Acta virol. **37:** 1-10, 1993

DETECTION OF IgM ANTIBODIES AGAINST COXSACKIE B VIRUSES BY A WESTERN BLOT TECHNIQUE

M. DEL ROSARIO ZUÑIGA, J. REICHARDT, W. BRAUN, B. WEBER, H. W. DOERR

Klinikum der Johann Wolfgang Goethe-Universität, Paul-Ehrlich Str. 40, 6000 Frankfurt a. M. 70, Germany

Received January 30, 1992; revised April 13, 1992

Summary. - We report the application of a modified Western blot (WB) micromethod basically relying on a diffusion-blotting technique (Modi-blot) combined with an immunological detection system using monoclonal antibodies and a biotinavidin amplification step for the detection of IgM antibodies against coxsackieviruses B1 (CBV1), CBV2 and CBV4. Fifty-one adult patients with clinical signs of coxsackievirus B infection (e. g. myocarditis and meningitis) were investigated. The test revealed a total of 31 (60 %) IgM positives. The majority of IgM antibodies were group reactive (22/31). Type-specific antibodies could be recognized in 9 cases (3 with CBV1, 5 with CBV2 and 1 with CBV4). The highest rate of antibody prevalence was found in sera from patients with acute meningitis (12/14). Controls [healthy adults (n=13) and individuals with other infections (n=13)] were all negative for specific IgM against coxsackievirus B1, B2 and B4. Further WB tests of 8 IgM positive specimens with coxsackievirus B4 revealed specific IgA responses in all cases, reinforcing the evidence for a recent infection. In addition, the patterns of IgG antibody subclasses, also investigated in this group, showed a clear predominance of specific IgG1 and IgG3 antibodies.

Key words: coxsackieviruses B; Western blot micromethod; immunoglobulin (sub)classes; acute infection

Introduction

Conventional procedures for laboratory diagnosis of CBV infections, e. g. virus isolation supported by the detection of a significant titer rise of virus-specific IgG antibodies against the isolated agent or a prototype strain, have proven laborious and time-consuming. Moreover, in some instances, no significant humoral immune response can be observed despite virus isolation (Pozzetto *et al.*, 1989), only one serum sample is available or the specimens are taken too late (Enders *et al.*, 1990). Considering these difficulties, alternative strategies

for a rapid diagnosis based on IgM antibody detection have been attempted (Pattison, 1983). Although almost all these techniques were based on the selective capture of μ-chain of human IgM (Bell *et al.*, 1986; Enders *et al.*, 1990; Frisk *et al.*, 1989; King *et al.* 1983; Pugh, 1984), IgG competitive inhibition or interference with rheumatoid factors could not completely be eliminated (Çhampsaur *et al.*, 1988). Moreover, those results vary in both specificity and sensitivity, and a widespread use in a routine context failed due to high costs (Bell *et al.* 1986) or other technical limitations (Enders *et al.*, 1990). This communication reports the use of a Western blot microtechnique as a complementary diagnostic tool for the detection of IgM antibodies against coxsackieviruses B in comparison with a μ-antibody capture ELISA. Assuming that IgM presumably persists for extended periods of time (Lennette *et al.*, 1961) and CBV-specific IgM could also be detected in healthy people, a verification of diagnosis with additional serological assays was carried out by the assesment of IgA (Angeretti *et al.*, 1987; Sarov *et al.*, 1984) and IgG antibody subclasses, which have already been introduced into different fields of serology (Doerr *et al.*, 1987).

Materials and Methods

Sera. The study included single sera from patients (n=51) with clinical manifestations of suspected CBV etiology [minor febrile illnesses (n=36), meningitis (n=14) and myocarditis (n=1)]. All serum samples were taken two to fifteen days after onset of disease. The sera obtained from the diagnostics laboratory of the Department of Medical Virology, University Clinics of Frankfurt (n=29), were previously tested for specific IgM against CBV1, CBV2 and CBV4 with a reverse ELISA according to King *et al.* (1983). Further samples (n=22) - also tested for IgM antibodies with a μ-antibody capture ELISA - were obtained from the Max-von-Pettenkofer Institute, University of Munich. In addition, serum specimens from 13 healthy adults of the laboratory staff were investigated.

