Laboratory techniques

Detection of cytomegalovirus by in situ hybridisation and immunohistochemistry using new monoclonal antibody CCH2: a comparison of methods

G NIEDOBITEK,* TERESA FINN,* H HERBST,* J GERDES,* LENA GRILLNER,† MARGARETA LANDQVIST,† BENITA ZWEYGBERG WIRGART,† H STEIN*

*Department of Pathology, Klinikum Steglitz, Freie Universität Berlin, Federal Republic of Germany, and the

†Institute of Clinical Microbiology, Section of Virology, Karolinska Hospital, Stockholm, Sweden

SUMMARY In situ hybridisation, immunohistochemistry, and morphological analysis for the detection of cytomegalovirus (CMV) were compared in routinely processed tissue sections from a patient with acquired immune deficiency syndrome (AIDS) and widespread CMV infection. Both in situ hybridisation and immunohistochemistry with the monoclonal antibody CCH2 labelled all “owl’s eye” cells intensely and, in addition, nuclei of some morphologically normal cells. Quantitative evaluation of the results showed that in situ hybridisation and immunohistochemistry with CCH2 were considerably more sensitive than purely morphological analysis, particularly in tissues with only a few cells infected by CMV.

It is further shown that immunohistochemistry with CCH2 detected a higher figure of CMV infected cells than in situ hybridisation. In conclusion, both in situ hybridisation and immunohistochemistry are rapid, sensitive, and specific methods for CMV detection. For routine purposes, however, immunohistochemistry seems to be more suitable.

The incidence of cytomegalovirus (CMV) infections associated with the dissemination of human immunodeficiency virus (HIV) infections is increasing,¹⁻³ thus making the rapid and sensitive diagnosis of CMV infections important. The commonly applied histopathological and virological techniques both have their respective drawbacks; the former is rapid, but not sensitive,⁴ and the latter is sensitive, but not rapid.⁵ Methods suitable for both rapid and sensitive detection of CMV infection are in situ hybridisation with biotinylated CMV-DNA probes⁶⁻⁸ and immunohistochemistry using CMV specific antibodies.⁹ To find out which approach was superior for routine purposes, we compared these two methods with each other and with routine CMV detection by histological examination.

Material and methods

PLASMID AND PROBES

PCM5018 (human CMV strain AD169 EcoRI-J-fragment subcloned into pACYC184), which does not share any sequence homologies with human DNA,¹⁰

was kindly provided by Dr B Fleckenstein, Erlangen, Federal Republic of Germany. For controls we used the Bam HI-W-fragment of Epstein-Barr virus, strain B95-8, subcloned in pBR322, obtained from Dr G W Bornkamm, Freiburg, Federal Republic of Germany.¹¹ Total plasmid DNA was labelled with biotinylated dCTP (Enzo) by nick translation.¹²

IN SITU HYBRIDISATION

In situ hybridisation was performed on routinely processed tissues as described.^{13,14} Tissue sections were collected on to poly-L-lysine coated slides¹⁵ and incubated at 65°C overnight. After dewaxing and rehydration the slides were pretreated in methanol/0.03% hydrogen peroxide, washed in 2 × SSC (0.3M NaCl/0.03M sodium citrate, pH 7.4), incubated in 0.02N hydrochloric acid for 10 minutes at room temperature, rinsed in 2 × SSC, incubated in 0.01% Triton X-100 at room temperature for one and a half minutes, and washed again in 2 × SSC. After incubation in pronase (10 mg/ml, Boehringer) (10 minutes, room temperature) and washing in 2 × SSC the slides were ready for hybridisation in a mixture of 2 × SSC, 50% deionised formamide, 10% dextran sulphate, 375 µg/ml herring sperm DNA,

and 2.5 µg/ml biotinylated probe. About 25 µl of hybridisation mixture was applied to each section. Cellular DNA and probe were denatured at 90°C for three minutes. After hybridisation for 15 minutes at room temperature the sections were washed for five minutes each in 50% deionised formamide/0.1 × SSC and in 2 × SSC/0.01% Triton-X. Immobilised biotin was detected with an avidin-horseradish peroxidase complex (Enzo, 1/200) for 30 minutes at room temperature and diaminobenzidine.^{16 17}

The monoclonal antibody CCH2 (IgG1, k) was obtained by immunisation with a lysate of cells infected with CMV and screened for selective reactivity with infected fibroblasts. It has been tested for cross reactivity against adenovirus, herpes simplex virus, Epstein-Barr virus, and varicella zoster virus with negative results (Landqvist *et al*, unpublished observations). CCH2 selectively precipitates a 43 kD protein from infected fibroblasts. Screening of CCH2 on human tissues from donors without CMV infection showed no cross reactivity (table 1).

