Laboratory techniques

Detection of cytomegalovirus antigens and DNA in tissues fixed in formaldehyde

N M JIWA,* A K RAAP,* F M van de RIJKE,* A MULDER,* J J WEENING,†
F E ZWAAN,‡ T H THE, M van der PLOEG* Departments of *Cytochemistry and Cytometry,
†Pathology, and ‡Haematology, the University of Leiden, and the Department of Clinical Immunology,
University of Groningen, The Netherlands

SUMMARY Immunohistochemical techniques with monoclonal antibodies against cytomegalovirus (CMV) immediate early (IEA) and early antigens (EA), and in situ hybridisation, were used to detect CMV infection in routinely obtained, formaldehyde fixed and paraffin wax embedded tissues taken from bone marrow transplant patients, who had died from interstitial pneumonia. To improve the rates of detection of CMV-IEA and EA the wax embedded material was pretreated with 0.4% pepsin/HCl at 37°C for 30 minutes. This pretreatment was also advantageous for in situ hybridisation. In the patients with histological evidence of CMV infection or positive viral culture from the lung tissue, or both, viral proteins and nucleic acids were detected in lung, as well as in other organs. Immunohistochemical techniques proved superior in heavily infected but necrotic tissues. In control patients (patients who had died from interstitial pneumonia without any evidence of CMV, or with no interstitial pneumonia at all) in situ hybridisation showed no positive signal, while immunohistochemical techniques showed only a few positive cells in lung tissue of one of nine patients. In addition to CMV-DNA analysis, formaldehyde-fixed, paraffin wax embedded tissue is amenable to immunohistochemical analysis with CMV monoclonal antibodies.

The course of cytomegalovirus (CMV) infection in man is mainly asymptomatic¹ but can be extremely harmful when the immune system is immature or compromised as is the case in patients with acquired immune deficiency syndrome (AIDS) and in allograft recipients.^{2,3} In patients who have undergone bone marrow transplantation interstitial pneumonitis is the most serious consequence of CMV infection. Mortality from this type of pneumonitis is about 60–80%.^{4,5} The pathogenesis of CMV interstitial pneumonitis is still obscure. To clarify the pathogenic process, therefore, it is necessary to document the infected cell type in relation to the overall histopathology. To confirm or exclude CMV as the causative agent of interstitial pneumonia, cytological examination of bronchoalveolar lavage fluid and histopathological examination of open lung biopsy specimens are important methods. Such histological examination is useful because CMV forms distinctive nuclear and cytoplasmic inclusion bodies but its sensitivity is low.⁶ Therefore, other rapid and sensitive

diagnostic methods are required. These include electron microscopy,⁷ in situ hybridisation,^{8–10} and viral antigen detection using monoclonal antibodies. This last technique is successful for use with frozen tissue sections,^{11,12} direct examination of bronchoalveolar lavage fluid,¹³ and peripheral blood leucocytes^{14,15} but has not until now been suitable for routinely formaldehyde fixed tissue sections.

In this study we describe a procedure for immunohistochemical detection of CMV-IEA and EA in routinely obtained formaldehyde fixed and paraffin wax embedded tissues, comparing the results with those of in situ hybridisation using acetylaminofluorene (AAF) modified DNA probes.^{10,16} Routinely obtained formaldehyde fixed necropsy material was used from patients who had had bone marrow transplantation and who had died of CMV interstitial pneumonia, idiopathic interstitial pneumonia, or other causes.

Material and methods

All patients (n = 16) had undergone allogeneic bone marrow transplantation for haematological malignancies. Grouping was as follows: group 1 (n = 7)

comprised patients who had died with confirmed interstitial pneumonitis, based on a clinical picture, positive viral culture from lung tissue or histological aberrations typical of CMV lung infection. Group 2 ($n = 4$) consisted of patients who had died of an interstitial pneumonia without evidence of a CMV infection (negative viral culture and absence of CMV histological aberrations). Patients of group 3 ($n = 5$) had died without interstitial pneumonia. At least four different, routinely obtained, formaldehyde fixed, paraffin wax embedded tissues were investigated from all patients, most often including liver, kidney, spleen and the adrenal glands.