The specificity of the Western blot was confirmed by assaying sera with known titers (1:10) of neutralizing IgM antibodies specific for CBV2 (n=1), CBV4 (n=1) and CAV9 (n=3) as well as serum samples from 10 patients with other viral infections. The latter were seropositive for IgM antibodies against different viral antigens as previously determined in our routine laboratory diagnostics by commercially available ELISA tests. These viruses were cytomegalovirus (n=3), rubella (n=4) and Epstein-Barr viruses (n=3).

Reference mouse antisera (mouse antiserum to CBV1, CB2 and CBV4) from a commercial source (MA Bioproducts, Maryland) with known complement-fixing antibody titers (32) were used to identify viral antigens.

Virus antigen and control antigen. For our study, we used clinical isolates of CBV1, CBV2 and CBV4 propagated in HeLa cells with infectious titers of approx. 5×10^7 $TCID_{50}$/ml. Infected cell cultures showing an extensive cytopathic effect were processed by three cycles of freeze-thawing and subsequently centrifuged at 4000 x g for 5 min to remove cellular debris. Control antigen was made using uninfected HeLa cells that were processed identically.

[^{35}S]-methionine labelling of CBV4 capsid proteins. Radioactive labelling of the viral proteins was basically performed as described by Chatterjee and Tuchowski (1981). Briefly, confluent monolayers of HeLa cells grown in Eagle's minimal essential medium (MEM) in a 175 cm^2 flask were infected with virus (5 $TCID_{50}$ per cell). After one hr adsorption, the medium was replaced by methionine-free MEM supplemented with 2 % foetal calf serum. [^{35}S]-methionine was then added 3 hr p. i. at a final concentration of 20 μCi/ml. Labelled virus was harvested 24 hr p. i. and purified as described below. Fractions exhibiting radioactivity were pooled and electrophoresed.

Virus purification. Viruses were purified essentially as described by Chatterjee and Tuchowski (1981). Briefly, after ultracentrifugation, an isopycnic centrifugation of the pelleted virus in a linear preformed CsCl gradient (1.2 - 1.4 g/ml) at 150 000 x g for 3 hr was carried out. Fractions containing non-labelled virus antigen were then identified by a sandwich ELISA as described by Katze and Crowell (1980). The protein content was then adjusted to 80 μg/ml. Aliquots were stored at -20 °C until use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the PhastSystem Unit (Pharmacia) with 8–25 % (w/v) polyacrylamide gradient gels (PhastGel gradient 8–25) and PhastGel sample applicators 12/0.3 (12 lanes, sample volume 0.3 μl/lane). Aliquots (10 μl) of purified virus or control antigen were suspended in an equal volume of a twofold concentrated sample buffer (10 mmol/l Tris-maleate, 2.5 % SDS, 2.5 % dithioerythritol, 1 mmol/l EDTA pH 6.8) and boiled at 100 °C for 5 min. Electrophoresis was carried out for 80 Volt. hr. Low molecular weight marker proteins phosphorylase b (94 K), bovine serum albumin (BSA, 67 K), ovalbumin (43 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (20.1 K) and Á-lactalbumin (14.4 K) (Pharmacia) were used.

