**Discussion**

In this communication is presented the successful application of AAF-labeled DNA probes for the non-radioactive detection of DNA sequences in routinely processed formalin fixed tissue sections as well as in cryostate sections of frozen tissues. The AAF labeling procedure has the following features. AAF binds mainly to the C-8 position of guanosine residues, a site not involved in base pairing. The melting temperature of AAF modified duplex DNA is lowered 1.1°C/% modification (taking in account all bases). Routinely we use a degree of modification of 1% to 5%. Both DNA and RNA, single- as well as double-stranded, can be modified reproducibly in both small and large quantities and the AAF-labeled probes can be stored indefinitely. High affinity poly- and monoclonal anti-AAF antisera are available.

The following methodological points concerning in situ hybridization to formalin fixed tissue sections deserve attention. First, care must be taken to prevent the loss of tissue sections from glass slides during the rather harsh in situ hybridization conditions. The use of glutaraldehyde activated gelatin-chrome alum and poly-lysine coating will circumvent this problem. Secondly, to promote diffusion of high molecular weight reactants (probes, antibodies) into the fixed tissue, protease treatment is a prerequisite. It is advised to titrate the protease concentration. The protease treatment, which gives an optimal balance between in situ hybridization signal and morphology should be used. A general protease protocol can not be given as the optimal treatment may depend on the type of tissue, the type of fixation, the fixation time and the nature of the target sequences and their surroundings. As an example, it is our experience that for visualizing mouse satellite DNA in formaldehyde fixed liver tissue a considerable stronger pro-
tease treatment is necessary than for the detection of viral sequences. Also the one order of magnitude difference in proteinase K concentration used for en bloc formalin fixed mouse liver and for isolated methanol/acetic acid fixed mouse liver nuclei is illustrative in this respect. An indication can, however, be given for virus infected, routinely processed pathological material. With our CMV positive material we got satisfactory results using 10–50 μg/ml proteinase K for 30 min at 37°C. Thirdly, it is our experience that prolonged formalin fixation periods have an adverse effect on the in situ hybridization results. It should be remarked that this effect is probably more pronounced for cellular genomic sequences than for viral sequences. Finally, the tissue denaturation step is critical. For formalin fixed tissue sections we found that thermal denaturations using hot formamide/SSC mixtures give better results than alkaline denaturations and that simultaneous heat denaturation of probe and tissue section under a coverslip is better than separate denaturations.

For cryostate sections a similar protocol can be followed, provided proteinase K treatment and denaturation time are optimized.

From the results it is clear that the present in situ hybridization procedure is more sensitive than conventional histopathology in detecting CMV infections. While evaluating the hybridized sections microscopically, one looks for signals (i.e. darkly colored cells) rather than morphology (i.e. cytopathological effect characteristic for CMV). This allows, even for inexperienced persons, a rapid and more objective screening than is possible with conventional staining techniques. In addition, it allows the identification of CMV positive cells which would have gone unnoticed by histopathological criteria. These facts, together with the speed with which results are obtained, show the feasibility of this AAF in situ hybridization procedure as a diagnostic tool in histopathology. It is at least as sensitive as procedures based on biotinated probes. This is in accordance with the idea that the various non-radioactive hybridization procedures based on hapten modification are of similar sensitivity, because the number of haptons introduced per nucleotide is approximately equal for all methods. If in practice, differences in sensitivity are noticed, they most probably are due to differences in the quality of the immunocytochemical detection systems.

Since now several non-radioactive hybridization techniques are available, it becomes feasible to perform double (Hopman et al. 1986a) and triple hybridizations (Nederlof et al., in preparation) so that tissue specimen can be screened by hybridization for the presence of more than one pathogen simultaneously. Furthermore it seems feasible to combine the present hybridocytocchemical technique with immunocytochemical identification of putative infected cells, e.g. endothelial cells with anti-Factor VII antibodies.

The objects used in this study contain an unknown, but in most cases probably high number of CMV-DNA copies per cell and an estimate of the absolute sensitivity of the non-radioactive in situ hybridization technique on routinely processed material is hardly possible. Continuing efforts to optimize the procedures may lead to a sensitivity that is comparable to the one obtainable with in situ hybridization of metaphase chromosome spreads in which low repetitive and unique DNA sequences can be located with a non-isotopic in situ hybridization procedure (Landeget al. 1985; Hopman et al. 1986b).

The reported method is now fully applicable for the routine diagnosis of CMV infections and it provides a practical alternative to, or supplementation of other diagnostic methods such as immunocytochemistry for CMV antigens, viral culture especially when fresh or frozen tissue is unavailable. Retrospective analysis of paraffin embedded tissues in archives of pathology departments is possible. With the described procedure also other viral infections (e.g. HSV-1 in Herpes encephalitis, JC virus in progressive multifocal leucoencephalopathy and parvo virus in a case of hydrops fetalis) have been detected with success.

With continued improvements of sensitivity, simplicity and speed of hybridization procedures and with the knowledge that more probes coding for known viral function will become available, the pathologist can look forward to the time when more subtle molecular genetic analysis of viral infections, including the latent stage (Schrier et al. 1985) in morphologically preserved tissues are possible on a routine basis.

Acknowledgements. The authors wish to express their gratitude to drs W. Ulrich, Vienna; H. Feichtinger, Innsbruck; J.H. van der Vegt, Deventer and Ph.J. Hoedemakers, Leiden for providing CMV positive material and to drs R.H. Boerman and M. Salimans, Leiden for providing JCV and parvo virus positive material as well as DNA probes for these viruses.

References

Loning T, Milde K, Foss HD (1986) In situ hybridization for
the detection of cytomegalovirus (CMV) infection. Application of biotinylated CMV-DNA probes on paraffin embedded specimen. Virchows Arch 409:777-790

Manuelidis L (1977) A simplified method for the preparation of mouse satellite DNA. Anal Biochem 78:561-568


