Immunological Studies of the Group B Coxsackieviruses by the Sandwich Enzyme-linked Immunosorbent Assay (ELISA) and Immunoprecipitation

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SUMMARY

A microplate double antibody sandwich ELISA was employed in an immunological study of the group B Coxsackieviruses. The assay, described in detail, detected high dilutions of virion antigen (less than 10 ng) in purified preparations and in crude infected cell extracts. Furthermore, by using a constant amount of antigen, group B virus antibodies in hamster antisera could be quantified with a sensitivity equivalent to the virus neutralization test. Titrations of virus antigens and antibodies were found to be type-specific when purified virions were employed in the assay. Urea disruption of virions exposed antigens common to all six group B viruses. The heterotypic reactivity of disrupted group B virions did not extend to the other viruses tested. Immunoprecipitation and SDS-PAGE analysis revealed that, of the four virion structural polypeptides (VPI to 4), VPI contained the major common antigenic determinants shared by members of the group B Coxsackieviruses.

INTRODUCTION

The double antibody sandwich ELISA is based on the principles outlined by Engvall and co-workers (Engvall et al. 1971; Engvall & Perlmann, 1972). Voller et al. (1976) first applied the test to the detection of viruses. More recently, the assay has been adapted for many virus–antibody systems including adenoviruses (Harmon et al. 1979), foot-and-mouth disease virus (Crowther & Abu-el Zein, 1979) and herpes simplex virus (Mills et al. 1978). We now report the use of the sandwich ELISA system and immunoprecipitation-SDS-PAGE analysis for assay of group B Coxsackievirus antigens and antibodies.

The group B Coxsackieviruses (B1 to B6) have been shown to share common antigens which were detected by a variety of immunological assays including immunodiffusion, complement fixation and immunofluorescence (Schmidt & Lennette, 1962; Schmidt et al. 1965; French et al. 1972). In a recent report from our laboratory, heterotypic group B antiviral antibodies were detected by an indirect ELISA test (Katze & Crowell, 1980). The present study uses purified viruses and hyperimmune animal sera to examine, in greater detail, the homologous and heterologous antigenic reactivity of the group B viruses. Both native and urea-disrupted virion antigens were studied (Philipson et al. 1973).

A number of investigators have assigned separate functions to the individual polypeptides of several picornaviruses, including the Coxsackieviruses (Beatrice et al. 1980), FMDV (Meloen et al. 1979), poliovirus (Breindl, 1971) and mengovirus (Lund et al. 1977). Immunoprecipitation studies were therefore performed to determine which of the four structural virion polypeptides, VP1 to 4 (Crowell & Philipson, 1971), contained the common antigenic determinants shared by members of the group B Coxsackieviruses.
METHODS

Cell cultures and viruses. Cell strains and procedures for virus propagation have been described previously (Katze & Crowell, 1980). The strains of the six group B Coxackieviruses used in the present study have also been described (Katze & Crowell, 1980). Poliovirus type 2 (MEF-1) and echovirus 6 (Charles) were propagated in a similar manner to the group B viruses. Coxackievirus A2 was grown in RD cells (Schultz & Crowell, 1980). Viruses were purified by consecutive banding in two cesium chloride gradients as described by Crowell & Philipson (1971). Purified human rhinovirus type 2 was obtained from Dr Karl Lonberg-Holm. Coxackievirus B3 was labelled with $^{35}$S-methionine (New England Nuclear, Boston, Mass.) by adding isotope 2 h after infection at a final concentration of 4 $\mu$Ci/ml. Labelled virus was purified as described above. Each group B virus preparation was tested for the presence of contaminating virions by reciprocal cross neutralization tests. All viruses and their antisera were found to be type-specific.

Crude virus-infected HeLa cell extracts were prepared as for the initial steps before virus purification. Infected cells were centrifuged at 900 $\times$ g for 15 min. Intracellular virus was recovered by three cycles of freeze-thawing. The cellular debris was removed by further centrifugation and the virus-containing supernatant fluids were stored at $-20^\circ$C until used.