Western blot assay. Diffusion blotting of the SDS-PAGE-resolved viral proteins was performed basically as described by Braun and Abraham (1989). Appropriate antigen concentration was previously determined by serially diluting the sample with sample buffer and by monitoring the results with positive control sera. After blotting, the protein-loaded membrane was dried and cut into strips. The strips were then incubated with a "blocking solution" (0.02 mol/l Tris, 0.2 mol/l NaCl, 10 % goat serum and 1 % BSA) at room temperature (RT) for 1 hr and then washed three times with Tris/Tween buffer (0.02 mol/l Tris, 0.2 mol/l NaCl, 0.2 % BSA and 0.2 % Tween-20 pH 7.4). Incubation with the diluted serum sample (1:200) took place at RT for 1 hr. For IgM detection, serum samples were pretreated with rheumatoid factor (RF) adsorbent (Behring) according to the manufacturer's instructions. Subsequent detection of CBV-specific immunoglobulin (sub) classes comprised incubation of the strips first with the appropriate monoclonal mouse anti-human immunoglobulin antibody (anti-IgG 1–4 from Nordic; anti-IgM and anti-IgA from Immunotech), secondly with a biotin-labelled monoclonal rat anti-mouse IgG antibody (Immunotech) and thirdly with streptavidin-horseradish peroxidase (Calbiochem). The optimal working dilutions were previously determined by checkboard titrations and all reagents were diluted in blocking solution. Each reaction was allowed to proceed at RT for 1 hr. After each step, extensive washing (see above) was carried out to remove unbound reactants.

The enzyme reaction, simultaneously performed for all blots with aminoethyl-carbazole/H_2O_2 as the substrate, was terminated after 15 min at 37 °C by washing the membranes with bidistilled water. The strips were air dried and photographed.

Results

Viral proteins

Preliminary experiments using labelled virus were performed to monitor viral proteins. In screening for CBV-specific antigen, the purification of [^{35}S]-methionine labelled CBV4 in caesium chloride gradient revealed two groups of fractions No. 12–16 (pool I) and fractions No. 19–21 (pool II), showing peaks of radioactivity which coincided with those exhibiting the highest absorbancy values in the sandwich ELISA.

The electrophoresis profile of the pool I (Fig. 1) revealed three major bands, corresponding in size to the three viral proteins (VP1–3), whereas the pool II exhibited only VP1 and some non-viral polypeptides. VP4 was not detected by this method probably due to its small size. The estimated molecular weights were 39.9 K, 37.8 and 28.8 K for VP1, VP2 and VP3, respectively.

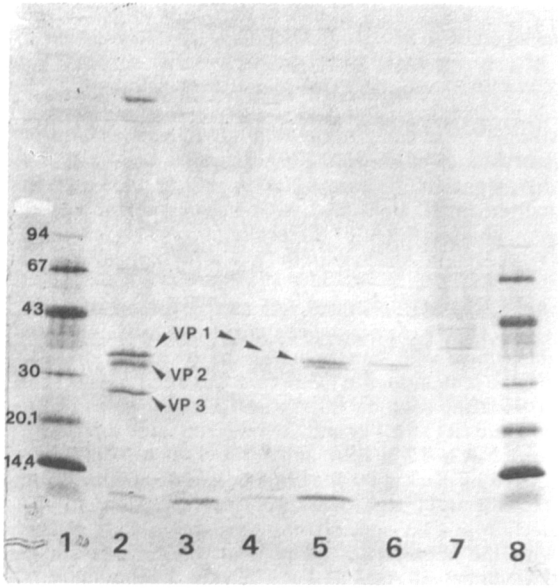

Fig. 1
SDS-PAGE polypeptide distribution of CBV4 capsid proteins after purification in caesium chloride gradient
Two pools of antigen-containing fractions (I, II) were investigated. Lane 1 and 8: low molecular weight markers proteins (in thousands); lane 2–4: pool I serially diluted twofold; lane 5–6: pool II serially diluted twofold; lane 7: control antigen, lysate of mock-infected HeLa cells.

By assaying proteins from the pool I of non-labelled virions with CBV4-specific mouse antiserum in the WB, antiviral antibodies reacted exclusively with VP1 (Fig. 2). Other viral polypeptides as VP2 and VP3 failed to be recognized even though these proteins could be observed in the silverstained gel. Similarities in band patterns, molecular weight values and reactivity in the WB were found when assaying CBV1- and CBV2-blots with the respective mouse antisera (data not shown).

