Specificity of IgM Antibodies in Acute Human Coxsackievirus B Infections, Analysed by Indirect Solid Phase Enzyme Immunoassay and Immunoblot Technique

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SUMMARY

The specificity of the IgM response in acute human coxsackievirus B infections was examined by indirect solid phase enzyme immunoassay and immunoblot techniques. IgM antibodies detected by ELISA were either strictly type-specific, type-predominant or group-reactive to coxsackieviruses B-1 to B-5. Homotypic and type-dominant responses were clearly correlated with the serotype isolated from the patient. Analysis of ELISA antigens by SDS-gel electrophoresis, protein transfer and subsequent immunodetection of individual virus polypeptides, revealed VP1 as the major antigen; it was detected by homotypic as well as heterotypic serum specimens.

INTRODUCTION

Coxsackieviruses are important human pathogens, causing a broad spectrum of diseases from minor common colds to fatal myocarditis and neurological disorders (Grist et al., 1978). Viral diagnosis of these disorders depends mainly on virus isolation and characterization of the host immune response. The latter is measured by different standard serological assays which are cumbersome, often without diagnostic value and usually requires paired sera from the patients. Therefore the application of more sophisticated assays could improve diagnostic efforts, especially where virus-specific IgG and IgM reactivity is determined.

It has been shown that enterovirus-specific IgG as well as IgM antibodies reveal type- or group-specificity, depending on the assay employed. In the neutralizing and haemagglutination inhibition reaction, homotypic and heterotypic antibodies are found in both immunoglobulin classes. However, using the technique of immunodiffusion it could be demonstrated that antibodies from early serum specimens precipitating complete infectious coxsackievirus B are of IgM nature, while group-specific precipitates are composed of non-infectious particles and IgG antibodies (Schmidt et al., 1968). These observations indicate that demonstration of type-specific antibodies is obviously correlated closely to the assay used, the antibody class looked for, and the time the blood samples are taken after exposure to the virus.

With the development of the radioimmunoassay and the enzyme-linked immunoassay, test systems are available which permit the measurement of specific IgG and IgM antibodies in viral infections (Voller et al., 1976; Bidwell et al., 1977; Ziegler, 1979). So far, however, these assay systems have not been applied to evaluate the type-specificity of the IgM reaction in naturally occurring enterovirus diseases. Only group-specific IgM responses have been determined in coxsackievirus B infections associated with cardiac and other diseases (El-Hagrassy et al., 1980).

In this study specimens from human coxsackievirus B infections were examined for the presence of type- and group-specific IgM antibodies by microimmunoassay and immunoblot analyses. In addition, experiments were carried out to identify those virus polypeptides which carry either type- or group-specific antigenic determinants. It could be shown that virus polypeptide VP1 contains type- and group-specific antigenic sites and that in the course of coxsackie B virus infections homotypic and heterotypic IgM responses occur. These findings demonstrate the difficulties associated with the application of microimmunoassay in the identification of the causative agent in human enterovirus infection.
**Methods**

**Serum specimens.** Sera were collected from patients with acute coxsackievirus B infection (meningo-encephalitides), as confirmed by a significant antibody titre change in neutralization assay (NA) and the isolation of virus. Only rheumatoid factor-negative sera were subjected to virus-specific IgM determination assays.

**Virus antigens and control antigens.** Coxsackievirus B strains 1 to 5 (all clinical isolates) were propagated in HeLa cells, as described previously (Dörries & ter Meulen, 1980). For preparation of antigen or control antigen, cell-free supernatant of infected or mock-infected tissue culture fluid was sedimented for 4 h through a 20% sucrose cushion, 25,000 rev/min in a SW28 Beckman rotator at 4 °C. Virus or control antigen pellets were resuspended in 0.5 ml phosphate-buffered saline (PBS) without calcium and magnesium, adjusted to 500 μg protein/ml and kept frozen at −20 °C until used in ELISA.