Sections of paraffin wax embedded tissue were dewaxed, trypsinised in a 1 mg/ml solution at 37°C for 15 minutes, and stained with CCH2 using the APAAP method¹⁸ and new fuchsin¹⁹ for immunohistological analysis.

In haematoxylin and eosin stained sections, cells infected with CMV were identified by their typical intranuclear inclusions. For comparison of the different methods, two sections of the lungs and one

Table 1 Immunohistochemical staining with antibody CCH2

<i>Tissue</i>	<i>Not infected with CMV</i>	<i>Infected*</i>	
Heart	Negative	Positive	
Lung			
Liver			
Kidney		Positive	
Spleen			
Lymph node			
Tonsil		Positive	
Bone marrow			
Skeletal muscle			
Brain			
Thyroid gland			Positive
Parathyroid gland			
Pancreas			
Adrenal gland			

*Positivity is restricted to cells with diagnostic nuclear inclusions and few normally appearing cells

parotid section were evaluated by using a graticule and counting nine fields at a × 400 magnification.

CASE REPORT

A 32 year old man with AIDS died with the clinical signs of pneumonia. Necropsy showed widespread pneumonia, extensive ulceration of anal mucosa, and oral candidiasis. Microscopically disseminated CMV infection in almost every organ was found. Lungs, parotid and thyroid glands, and the whole gastro-intestinal tract were most severely infected (table 2).

Fig 1 (a) CMV infected cells by in situ hybridisation in a lung section. (Haematoxylin counterstaining.) (b) Demonstration of one CMV infected cell in a glomerulus by in situ hybridisation. (Haematoxylin counterstaining.)

Results

DETECTION OF INFECTED CELLS BY ROUTINE HISTOLOGICAL ANALYSIS

Routinely stained sections of this case showed numerous cells with "owl's eyes inclusions" in the

lungs, parotid glands, and the gastrointestinal tract. Other tissues, including renal glomeruli and liver, showed occasional infected cells (table 2).

DETECTION OF CMV BY IN SITU HYBRIDISATION
Staining results are summarised in table 2. In situ

Fig 2 Immunohistochemical identification of CMV infected cells using monoclonal antibody CCH2 (a) in a section of a parotid gland (b) in a lung section (c) in a section of a kidney. (Haematoxylin counterstaining.)

hybridisation with the biotinylated CMV specific probe stained nuclei of all cells with diagnostic nuclear inclusions. Most of these cells were also labelled in the cytoplasm. The nuclear inclusions stained homogeneously brown with differing intensity. The cytoplasmic reactivity consisted predominantly of intensely stained granules of varying size (fig 1a).

A few cells showed cytoplasmic labelling alone. Serial sections showed that this was usually due to the absence of the nuclear inclusion from the level of the section. Occasionally, morphologically normal cells displayed a nuclear labelling (fig 1b). Such cells were found particularly in tissues containing a small number of cells infected by CMV. In some organs, especially lung and liver, the presence of an endogenous or exogenous brownish pigment made the evaluation of the specific signal difficult. Background staining was generally of very low intensity.

DETECTION OF CMV BY MONOCLONAL ANTIBODY CCH2

Staining results are summarised in table 2. CCH2 labelled "owl's eye" cells and a few morphologically normal cells in sections from infected tissues (figs 2a-c). The staining was usually intense and easily

detectable. In most positive cells CCH2 stained the nuclei as well as the cytoplasm. Sometimes, however, either the nuclei or the cytoplasm was stained. The cytoplasmic labelling was usually diffuse or in some instances foamy, but never granular, as seen with in situ hybridisation. Serial sections showed that nuclear staining was usually missing because the level of sectioning did not meet the intranuclear inclusions.