Specificity of the WB assay

The specificity of the test was controlled by assaying sera from 2 individuals with known titers (1:10) of neutralizing IgM antibodies specific to CBV2 and CBV4 and CAV9 (n=3) as well as serum samples from 10 individuals with other viral infections, which were positive for IgM antibodies against different viral antigens (see *Materials and Methods*). An IgM-RF positive serum was also included in the control group to check the activity of the RF absorbent. As Fig. 2. shows, both samples with neutralizing IgM responses against CBV were positive in the WB, revealing only one band corresponding to the viral polypeptide VP1, as previously seen with mouse antiserum. In contrary to this, neither Coxsackievirus A9 IgM positives nor patients with other viral infections reacted with any of the CBV polypeptides in the WB.The IgM-RF positive serum pretreated with RF adsorbent did not react in the WB.

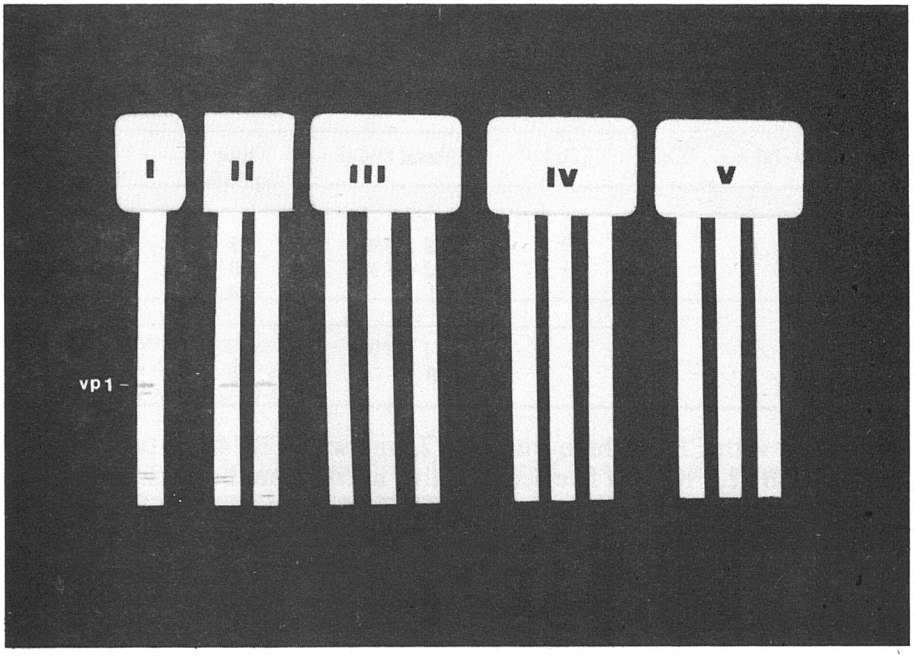

Fig. 2

WB assays with selected sera tested for IgM antibodies against capsid proteins of CBV4
I: mouse antiserum to CBV4; II: human sera with IgM neutralizing antibodies against CBV4 and CBV2, respectively; III: IgM positive sera for CAV9; IV: human sera positive for cytomegalovirus-specific IgM; V: human sera positive for IgM against Epstein-Barr virus.

Clinical specimens

Serum samples from 51 patients with clinical manifestations of suspected CBV etiology and 13 healthy adults (controls) were investigated for viral-specific IgM response by WB assay. As Table 1 shows, it detected 31 IgM positives in this group. Type-specific IgM response, i. e. IgM antibodies reacting with only one CBV-serotype could be observed in a lesser extent (9/31) against at least one serotype, the distribution being as follows: three with CBV1, five with CBV2 and one with CBV4. In general, the prevalence of IgM antibodies was higher in the patients with meningitis (12/14) than in the patients with minor illnesses (18/36) or in healthy adults (0/13).