**[^1^] Methionine labelling of coxsackievirus B-2-specific proteins.** Confluent monolayers of HeLa cells in a 25 cm² tissue culture flask were either infected with coxsackievirus B-2 (10⁶ TCID₅₀) or mock-infected with PBS. The inoculum was removed 1 h after infection and 2 ml of minimal essential medium (MEM) with 2% foetal calf serum (FCS) was added to the cultures. The medium was replaced 5 h post-infection by 2 ml MEM without methionine or FCS for 1 h, followed for 2 h by 2 ml of fresh MEM, containing 200 μCi/ml[^1^]methionine and 2% dialysed FCS. Subsequently, the radioactive medium was replaced by 2 ml of MEM (2% FCS). The cultures were frozen at −20 °C 10 h after infection. Next day, cultures were thawed and frozen twice, and cell debris was removed by a 12000 g centrifugation for 5 min in an Eppendorf centrifuge. Aliquots of the supernatant were used for protein-blot studies. To obtain purified virus particles, portions of the supernatant were further processed by pelleting the virus (2 h in a Ti50.1 Beckman rotor, 30000 rev/min, 4 °C) and subsequently by sucrose gradient purification (2 h in a vertical 65Ti Beckman rotor, 14000 rev/min, 4 °C, 11 to 45% (w/v) sucrose in PBS).

**Antibody to human immunoglobulin.** Heavy-chain-specific anti-human-IgM antibody was affinity-purified from the corresponding goat antiserum (Meloy, Aschaffenburg, F.R.G.) as described previously (Dörries & ter Meulen, 1980).

**Alkaline phosphatase-conjugated anti-human-immunoglobulin (AP-aIgM).** Affinity-purified anti-IgM antibody was labelled with alkaline phosphatase (Sigma) according to the method of Engvall & Perlman (1972). Specific volume activities of freshly labelled antibodies were about 320 enzyme units/ml at 25 °C.

**[^1^] I-conjugated anti-human-IgM antibody (I-algM).** Affinity-purified anti-IgM antibody was labelled with[^1^]I (Amersham Buchler) as described previously (Dörries & ter Meulen, 1980). Specific activities of labelled antibodies were about 20 μCi/μg antibody.

**Solid phase enzyme immunoassay (ELISA).** To identify enterovirus-specific antibodies in human serum specimens, a modified microplate assay (Voller et al., 1976) was used. For reproducible sensitivity of the assay, antigen- and enzyme-labelled indicator immunoglobulin were calibrated by block titration experiments. A concentration of 25 to 50 μg of protein/ml of virus antigen and a 1 : 100 to 1 : 400 dilution of enzyme-labelled anti-IgM antibody was determined to be a reasonable balance between sensitivity of the assay and economical use of reagents. Briefly, 0-1 ml of antigen or control antigen diluted in 0-05 m-sodium carbonate buffer, pH 9-6 were adsorbed to flat bottom microtitre plates ("Immunolon", Dynatech, Nütingen, F.R.G.) overnight at 4 °C. Unbound proteins were removed next day by washing the wells three times with deionized water containing 0-1% Tween 20. Subsequently 0-1 ml diluted serum specimens in dilution buffer (0-05 m-Tris–HCl, 5% new born calf serum, 0-1% Tween 20, 0-002% phenol red, 0-02% sodium azide) were incubated in antigen- and in control antigen-coated wells for 1 h at 37 °C. After washing the wells again, 0-1 ml of AP-aIgM in dilution buffer was added to the wells for 1 h at 37 °C. Unbound enzyme activity was removed by washing, and 0-15 ml of p-nitrophenyl phosphate (1 mg/ml) in substrate buffer (10% diethanolamine pH 9-8, 1 mM-MgCl₂) was added as a substrate for antibody-coupled alkaline phosphatase. The enzyme reaction was allowed to proceed for 1 h at 25 °C and stopped by the addition of 0-03 ml of 5 m-NaOH. Released p-nitrophenol was measured at 405 nm in an eight-channel vertical spectrophotometer (Titertek, Flow Laboratories), linked to a computer system (MiniMinc, Digital) for data storage and processing. A net absorbance (A₄₅₀) was calculated from the equation net A₄₅₀ = mean A₄₅₀ antigen well - mean A₄₅₀ control antigen well and plotted versus the dilution of serum.