Seven CMV-DNA fragments were cloned in single stranded DNA phage M13 and were a generous gift of Dr PF Lens (Organon Teknica, Oss, The Netherlands). The CMV-DNA inserts of these fragments span 29.9 kilobase pairs of the CMV genome (237 kilobase pairs) and do not cross react with human DNA. HSV-1 DNA, human parvovirus B19 DNA, pBR322 and phage M13, used as negative controls, never gave any signals in material positive for CMV-DNA. DNA samples were modified with acetylaminofluorene according to the method of Landegent *et al.*¹⁶ For in situ hybridisation the probe was fragmented to average lengths of 200–400 base pairs by sonication.

Acetoxy-acetylaminofluorene and monoclonal mouse anti-AAF were generous gifts of Dr RA Baan (Medical Biology Laboratory, Rijswijk, The Netherlands). Murine anti-CMV-IEA monoclonal antibodies C-10, C-11, and C-12, which recognise 70 kilodalton IEA proteins, were developed by The *et al* and have been described elsewhere.¹⁷ Murine anti-CMV-IEA was developed by Rothbarth *et al* and murine E13 monoclonal against a 68 kilodalton CMV-IEA was obtained from Sanbio (Sanbio Biosoft, France).

Peroxidase conjugated rabbit anti-mouse immunoglobulin was obtained from Dakopatts (Denmark). SSC was 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5; phosphate buffered saline (PBS), pH 7.4, and PBS containing 0.05% Tween-20 (Sigma Chemicals, USA) (PBST) were also used. Bovine serum albumin (BSA) (fraction 5) was obtained from BDH Chemicals Ltd, Poole, England.

IMMUNOHISTOCHEMICAL PROCEDURES

Formaldehyde fixed, paraffin wax embedded tissue sections (5 μ m) were mounted on glass slides cleaned with alcohol and ether. After deparaffinisation in xylene the tissue sections were placed in prewarmed 0.4% pepsin (Sigma, USA) in 0.2N HCl at 37°C for 30 minutes. Formaldehyde fixed tissues were blocked with 1% hydroxylammoniumchloride in PBS for 15 minutes. The slides were incubated with the different

monoclonal antibodies against CMV-IEA or EA at 37°C for 45 minutes. After washing in PBS the slides were incubated with rabbit-anti-mouse-peroxidase 1/100 at 37°C for 45 minutes. Visualisation of the peroxidase and counterstaining procedures are described below.

NON-RADIOACTIVE IN SITU HYBRIDISATION PROCEDURE

In situ hybridisation procedures were performed according to Raap *et al.*¹⁰ with the modification that pepsin¹⁸ was used instead of proteinase K. Briefly, formaldehyde fixed, paraffin wax embedded tissue sections (5 μ m) were mounted on polylysine coated coverslips and baked for one hour at 56°C. After deparaffinisation in xylene the slides were treated with prewarmed 0.4% pepsin in 0.2N HCl for 30 minutes at 37°C. Hereafter the slides were briefly washed in PBS, incubated for 15 minutes in 1% hydroxylammonium-chloride in PBS, and finally dehydrated in graded alcohol and air dried. The probe mixture (10 ng/ μ l) was dissolved in 50% formamide/5 $\times$ SSC, 25 mM Na₂HPO₄, and 250 μ g/ml sonicated salmon sperm DNA, pH 6.8. This mixture was put on the tissue sections and target and probe DNA sequences were denatured at 80°C for 10 minutes. The slides were hybridised at 37°C for 16 hours, washed several times with 2 $\times$ SSC (room temperature) followed by 0.1 $\times$ SSC at 50°C, and finally rinsed in 2 $\times$ SSC.

After blocking with 2% BSA in PBST at 37°C for 20 minutes the slides were treated with monoclonal anti-acetylaminofluorene diluted 1/1000 in 2% BSA-PBST for 45 minutes at 37°C, washed in PBST, and incubated with rabbit anti-mouse IgG peroxidase diluted 1/100 in 2% BSA-PBST at 37°C for 45 minutes. Peroxidase activity was visualised by incubation for five minutes in 0.5 mg/ml diaminobenzidine (DAB, Sigma Chemicals, USA), 0.05% H₂O₂ in 50 mM Tris-HCl, pH 7.4, containing 0.01M imidazole, pH 7.4. Slides were briefly counterstained with Mayer's haematoxylin. When haemosiderin or other iron pigments were present in the tissue sections, Perls's Prussian Blue counterstaining was performed.