Comparison of μ-antibody capture ELISA and WB results

Concording results between both tests were found in 37 (22 positive and 15 negative) cases. Similarities and discrepancies of both tests are illustrated in Table 2. It shows contradictory results [ELISA negative, but WB positive (n=9) and vice versa (n=5)] in 14 cases. From all nine sera positive in the WB only,

Table 1. IgM antibody prevalence in clinical groups determined

Clinical group	(n)	No. of seropositives		
		total (%)	type specific	group specific
minor illnesses	(36)	18 (50 %)	9	9
meningitis	(14)	12 (85 %)	0	12
myocarditis	(1)	1	0	1
	51	31 (60 %)	9	22
healthy adults	(13)	0	0	0

three reacted with CBV1, three with CBV2, one with CBV4 and two with both CBV1 and CBV2. From all five sera positive in the μ-antibody capture ELISA only, two reacted with CBV4, one with CBV1/CBV2 and one with CBV1/CBV4. False positive results in the WB could be excluded by incubation with RF adsorbent.

Other serological parameters

Considering that IgM antibodies may persist after the acute phase of infection (Lenette *et al.*, 1961; Pozzetto *et al.*, 1989), additonal WB assays using CBV4 as antigen for the determination of other seroparameters (e. g. IgG antibody subclasses and IgA) were performed. Three different groups of sera were investigated: (1) eight sera exhibiting IgM and IgG antibodies specific for CBV4 (Fig. 2), (2) four sera positive for CBV4-IgG only and (3) four CBV-seronegative controls. As the Table 3 shows, all IgM positive sera exhibited specific IgA antibodies and IgG antibody subclass 1 and 3, whereas only three specimens

Table 2. Comparison of the results obtained by the μ-antibody capture ELISA and the WB for IgM antibodies against CBV1, CBV2 and CBV4

Clinical group	(n)	No. of seropositives			
		total	both tests	WB only	μ-ELISA only
minor illnesses	(36)	23	9	9	5
meningitis	(14)	12	12	0	0
myocarditis	(1)	1	1	0	0
total	51	36	21	9	5

Table 3. Distribution of CBV-specific IgG subclass IgA antibodies in selected human sera

Clinical group	(n)	No. of seropositives					
		IgA	IgG1	IgG2	IgG3	IgG4	IgM
I	(8)	8	8	3	8	3	0
II	(4)	0	4	0	0	0	0
III	(4)	0	0	0	0	0	0

Group I: CBV-IgG and -IgM positives
Group II: CBV-IgG positives
Group III: CBV seronegatives

revealed IgG2 and IgG4 antibodies. In contrast to the first group, none of the sera from the second group contained IgA antibodies and the IgG subclass response was confined to IgG1. None of the sera from the third group were positive in any test.

Discussion

In the present communication, a Western blot microtechnique has been used to detect viral-specific IgM as a complementary test for a serologic diagnosis of active infections with CBV.

Considering that (1) our data for the molecular weight of VP1-3 are in good agreement with previously reported values (Chatterjee and Tuchowski, 1981), (2) the electrophoretic profile observed by us corresponds to the well documented typical pattern for enteroviruses (Dörries and ter Meulen, 1983; Katze and Crowell, 1980; Mertens *et al.*, 1983; Pozzetto *et al.*, 1990) and (3) these proteins were recognized by viral-specific mouse antibodies in the WB, we conclude that these proteins represent the three major viral capsid polypeptides of CBV.

As it was shown in experiments with human sera containing neutralizing IgM antibodies specific for CBV2 and CBV4, these immunoglobulins seem to be directed essentially to VP1, since IgM against VP2 and VP3 were not found. These results, similar to those reported by Pozzetto *et al.* (1990) may reflect a relatively low avidity of these antibodies or an alteration of the antigenic properties during electrophoresis or blotting. Nevertheless, the lack of VP2- and VP3-specific IgM antibodies does not seem to be crucial for diagnostic purposes, since the majority of group and type specific antigenic determinants is located on VP1 (Dörries and ter Meulen, 1980; Katze and Crowell, 1980; Mertens *et al.*, 1983). The analysis of human sera from patients with clinical manifestations of suspected CBV etiology revealed a relatively high prevalence of IgM antibodies