**Gel electrophoresis and protein-blot technique.** For separation of coxsackievirus-specific proteins by vertical polyacrylamide slab gel electrophoresis, the discontinuous system of Laemmli (1970) was used. ELISA antigens and control antigens (1-0 mg/ml protein), were mixed in a 1 : 2 ratio with sample buffer (0-02 m-Tris–HCl pH 6-8, 20% glycerol, 2% SDS, 2% 2-mercaptoethanol (ME), 0-05% bromophenol blue) and boiled for 5 min in a water bath. Disrupted antigens (0:05 ml/track) were separated on a vertical 13% polyacrylamide slab gel (total concentration = 13% crosslinker = 2-7%, 1-5 mm thick, 40 V, 16 h).

Transfer of separated proteins to a nitrocellulose filter and subsequent immunodetection of virus-specific antigens was as described by Burnette (1981). Briefly, polyacrylamide gel-separated proteins were transferred to a nitrocellulose sheet (type BA 83, Schleicher & Schüll, Dassel, F.R.G.) by electroblotting overnight at room temperature and a voltage gradient of 6 V/cm. Protein-loaded filters were incubated with 15 ml of a 1 : 25 to 1 : 50 dilution of an acute serum (1 h, room temperature). IgM, bound to individual coxsackievirus polypeptides was
monitored by treatment of the filter with $^{125}$I-labelled anti-human-IgM antibody (5.0 × 10$^5$ ct/min/ml, 15 ml, 1 h, room temperature) and exposing a Kodak X-ray film (type AR) to the filter for at least 3 h at -70 °C using a Kodak intensifying screen (X-Omat).

Other tests. Protein determinations were done by the Bio-Rad protein assay. Presence of rheumatoid factor in serum specimens was checked using the Cellognost-RF reagent (Behringwerke, Marburg, F.R.G.). Infectivity assays and neutralization assays (NA) were carried out according to standard techniques (Philips, 1976).

RESULTS

Specificity of enzyme-labelled anti-IgM antibody (AP-algM)

To exclude false positive results in virus-specific IgM titration experiments due to cross-reactivity of enzyme-labelled anti-IgM antibody preparations, mono-specificity of AP-algM was checked by ELISA. Binding of AP-algM, diluted in twofold steps, was measured on a constant amount of electrophoretically pure human myeloma IgG as well as myeloma IgM. As shown in Table 1, cross-reactivity of AP-algM, diluted over a range from 1 : 50 to 1 : 6400, was insignificant. Using a 1 : 100 dilution of the conjugate, IgM was recognized specifically between 3 and 1.5 ng/ml without cross-reactivity to IgG (Table 2).

Titration of virus-specific IgM

Serum specimens of acute human enterovirus infections were examined for the presence of virus-specific IgM-antibodies. A representative titration is shown in Fig. 1. The tested serum exhibited a highly specific IgM binding on coxsackievirus B-3 antigen, but almost no detectable difference between binding on coxsackievirus B-1 and HeLa control antigen. To determine antibody titres, background corrected absorbances ($A_{405}$) were plotted against the dilution of serum. The endpoint titre was standardized as the last serum dilution showing a net $A_{405} \geq 0.25$.

Table 1. Monospecificity of Ap-algM*

<table>
<thead>
<tr>
<th>Dilution of Ap-algM</th>
<th>IgG (100 ng/ml)</th>
<th>IgM (100 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 50</td>
<td>0.147†</td>
<td>2.000</td>
</tr>
<tr>
<td>1 : 100</td>
<td>0.099</td>
<td>2.000</td>
</tr>
<tr>
<td>1 : 200</td>
<td>0.068</td>
<td>2.000</td>
</tr>
<tr>
<td>1 : 400</td>
<td>0.079</td>
<td>1.410</td>
</tr>
<tr>
<td>1 : 800</td>
<td>0.036</td>
<td>0.749</td>
</tr>
<tr>
<td>1 : 1600</td>
<td>0.020</td>
<td>0.380</td>
</tr>
<tr>
<td>1 : 3200</td>
<td>0.007</td>
<td>0.211</td>
</tr>
<tr>
<td>1 : 6400</td>
<td>0.048</td>
<td>0.134</td>
</tr>
</tbody>
</table>

* Alkaline phosphatase-labelled anti-human-IgM.
† Absorbance at 405 nm.