Results

LUNG SAMPLES

At light microscopical examination, lung tissue of patients of group 1 showed interstitial pneumonia with a variable degree of severity. Alveolar septa were oedematous with foci of fibrinous exudation, extravasation of erythrocytes, and haemorrhage. Only sparse inflammatory infiltrates were present. Four patients showed hyalin membranes and in six (numerous) nuclear inclusion bodies typical of CMV infection were seen. In one patient (case 7), no CMV

Fig 1 Section (5 µm) of lung tissue of case 4. Immunoperoxidase-DAB detection of CMV-IEA antigens using monoclonal antibodies. Slightly counterstained with haematoxylin. Arrows: positive cells.

cytopathic effects were seen in the necropsy material despite the fact that these effects were clearly visible in an open lung biopsy specimen taken several days before the patient died. Morphologically changed cells were not seen in groups 2 and 3 patients.

Immunohistochemical techniques failed to give positive signals when formaldehyde fixed, paraffin wax embedded tissue sections were used without any prior treatment. When the tissue sections were pretreated with pepsin, positive nuclear and cytoplasmic signals were obtained in all cells with morphological aberrations with all the CMV-IEA and EA monoclonals tested. In addition to these, positive signals were also obtained in cells without any morphological aberration, indicating that more cells were infected than histological analysis indicated (fig 1). Such observations were made for all patients of group 1. In group 2 only one patient showed weak positive signals in a few cells of the lung when immunohistochemistry was

Fig 2 Section of same tissue but hybridised with 29.9 kilobase inserts of CMV AD169 and slightly counterstained with haematoxylin. Arrows: positive cells.

used, indicating that CMV antigens, in spite of the negative viral culture and lack of morphologically changed cells, were present in the affected lung tissue. Lungs from patients in group 3 were negative for antigens. Using pepsin/HCl as pre-digestion for in situ hybridisation, the morphological detail was better preserved than after treatment with proteinase K in all tissues examined. Positive signals were obtained in the same population of cells as those described for immunohistochemistry (fig 2), although the patient in group 2 failed to show any positive signal: patients in group 3 were negative.

OTHER ORGANS

The organs examined from patients of group 1 are listed in the table which also gives the comparison between histological, in situ hybridisation, and immunohistochemical results. These organs were also

Table Comparison between histological, in situ hybridisation, and immunohistochemical results of bone marrow transplant patients who died with interstitial pneumonitis

Case No	Tissue examined	Histological aberrations	In situ hybridisation	CMV-IEA
1	Lung	++	++	++
	Liver	-	+	+
	Spleen	-	+	+
	Kidney	-	+	+
	Adrenal gland	+	++	+++
2	Lung	++	++	++
	Liver	Not available	-	-
	Spleen	Not available	-	-
	Kidney	-	-	-
	Adrenal gland	-	-	-
	Salivary gland	-	-	-
3	Lung	++	++	++
	Liver	-	+	+
	Spleen	-	-	+
	Kidney	-	+	+
	Adrenal gland	-	+	+
	Small intestine	+++	+++	+++
4	Lung	++	++	++
	Liver	-	+	+
	Spleen	-	-	-
	Kidney	-	+	+
	Pancreas	-	+	+
	Adrenal gland	+	+	+
5	Lung	+	++	++
	Liver	-	+	+
	Spleen	-	+	+
	Adrenal gland	+	+	+
	Salivary gland	+	+	+
	Thyroid gland	-	+	+
6	Lung	+	+	+
	Liver	-	+	-
	Spleen	-	+	+
	Kidney	-	+	+
7	Lung (biopsy)	+	++	++
	Lung (necropsy)	-	+	+
	Liver	-	-	-
	Spleen	-	-	-
	Adrenal gland	+	+	++

- negative; + few positive cells; ++ large number of positive cells; +++ foci heavily infected.

Fig 3 (a) *In situ hybridisation of adrenal gland of case 1;*
(b) *details, note typical nuclear DAB staining.*

investigated for patients of the two other groups. In most patients cells specifically changed by CMV were scattered in one or more other organs. Only in cases 2 and 6 did no other organ than the lungs show these cells. Case 7 had no typically changed cells in the lung tissue at necropsy although the adrenal gland showed some suspicious cells. In only one organ (small intestine of case 3) were foci of heavily infected cells observed in the section stained with haematoxylin and eosin. Both immunohistochemical techniques and *in situ* hybridisation detected positive cells in organs which had been interpreted as negative by histological examination. This was especially true of the adrenal glands, liver, splenic and kidney tissues which often became positive using these methods. In some sections results of *in situ* hybridisation and immunohistochemistry were not identical. This was observed when few CMV infected cells were present in the tissue sections examined, indicating that this discrepancy could be due to sampling error. In tissues seriously affected by CMV and showing necrosis, immunohistochemical techniques identified two to three times as many positive cells than *in situ* hybridisation (figs 3a–b

and 4a–b). This was particularly observed in the adrenal glands of cases 1 and 7.