with a clear predominance of "group-specific" response (i. e. IgM antibodies reacting with more than one CBV serotype), especially in patients with aseptic meningitis. This high incidence of heterotypic IgM antibodies, reported also by other investigators (Bell *et al.*, 1986; King *et al.*, 1983; Pozzetto *et al.*, 1989), could partially be due to the induction by group-specific epitopes released after boiling the antigen for the SDS-PAGE. Similar outcomes could also be established across all enterovirus genus for IgG and IgM antibodies with other methods (Enders *et al.*, 1990; Pattison, 1983; Pozzetto *et al.*, 1990). On the other hand, the high frequency of group reactive IgM antibodies could also be considered as the result of (1) simultaneous infections with more than one serotype or (2) the expression of an anamnestic response to a different serotype involved in a previous infection, as it may occur with neutralizing antibodies (Lennette *et al.*, 1961). Moreover, a correlation with the duration of illness has also been postulated, so that homotypic responses appear at the early phase of infection and are progressively replaced by heterotypic antibodies (Enders *et al.*, 1990). To date, however, a definite explanation for this phenomenon cannot be given, because some aspects in the development of the IgM response such as the type and the magnitude as well as a presumed correlation with age and illness, remain to be investigated.

Although the results obtained by WB and ELISA were compatible in most of the samples, several cases exhibiting discordant results (WB positive and ELISA negative and vice versa) could be observed. These features could be due to a low concentration of IgM antibodies resulting in critical absorbancy values (e. g. equal or under the cut off) or nonspecific reactions in the μ-antibody capture ELISA, described by Champsaur *et al.* (1988).

Although the mere presence of CBV-IgM antibodies indicates an active infection, other serological parameters (IgG antibody subclasses and IgA) have been determined to support this assumption and to estimate the use of these parameters as markers of acute infections. According to our data, IgA may be considered as a potential indicator of recent infections. Even though IgA response to non-polio enteroviruses has been poorly investigated, seroepidemiological studies with other viruses (Angeretti *et al.*, 1987; Sarov *et al.*, 1984) support this thesis. Moreover, similar features have been described by Pozzetto *et al.* (1990), who found a significant difference in the distribution of IgA-specific antibodies to VP1 by testing patients with confirmed echovirus infections and control healthy adults.

Our data on the restriction pattern of IgG antibodies indicating a predominance of subclass 1 and 3, are partly similar to the results reported by Torfasson *et al.* (1987), who found an equal prevalence of IgG1 and IgG3 and an absence of IgG2 and IgG4. The fact that IgG3 was found in all 8 IgA and IgM positives and in view of its biological properties i.e. short lifetime and neutralizing activity (Schur, 1987), it is likely that this antibody subclass may also be elicited in the early phase of patients are currently performed to support this thesis.

Even though our WB assay shows some limiting factors, namely the use of

monovalent antigens or antigen purification by caesium chloride gradient, we think that this microtechnique may be considered as an alternative tool for routine diagnosis. Our test displays a sensitivity comparable to that showed by the μ-antibody capture ELISA and enables at the same time the assessment of other serological parameters, e. g. IgA as well as IgG antibody class and subclasses. Moreover, the use of the Modi-blot system – employing an integrated electrophoresis system for the preparative separation of viral proteins and their subsequent transfer by diffusion – allows a simple and rapid method for the mass production of virtually identical blot strips, which in turn enables a large scale testing with reproducible results (Braun and Abraham, 1989).

Acknowledgements. We wish to thank Prof. Dr. M. Roggendorf, Munich, for providing serum samples and G. Bauer and M. Besel for technical assistance.

References

Angeretti, A., Magi, M., Merlino, C., Ferrara, B., and Ponzi, A. (1987): Specific serum IgA in rotavirus gastroenteritis. *J. med. Virol.* **23**, 345.

Banatvala, J., Bryant, J., Schernthaner, G., Borkenstein, M., Schober, E., Brown, D., De Silva, L., Menser, M., and Silink, M. (1985): Coxsackie B, mumps, rubella and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria and Australia. *Lancet* **1**, 1409.

Bell, E., Mc Cartney, R., Basquill, D., and Chaudhuri, A. (1986): μ-antibody capture ELISA for the rapid diagnosis of enterovirus infections in patients with aseptic meningitis. *J. med. Virol.* **19**, 213.