Table 2. Sensitivity of IgM detection by AP-algM*

<table>
<thead>
<tr>
<th>Immunoglobulin (ng/ml)</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.00</td>
<td>0.248†</td>
<td>2.000</td>
</tr>
<tr>
<td>50.00</td>
<td>0.120</td>
<td>1.756</td>
</tr>
<tr>
<td>25.00</td>
<td>0.107</td>
<td>0.967</td>
</tr>
<tr>
<td>12.50</td>
<td>0.110</td>
<td>0.578</td>
</tr>
<tr>
<td>6.25</td>
<td>0.120</td>
<td>0.346</td>
</tr>
<tr>
<td>3.13</td>
<td>0.088</td>
<td>0.240</td>
</tr>
<tr>
<td>1.57</td>
<td>0.098</td>
<td>0.171</td>
</tr>
<tr>
<td>0.00</td>
<td>0.099</td>
<td>0.138</td>
</tr>
</tbody>
</table>

* Alkaline phosphatase-labelled anti-human-IgM; binding was tested in a 1 : 100 dilution.
† Absorbance at 405 nm.
Fig. 1. Titration of coxsackievirus B-3 specific IgM. (a) Serially diluted serum from an acute coxsackievirus B-3 infection was examined for the presence of B-3-specific IgM on coxsackievirus B-3 (■), coxsackievirus B-1 (●), and HeLa control antigen (▲). (b) Background-corrected absorbances were plotted versus the dilution of serum for coxsackievirus B-3 (■) and coxsackievirus B-1 (●).

Fig. 2. Specificity of coxsackievirus B IgM from acute human sera. (a) Heterotypic serum, (b) predominant serum and (c) homotypic serum were tested at 1:100 dilution on coxsackievirus B-1 to B-5 and HeLa control antigen. Background-corrected values are shown for each serotype. The columns shaded by triangles indicate the serotype isolated from the patient.
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A value which represents the mean net $A_{405} + 3 \times$ standard deviation of a 1:100 dilution of 40 sera from healthy newborn children. In view of this cut-off point, the coxsackievirus B-3 specific IgM titre was determined as 1:800.

Type-specificity of IgM antibodies detected by ELISA

Since the presence of virus-specific IgM indicates the acute stage of the infection only a single dilution of the specimens (1:100) was tested on coxsackievirus B strains 1 to 5 and HeLa control antigen for the screening of sera for type-specificity. Three types of IgM response were found (Fig. 2). These were a heterotypic IgM response against the group-specific antigen, a predominant IgM response against the isolated virus strain, accompanied by a slight cross-reaction against related strains, and a strictly homotypic response to the causative agent.

Type- and group-specific antigenic determinants in ELISA antigen preparations

The finding that both homotypic as well as heterotypic IgM responses to coxsackievirus could be seen in ELISA using the same antigen preparation indicated the presence of type-specific and group-specific antigenic determinants in these antigen preparations. As type-specific antigenicity of enteroviruses is ascribed to the complete infectious virus particle and group-specific antigenicity to incomplete provirions or damaged particles, the structural composition of [$^{35}$S]-methionine-labelled coxsackievirus B-2, which was propagated exactly as an ELISA antigen, was examined by sucrose gradient centrifugation and subsequent gel electrophoresis. As shown in Fig. 3, virus banded as a broad peak on the 11 to 45% sucrose gradient. Electrophoresis of the peak fractions revealed the presence of high amounts of VP0, 1 and 3, but little VP2 and no VP4. Because VP0 is cleaved to VP2 and VP4 in the assembled provirion (Rueckert, 1976), it was concluded that ELISA antigens consisted of intact infectious virus and incomplete provirions, offering type- as well as group-reactive determinants to the antibody in ELISA.