COMPARISON OF IN SITU HYBRIDISATION, IMMUNOHISTOLOGICAL, AND MORPHOLOGICAL ANALYSIS FOR DETECTION OF CELLS INFECTED WITH CMV

For comparison of the sensitivity of in situ hybridisation, immunohistological, and purely morphological criteria, we counted the number of cells positive with either of these methods. Both in situ hybridisation and immunohistological analysis with CCH2 showed many more infected cells than routine histological analysis. Furthermore, immunohistochemistry produced a slightly higher number of positive cells than in situ hybridisation (table 3).

Discussion

We evaluated the efficiency of three rapid methods for showing the presence of CMV infection in routinely processed human tissues. Both in situ hybridisation and immunohistochemistry with CCH2 labelled all diagnostic nuclear inclusions and the cytoplasm of these cells. In addition, both methods stained cells lacking the diagnostic nuclear inclusions, mainly because the level of section did not meet the nuclear inclusion. In some instances, however, the signal was present in nuclei of morphologically normal cells. This agrees with the results of previous observations.^{6,7}

Quantitative evaluation of the number of infected cells with in situ hybridisation or immunohistochemistry and by the application of morphological criteria show that:

- (i) the detection of cells infected with CMV is much easier with in situ hybridisation or immunohistochemistry than by screening for nuclear inclusions;
- (ii) the number of cells scored as infected was much higher with in situ hybridisation and with immunohistochemistry than with purely morphological criteria; and
- (iii) immunohistochemistry with CCH2 detected a higher number of infected cells than in situ hybridisation.

These findings clearly indicate that both in situ hybridisation and immunohistochemistry with CCH2 are rapid and sensitive methods. The slightly higher figures seen with immunohistochemistry might be due to the cross reactivity of CCH2 with structures unrelated to CMV, but this seems to be unlikely as

Table 2 *Detection of CMV in necropsy tissues of an AIDS case by routine histological analysis, in situ hybridisation, and immunohistochemical techniques*

Tissue	Routine histology	In situ hybridisation	Immunohistochemistry (CCH2)
Heart	-	+	
Lung	++	+++	+++
Liver	(+)	++	++
Kidney	(+)	+	++
Adrenal	(+)		
Parotid	++	+++	+++
Thyroid	+	Not investigated	++
Parathyroid	-	+	+
Brain	-	-	+
Testis	(+)	+	+
Lymph node	-	-	+
Gastrointestinal	++		

Key: - negative; (+) few infected cells; + low amount of infected cells; ++ moderate amount of infected cells; +++ high amount of infected cells.

Table 3 *Detection of CMV infected cells: quantitative evaluation of routine histological analysis, in situ hybridisation, and immunohistological techniques*

Organ	Routine histology	In situ hybridisation	Immunohistochemistry
Parotid gland	142	323	341
Left lung	14	36	52
Right lung	21	48	79

The numbers indicate the total number of CMV infected cells in nine fields at a 400 × magnification.

CCH2 reacted specifically with fibroblasts infected with CMV. Furthermore, CCH2 did not produce any staining of human tissues from donors without CMV infections. The slightly higher figures of infected cells observed with CCH2 might be due to the use of the APAAP method, which, in conjunction with a new fuchsin development, is one of the most sensitive immunoenzymatic detection methods.²⁰ Alternatively, infected cells could contain larger amounts of accessible CMV proteins than CMV-DNA.

In the sections of the brain and the lymph node very few cells were CMV positive by immunohistochemistry, but not with in situ hybridisation (table 2). These immunohistologically positive cells showed typical "owl's eye" morphological detail. Therefore, it was considered most likely that the negative results of in situ hybridisation were due to the absence of CMV infected cells from the level of the section rather than the failure to detect infected cells.

In conclusion, detection of CMV proteins is of comparable sensitivity as demonstration of CMV DNA by in situ hybridisation. The detection of CMV in routine formalin fixed paraffin wax embedded tissue specimens with immunohistochemistry using CCH2 is well within the scope of routine pathologists, as this technique is identical with methods for detecting intermediate filaments, lymphoid-associated antigens, and other diagnostically important molecules.

This work was supported by the Bundesministerium für Forschung und Technologie, grant 016A8501.

The tissues were kindly provided by Prof F Niedobitek. We thank Sylvia Habedank and Heidrun Steeger for excellent technical assistance and Benjamin Young for secretarial assistance.