Braun, W., and Abraham, R. (1989): Modified diffusion blotting for rapid and eficient protein transfer with PhastSystem. *Electrophoresis* **10**, 249.

Champsaur, H., Fattal-German, M., and Arranhado, R. (1988): Sensitivity and specificity of viral immunoglobulin M determination by indirect enzyme-linked immunosorbent assay. *J. clin. Microbiol.* **26**, 328.

Chatterjee, N., and Tuchowski, C. (1981): Comparison of capsid polypeptides of group B coxsackieviruses and polypeptide synthesis in infected cells. *Arch. Virol.* **70**, 255.

Doerr, H. W., Rentschler, M., and Scheifler, G. (1987): Serologic detection of active infections with human herpes viruses (CMV, EBV, HSV, VZV): Diagnostic potential of IgA class and IgG subclass-specific antibodies. *Infect.* **15**, 93.

Dörries, R., and ter Meulen, V. (1980): Detection of enterovirus specific IgG and IgM antibodies in humans by an indirect solid phase radioimmunoassay. *Med. Microbiol. Immunol.* **168**, 159.

Dörries, R., and ter Meulen, V. (1983): Specificity of IgM antibodies in acute human Coxsackievirus B infections, analysed by indirect solid phase enzyme immunoassay and immunoblot technique. *J. gen. Virol.* **64**, 159.

Enders, G., Bäder, U., Nickerl, U., and Gärtner, L. (1990): Diagnostik bei Enterovirus-Infektionen. *Münch. Med. Wschr.* **132**, 360.

Frisk, G., Nilson, E., Ehrnst, A., and Diderholm, H. (1989): Enterovirus IgM detection: specificity of μ-antibody-capture radioimmunoassays using virions and procapsids of Coxsackie B virus. *J. virol. Meth.* **24**, 191.

Katze, M., and Crowell, R. (1980): Immunological studies of the group B Coxsackieviruses by the sandwich enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. *J. gen. Virol.* **50**, 357.

King, M., Shaikh, A., Bidwell, D., Voller, A., and Banatvala, J. (1983): Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. *Lancet* **1**, 1397.

Lennette, E., Shinomoto, T., Schmidt, N., and Magoffin, R. (1961): Observations on the neutralizing antibody response to group B coxsackieviruses in patients with central nervous system disease. *J.*

Immunol. **86**, 257.
Mertens, T., Pika, U., and Eggers, H. (1983): Cross antigenicity among enterovirus as revealed by immunoblot technique. *Virology* **129**, 431.
Pattison, J. R. (1983): Tests for Coxsackie B virus-specific IgM. *J. Hyg.* **90**, 327.
Pozzetto, B., Gaudin, O., Lucht, F., Hafid, J., and Ros, A. (1990): Detection of immunoglobulin G, M, and A antibodies to enterovirus structural protein by immunoblot technique in echovirus type 4-infected patients. *J. virol. Meth.* **29**, 143.
Pozzetto, B., Gaudin, O., Aouni, M., and Rois, A. (1989): Comparative evaluation of immunoglobulin M neutralizing antibody response in acute-phase sera and virus isolation for the routine diagnosis of enterovirus infections. *J. clin. Microbiol.* **27**, 705.
Pugh, S. (1984): Heterotypic reactions in a radioimmunoassay for coxsackie B virus specific IgM. *J. clin. Pathol.* **37**, 433.
Sarov, I., Insler, V., Sarov, B., Ceverini, R., Rumpianesi, F., Donati, M., Kleinman, D., Piura, B., Lieberman, J., Kimmel, N., Friedmann, M., and La Placa, M. (1984): Specific serum IgA antibodies in the diagnosis of acute viral and chlamydial infection, p. 157. In S. Sanna, and R. Morace (Eds): *New Horizonts in Microbiology*, Elsevier, Amsterdam.
Schur, P. (1987): IgG subclasses - a review. *Annals of Allergy* **58**, 89.
Torfason, E., Reimer, C., and Keyserling, H. (1987): Subclass restriction of human enterovirus antibodies. *J. clin. Microbiol.* **25**, 1376.