To localize these antigenic determinants on virus structural proteins, the reaction of virus-specific IgM with individual virus polypeptides was examined by immunoblot analysis. To ensure that virus-specific proteins were transferred from the gel to the filter, [$^{35}$S]methionine-

Fig. 3. Viral polypeptides of coxsackievirus B-2 ELISA antigen. (a) [$^{35}$S]methionine-labelled coxsackievirus B-2 and HeLa control antigens were subjected to sucrose gradient centrifugation. The symbols represent (●) coxsackievirus B-2, (□) HeLa control antigen and (●) sucrose concentration. (b) Peak fractions 10, 11 and 12 of the HeLa (H) and virus (C) gradients were analysed by SDS-PAGE. The positions of the virus proteins VP0 to VP3 are shown at the right of the gel, mol. wt. standards (kdal) at the left.
Fig. 4. Protein blot of coxsackievirus B-2 polypeptides. [35S]Methionine-labelled coxsackievirus B-2 (C) and HeLa (H) control antigen were purified by sucrose gradient centrifugation, subjected to slab gel electrophoresis and electroblotted on to a nitrocellulose filter. The positions of virus proteins VP0 to VP4 and mol. wt. standards (kDal) are shown.

Fig. 5. Immunodetection of IgM specific for individual virus polypeptides of the coxsackie B virus group. Control HeLa (H) and ELISA antigens separated by slab gel electrophoresis were blotted on to a nitrocellulose filter. IgM from an acute coxsackievirus A-9 infection, bound to virus polypeptides, was detected by 125I-labelled anti-human IgM. The positions of coxsackie B-2 virus proteins VP1 to VP3 and mol. wt. standards (kDal) are shown.

Fig. 6. Immunodetection of IgM specific for individual virus polypeptides of the coxsackie B virus group. Control HeLa (H) and ELISA antigens, separated by slab gel electrophoresis were blotted on to a nitrocellulose filter. IgM from an acute coxsackievirus B-2 infection, bound to virus polypeptide VP1 of coxsackievirus B-2, was detected by 125I-labelled anti-human IgM. Standards as in Fig. 5.
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labelled coxsackievirus B-2 was subjected to gel electrophoresis and subsequent blotting. A typical experiment is shown in Fig. 4. Compared to uninfected HeLa cells, virus-specific proteins VP1 to 4 were clearly identified in gradient-purified virus preparations. However, due to the presence of incomplete provirions, indicated by the VP0 band, the VP2 and VP4 bands are less pronounced than VP1 and VP3. Having established that virus-specific polypeptides were transferred during the blotting procedure, filters loaded with unlabelled coxsackievirus B polypeptides from ELISA antigens were incubated either with a heterotypic or a homotypic IgM positive serum. Virus-specific IgM bound to individual polypeptides was monitored by I-aIgM.

In experiments which used the serum of an acute coxsackie A-9 infection for immunodetection of virus polypeptides, the VP1 of all five coxsackievirus B strains was detected as the major antigen (Fig. 5). In contrast, the homotypic VP1 was recognized preferentially by incubating the filter with an acute coxsackievirus B-2 serum, which was shown to be type-specific in ELISA (Fig. 6). This result indicated that type- as well as group-specific antigenic determinants are located on VP1.