The presence of iron pigments in the tissue sections makes it difficult to distinguish these pigments from the DAB precipitates. Therefore, the iron staining Perl's Prussian Blue in combination with haematoxylin was used as an alternative. This resulted in a very good distinction between the bright blue-stained iron pigments, the brown DAB precipitates, and the purple-blue haematoxylin.

The same organs of the patients in the two other groups were also investigated, but no positive results for CMV were obtained.

Discussion

For a definite diagnosis of CMV infections, typical histological signs are of limited value because histopathological changes are seen only in a small percentage of cases in which CMV disease is suspected (six in this study). For that reason other detection methods with preservation of morphological detail have been developed. *In situ* hybridisation has proved

Fig 4 (a) *Detection of CMV-IEA antigens in tissue section of same adrenal gland as in fig 3. Note higher numbers of positive cells and mainly cytoplasmic DAB staining.*
(b) *Details.*

Detection of CMV antigens and DNA in tissue fixed in formaldehyde

especially useful for analysis of paraffin wax embedded material. Immunohistochemical procedures for the detection of CMV antigens in tissues have been successfully used on frozen tissue sections. Naoumov *et al*¹⁹ and Nelson *et al*²⁰ used formaldehyde fixed, paraffin wax embedded tissue sections for in situ hybridisation and immunohistochemistry for CMV detection. The latter technique gave much less reliable results than in situ hybridisation, but tissue sections were not pretreated for immunohistochemical analysis. We used proteolytic enzymes successfully to unmask different kinds of antigens in formaldehyde fixed material,^{21,22} but until now, not for the detection of CMV antigens. The results obtained with immunohistochemistry and in situ hybridisation using this protocol proved similar in most cases.

The discrepancy between the results of antigen detection and in situ hybridisation in certain tissues (adrenal glands of cases 1 and 7) could have occurred for several reasons. Antigen detection might be more sensitive than in situ hybridisation as only a few DNA molecules can be the source of a large number of protein molecules for detection by immunological procedures. In our study we used a probe of 29 kilobase pairs from the entire CMV genome which is 237 kilobase pairs. Even when the entire genome was used, we were not able to show the presence of additional positive cells (data not shown). The most likely explanation for the discrepancy observed is that necrotic areas are full of lytic enzymes which degrade DNA, while proteins are more resistant, at least for some time. This could explain why antigen detection was still possible while in situ hybridisation is unable to detect CMV-DNA in tissues with lytic components.

With the in situ hybridisation and immunohistochemical techniques, CMV was detected in all patients who had died having been diagnosed as having CMV pneumonia. The relatively low prevalence of cells positive for CMV-DNA or antigen in lungs which had obviously been affected, supports the view that the presence of replicating virus or viral antigens could not fully explain the pathogenesis of the CMV interstitial pneumonia. It is well known that this type of pneumonia is associated with severe graft-versus-host disease.²³ Indeed, in patients with autologous and syngeneic bone marrow transplantations CMV interstitial pneumonia is rare,²⁴ although CMV infections occur frequently. Two of the patients described (cases 6 and 7) were treated with the anti-viral drug 9-[1,3-dihydroxy-2-propoxymethyl] guanine (DHPG). In case 6 only a few cells were CMV positive at histological examination, immunohistochemistry, or in situ hybridisation, in spite of the severe lung disease. Case 7 was clearly positive for CMV in all three tests in a lung biopsy specimen but in necropsy material no evidence for CMV could be found. These observations

agree with those of Shepp *et al*,²⁵ who described that 90% of patients with CMV interstitial pneumonia died from their pulmonary complications in spite of the fact that administration of DHPG resulted in an almost complete reduction of CMV in their lung tissues.

In this study we have shown that immunohistochemistry and in situ hybridisation are useful for elucidating the role of CMV infection in the pathogenesis of interstitial pneumonia, especially when tissues are examined before administration of antiviral drugs. For rapid diagnosis in routine pathology, we prefer the immunohistochemical method because results are obtained within four hours, and even in necrotic tissues, good results can be obtained.

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Requests for reprints to: Dr N M Jiwa, Department of Cytochemistry and Cytometry, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.