Disruption of virions. Equal volumes of undiluted purified virions and 6 M-urea (Ultra-pure, Mann Research Labs, Orangeburg, N.Y.) prepared immediately before use in tris-saline buffer (0.1 M) at pH 9.0, were incubated at 37 $^\circ$C for 60 min. The mixture was placed in an ice bath and immediately diluted in the appropriate ELISA buffer. $^{35}$S-methionine-labelled virus was disrupted with SDS prior to use in immunoprecipitation studies as follows. Equal volumes of purified virus were mixed with buffer (0.05 M-tris, 0.14 M-NaCl, pH 9.0) containing 10% sodium deoxycholate (DOC), 1% Nonidet P-40 (NP40) and 3% SDS, and boiled at 100 $^\circ$C for 5 min. Following a further dilution in reaction buffer, the disrupted virus preparation was centrifuged at approximately 15,000 $g$ for 3 h. The supernatant, containing the dissociated virus polypeptides, was used as antigen in the immunoprecipitation study.

Protein determinations. The protein content of the purified virions and $\gamma$-globulin preparations was determined by a modification of the micromethod of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Virus antisera. Rabbit hyperimmune antisera to the purified group B viruses and poliovirus type 2 were prepared as previously described (Katze & Crowell, 1980). The $\gamma$-globulin fraction of the rabbit antiserum was prepared by precipitation with saturated ammonium sulphate (Stelos, 1967). Hamster antisera to the purified Coxackieviruses and poliovirus type 2 were prepared as follows. Adult female Syrian golden hamsters were given weekly intraperitoneal (i.p.) injections of 0.5 ml of purified virus. The first injection contained 106 p.f.u., followed by 107 p.f.u. on week 2, and 108 p.f.u. on weeks 3, 4 and 5. Two weeks after the final injection, the hamsters were sacrificed and bled out by cardiac puncture. The hyperimmune sera were divided into aliquots and frozen at $-70^\circ$C.

Virus plaque assay and neutralization test. Virus was titrated on HeLa cell monolayers as described previously (Crowell & Syverton, 1961). The virus neutralization test was performed as already described (Katze & Crowell, 1980). Serum neutralization titres were defined as the serum dilution which reduced the plaque forming titre by one-half as obtained from interpolation of the data plotted in graphic form (Svehag & Mandel, 1964).

Immunoprecipitation. Native or disrupted Coxackievirus was immunoprecipitated first with normal serum and then with the hyperimmune antiserum. Virus and antiserum were diluted before use in a reaction buffer (RB) consisting of 0.02 M-tris, pH 7.0, 0.14 M-NaCl, 0.5% DOC and 0.5% NP40. Pre-immune rabbit serum (final dilution of 1:100) was added.
to virus preparations for 1 h at 0°C. Each sample then received a 10-fold excess of a 10% suspension of formalin-inactivated *Staphylococcus aureus* (strain, Cowan 1) for 30 min at 0°C (Kessler, 1975). Following incubation, the samples were centrifuged at 3800 g for 15 min. Specific immunoprecipitation was carried out by adding to the supernatant the appropriate antiviral and normal antisera for 90 min at 0°C. A 10-fold excess of *S. aureus* was added to the virus–antibody mixture for an additional 30 min. The sample was then layered on to a 1 m-sucrose cushion (diluted in RB) and centrifuged at 3800 g for 15 min. The pellet was washed twice in RB and, finally, resuspended in gel sample buffer and boiled for 5 min at 100°C. The sample was then centrifuged once more and the supernatant analysed by SDS–PAGE on slab gels.

*Polyacrylamide gel electrophoresis.* Immune precipitates were subjected to SDS disc electrophoresis in slabs of 12% acrylamide cross-linked with 0.32% *N,N*-methylenebisacrylamide (Maizel, 1971). Stacker gels contained 3% acrylamide cross-linked with 0.08% bisacrylamide. The sample buffer was 0.1 M-tris (pH 6.7), 5% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue and 10% glycerol. The slab gels were run at 25 mA for 2 to 3 h. For fluorography, gels were treated as described by Bonner & Laskey (1974), placed in contact with Kodak XR-2 film and stored at -70°C until developed.