DISCUSSION

In the present study, enzyme-linked microimmunoassay and immunoblot analysis have been applied to the examination of coxsackievirus B-specific IgM responses in acute human infections. Specificity of enzyme-labelled anti-IgM antibodies had proved to be crucial for detection of virus-specific IgM in serum specimens. Moreover, the ratio between alkaline phosphatase labelled anti-heavy-chain-specific antibodies and virus antigens had to be balanced carefully before IgM titration experiments. According to Voller et al. (1976) and our own standardization experiments done earlier in microradioimmunoassay (Dörries & ter Meulen, 1980), calibration of enzyme-labelled anti-IgM and coxsackievirus B antigens resulted in a sensitive assay system for virus-specific IgM. Although possible competition of virus-specific IgM and IgG for limiting antigenic determinants on the surface of the microtitre well (Ziegler, 1979) was not evaluated extensively, pre-treatment of serum specimens with Sepharose-bound protein A to remove the bulk of virus-specific IgG, seemed to lower the chance of false positive IgM determinations which could arise from the presence of 19S rheumatoid factor or a slight cross-reactivity of AP-aIgM in serum specimens containing high levels of virus-specific IgG in the lowest serum dilution of 1:100. Patterns of coxsackievirus B-specific IgM response detected by ELISA varied from strictly homotypic, through type-predominant to highly heterotypic. The specificity of homotypic and type-predominant IgM antibodies was in good agreement with virus serotypes isolated from the patient, thereby offering the opportunity of typing the aetiological agent in one serum specimen, tested in a single dilution. This identification was possible providing the type-predominant response was not overwhelmed by cross-reactive IgM clones. However, since the transient occurrence of type-specific IgM is obviously correlated to very early times after infection, as has been shown by Schmidt et al. (1968), identification of the infecting virus type by homotypic IgM would only be possible in serum specimens taken as early as possible after exposure to the virus. Although heterotypic IgM antibodies have proved to be helpful in diagnosing a primary infection by enterovirus, they did not contribute to the type-specific diagnosis of the infection. Even titration of two subsequent serum specimens revealed no preferential change in the titre of antibodies directed against the isolated virus type compared to related virus strains (data not shown).

Problems associated with the type-specificity of coxsackievirus-specific immunoglobulins detected by ELISA are well known in assay systems looking for virus-specific IgG from hyperimmune rabbit sera. As has been shown by Katze & Crowell (1980), heterotypic antibodies reacting with urea-disrupted virus could be excluded from binding to the common group antigen by presenting highly purified, infectious virus particles to the antibodies. However, coupling of these purified antigens directly to the surface of the microtitre well (Katze & Crowell, 1980) or partial purification of virus antigen by column chromatography (Herrmann et al., 1979) resulted immediately in cross-reaction. Only coupling of highly purified virus particles through catching antibody will allow detection of virus-type-specific IgG antibodies by ELISA, since this
procedure does not lead to a disruption of the complete enterovirus particle. In such complete virus particles the group-specific antigen is not accessible to cross-reacting antibodies (Katze & Crowell, 1980). However, this coupling procedure requires for each enterovirus strain a potent monospecific antiserum, and hence is not applicable to mass screening. It would therefore be of great advantage if type- and group-specific antigens were available separately for enterovirus diagnosis.

The application of this method of protein transfer to a nitrocellulose filter in combination with subsequent immunodetection of individual coxsackievirus polypeptides by heterotypic virus-specific IgM antibodies demonstrated that cross-reactive virus antibodies were almost exclusively directed against the VP1 protein of all five virus strains tested. These results are in accordance with the findings of Katze & Crowell (1980) who showed that cross-reactive group antigenic determinants of coxsackie B viruses are located mainly on VP1, using urea-disrupted, radioactively labelled virus antigens for immunoprecipitation studies with hyperimmune (IgG) rabbit sera. However, using human sera in the immunoblot system which were strictly homotypic in ELISA we have observed that type-specific IgM is also directed against the VP1 protein, but preferentially to the VP1 of the causative agent. Although some slight cross-reactivity with VP1 of related strains was detectable, one would assume that type-specific antigenic determinants are located together with group-reactive determinants on the VP1 protein. These findings make it necessary to analyse the antigenic fine structure of the VP1 polypeptide more carefully in order to map its type-specific and group-specific domains. Application of monoclonal antibodies will help to characterize these proteins.

The data obtained demonstrate that it is relatively easy to detect and measure a heterotypic IgM immune response in acute coxsackievirus B infection with microenzyme immunoassay. With this technique, it will be possible to associate a group of enteroviruses with a disease process. However, it is only occasionally that identification of the infecting serotype can be achieved. This will only be feasible when the patient either mounts a homotypic IgM response or when in the ELISA a type-specific antigen is employed which excludes heterotypic antibodies from binding to the antigen (Katze & Crowell, 1980). It should be the aim of virus diagnostic laboratories to develop techniques in the preparation of type-specific enterovirus antigens.

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