References

- 1 Marchevsky A, Rosen MJ, Chrystal G, Kleinerman J. Pulmonary complications of the acquired immunodeficiency syndrome: a clinicopathologic study of 70 patients. *Hum Pathol* 1985;16:659-70.
- 2 Nash G, Fligiel S. Pathologic findings in the acquired immune deficiency syndrome (AIDS): an autopsy study of seventeen homosexual males. *Am J Clin Pathol* 1984;81:6-12.
- 3 Welch K, Finkbeiner W, Alpers C, et al. Autopsy findings in the acquired immune deficiency syndrome. *JAMA* 1984;252:1152-9.
- 4 Macaset FF, Holley KE, Smith TF, Keys TF. Cytomegalovirus studies of autopsy tissue. II. Incidence of inclusion bodies and related pathologic data. *Am J Clin Pathol* 1975;63:859-65.
- 5 Smith TF, Holley KE, Keys TF, Macaset FF. Cytomegalovirus studies of autopsy tissue. I. Virus isolation. *Am J Clin Pathol* 1975;63:854-8.
- 6 Myerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK. Widespread presence of histologically occult cytomegalovirus. *Hum Pathol* 1984;15:430-9.
- 7 Löning T, Milde K, Foss H-D. In situ hybridization for the detection of cytomegalovirus (CMV) infection. *Virchows Arch (Pathol Anat)* 1986;409:777-90.
- 8 Myerson D, Hackman RC, Myers JD. Diagnosis of cytomegaloviral pneumonia by in situ hybridization. *J Infect Dis* 1984;150:272-7.
- 9 Sacks SL, Freeman HJ. Cytomegalovirus hepatitis: evidence for direct hepatic viral infection using monoclonal antibodies. *Gastroenterology* 1984;86:346-50.
- 10 Rueger R, Bornkamm GW, Fleckenstein B. Human cytomegalovirus DNA sequences with homologies to the cellular genome. *J Gen Virol* 1984;65:1351-64.
- 11 Polack A, Hartl G, Zimmer U, et al. A complete set of overlapping cosmid clones of M-ABA virus derived from nasopharyngeal carcinoma and its similarity to other Epstein-Barr virus isolates. *Gene* 1984;27:279-88.
- 12 Rigby PW, Dieckmann M, Rhodes C, Berg P. Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J Mol Biol* 1977;113:237-51.
- 13 Brigati DJ, Myerson D, Leary JL, et al. Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* 1983;126:32-50.
- 14 Niedobitek G, Finn T, Herbst H, Bornhöft G, Gerdes J, Stein H. Detection of viral DNA by in situ hybridization using bromodeoxyuridine-labeled DNA probes. *Am J Pathol* 1988;31:1-4.
- 15 Huang WM, Gibson SJ, Facer P, Gu J, Polak JM. Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. *Histochemistry* 1983;77:275-9.
- 16 Graham RC, Karnovsky MJ. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J Histochem Cytochem* 1966;14:291-302.
- 17 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577-80.
- 18 Cordell JL, Falini B, Erber WN, et al. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;32:219-29.
- 19 Stein H, Gatter K, Asbahr H, Mason DY. Use of freeze-dried paraffin-embedded sections for immunohistologic staining with monoclonal antibodies. *Lab Invest* 1985;52:676-83.
- 20 Stein H, Dallenbach F. Monoclonal antibodies: Characterization and diagnosis of malignant lymphomas and other skin neoplasms. In: Orfanos CE, Stadler R, eds. *Dermatology in five continents*. Heidelberg: Springer Verlag 1988:(in press).

Requests for reprints to: Dr G Niedobitek, Department of Pathology, Klinikum Steglitz, Hindenburgdamm 30, 1000 Berlin 45, Federal Republic of Germany.

Notices

Methods of Immunological Research and Diagnosis

(An at-the-bench laboratory training programme).

The Ernest Witebsky Center for Immunology, University at Buffalo, State University of New York.
June 4–17 1989.

A two week program consisting of daily practical laboratory exercises personally carried out by participants, accompanied by lectures, demonstrations and discussions.

Contact James F Mohn, Director, The Ernest Witebsky Center for Immunology, 234 Sherman Hall, Buffalo, New York 14214.

Inquiries should be received by 10 March 1989 to ensure consideration for acceptance.

Second International Conference on Gastrointestinal Cancer

August 27–September 1, 1989
Jerusalem, Israel

For further information please contact:
GIA Secretariat, PO Box 50006, 61500
Tel Aviv, Israel.

The symposium will be preceded by:
Clinical applications of a long-acting
somatostatin analogue on August 27 at
3.30 pm, The Jerusalem Hilton.

Fourth South-East-European Symposium of Paediatric Surgery

Graz, Austria, September 8–9, 1989
on normal and disturbed oesophageal
function in childhood
(Simultaneous translation)

Inquiries to: Professor Dr H Sauer,
Department of Paediatric Surgery, the
University of Graz, Heinrichstraße 31,
A-8010 Graz, Austria.

Corrections

The letter: False positive results in the Toxoreagent test for *Toxoplasma gondii* in immunocompetent patients (*J Clin Pathol* 1988;41:1135–6) should have indicated the first author to have been JK Wood. We apologise for this oversight.

Similarly, an error was made in the legend to Fig. 1a in the laboratory technique: Detection of cytomegalovirus by in situ hybridisation and immunohistochemistry using new monoclonal antibody CLH2 (*J Clin Pathol* 1988;41:1005–9). The legend should have read: Detection of CMV infected cells by in situ hybridisation in a lung section.

FUTURE IMLS SYMPOSIA

Date/Location	Subject/Title	Organiser
4 March 1989 Southampton	<i>Conception to cradle—fetal and neonatal diagnosis and monitoring</i>	Dr D McLellan, School of Pharmacy, Portsmouth Polytechnic, Park Building, King Henry I Street, Portsmouth PO1 2DZ.
18 March 1989 Cambridge	<i>Special coagulation review—educational seminar</i>	Mr D M Reardon, Haematology Department, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ.
March 1989 Nottingham	Microbiology/virology	Mr A Pawley, Virology Department, PHL, Queens Medical Centre, University Hospital, Nottingham.
8 April 1989 Sheffield	<i>Haematology—in a lighter vein</i>	Mr N R Porter, Haematology, Floor H, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF.
26 April 1989 Liverpool	<i>Laboratory developments in paediatric biochemistry</i>	Mr D Kilshaw, Pathology Department, Arrowe Park Hospital, Upton, Wirral, Merseyside L49 5PE
2–9 September 1989 Coventry	<i>IMLS Triennial Conference</i>	Mr C G Smith, Pathology Department, Dudley Road Hospital, Summerfield, Birmingham B18 7QH
17 March 1990 Cambridge	<i>Special red cell review—educational seminar</i>	Mr D M Reardon, Haematology Department, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ.
30–31 March 1990 York	Microbiology/histology/cytology/immunology	Mr B Jones, Pathology Department, Royal Halifax Infirmary, Free School Lane, Halifax HX1 2YP.
April 1990 Durham	<i>Blood Group Serology '90</i>	Miss D L Trattles, Blood Transfusion Laboratory, Department of Clinical Pathology, General Hospital, Ayresome Green Lane, Middlesbrough, Cleveland TS5 5AZ.
13–16 September 1990 Sheffield	<i>Microbe '90</i>	Mr C J P Brazier, Public Health Laboratory, Northern General Hospital, Herries Road, Sheffield S3 7AU.
September 1990 Birmingham	Cellular pathology	Miss L Grosvenor, Histopathology Department, General Hospital, Steelhouse Lane, Birmingham B4 6NH.
April 1991 Durham	Microbiology	Mr G P Hedley, Regional Transfusion Centre, Holland Drive, Barrack Road, Newcastle upon Tyne NE2 4NG.
April 1991 Sheffield	<i>Haematology—in a different vein</i>	Mr N R Porter, Haematology, Floor H, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF.
September 1991 Birmingham	Microbiology	Mr T Johnson, Microbiology, Department, Selly Oak Hospital, Raddlebarn Road, Selly Oak, Birmingham B29 6JD.
September 1992	<i>IMLS Triennial Conference</i>	
September 1993 Birmingham	Microbiology	Mr T Johnson, Microbiology Department, Selly Oak Hospital, Raddlebarn Road, Selly Oak, Birmingham B29 6JD.
April 1994 Durham	Microbiology	Mr G P Hedley, Regional Transfusion Centre, Holland Drive, Barrack Road, Newcastle upon Tyne NE2 4NG.