*Sandwich enzyme-linked immunosorbent assay (ELISA).* A modification of the sandwich assay described by Voller *et al.* (1976) was performed as follows. Polystyrene microtitre plates (Cooke MicroElisa, Dynatech Labs, Alexandria, Va.) were coated with 0.2 ml γ-globulin from hyperimmune rabbit virus antisera in 0.06 M-carbonate buffer, pH 9.6. Plates were sealed and incubated in a humidified chamber for 5 to 6 h at 37°C. Before use in the assay, antibody-coated plates were washed three times with PBS, pH 7.2, containing 0.05% Tween-20 (PBS/Tween). For antigen detection, serial dilutions of virus antigen in PBS/Tween were added in 0.2 ml amounts followed by an overnight incubation at 6°C. After three additional washes, a fixed dilution of 0.2 ml hamster antiviral antisera diluted in PBS/Tween was added for 2 h at room temperature. The peroxidase-labelled rabbit anti-hamster immunoglobulin (Cappel Lab., Cochranville, Pa.) was then added for 2 h at room temperature. After a final washing, the amount of peroxidase bound was determined by addition of 0.2 ml of o-phenylenediamine (OPD; Eastman Kodak, Rochester, N.Y.) for 30 min at room temperature. The substrate solution, prepared immediately before use, consisted of 0.003% H$_2$O$_2$ and 0.01% OPD (w/v). After the enzyme–substrate reaction was stopped by 4 M-H$_2$SO$_4$, the yellow-brown colour produced was measured at 490 nm in a Beckman DBG spectrophotometer. For titration of antibody, the assay was performed similarly except that a fixed dilution of virus antigen was used and the hamster antisera were serially diluted.

The ELISA endpoint titre was defined as the highest virus (or antibody) dilution giving a value of 0.25 absorbance (A) units above control levels. All samples were run in duplicate with the value reported being the average of the two wells. The duplicate values agreed within 0.05 A units. Day to day reproducibility of the ELISA titres was greater than 90% within one twofold dilution of the virus and antiserum used. Controls in the ELISA system consisted of the reaction of hyperimmune hamster antisera and rabbit γ-globulin with an uninfected HeLa cell extract. The cell extract was reacted both as a crude preparation and as a purified antigen prepared through CsCl gradients as for virus purification. Additional controls included the reactions of virus antigen with normal hamster antisera and with γ-globulin prepared from pre-immune rabbit antisera. All control reactions were negative, resulting in absorbance levels from 0.02 to 0.10 A units.
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![Graph showing standard curve for Coxsackievirus B1 antigen detection. Wells were coated with γ-globulin (10 μg/ml) from rabbit antiserum prepared against Coxsackievirus B1 and reacted with serial dilutions of purified Cox B1 virions (Δ), or an uninfected HeLa cell extract (○). Hamster Cox B1 antiserum was added at 1:500 dilution. The ELISA was performed as described in Methods.](image)

Fig. 1. Sandwich ELISA: standard curve for Coxsackievirus B1 antigen detection. Wells were coated with γ-globulin (10 μg/ml) from rabbit antiserum prepared against Coxsackievirus B1 and reacted with serial dilutions of purified Cox B1 virions (Δ), or an uninfected HeLa cell extract (○). Hamster Cox B1 antiserum was added at 1:500 dilution. The ELISA was performed as described in Methods.

Table 1. Comparative sandwich ELISA titres of purified and unpurified preparations of Coxsackieviruses B1 to B6 and poliovirus type 2

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Purified virus</th>
<th>Unpurified virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infectivity†</td>
<td>Protein ELISA‡</td>
</tr>
<tr>
<td></td>
<td>(p.f.u./well)</td>
<td>(μg/well) titre</td>
</tr>
<tr>
<td>Cox B1</td>
<td>8.0 × 10¹⁰</td>
<td>100</td>
</tr>
<tr>
<td>Cox B2</td>
<td>5.0 × 10⁹</td>
<td>60</td>
</tr>
<tr>
<td>Cox B3</td>
<td>3.5 × 10⁹</td>
<td>80</td>
</tr>
<tr>
<td>Cox B4</td>
<td>4.0 × 10⁹</td>
<td>90</td>
</tr>
<tr>
<td>Cox B5</td>
<td>4.5 × 10⁹</td>
<td>100</td>
</tr>
<tr>
<td>Cox B6</td>
<td>5.0 × 10⁹</td>
<td>90</td>
</tr>
<tr>
<td>Polio</td>
<td>3.5 × 10¹⁰</td>
<td>120</td>
</tr>
</tbody>
</table>

* Microtitre wells were coated initially with homologous γ-globulin (10 μg/ml) of the hyperimmune rabbit sera. After addition of virus, homologous hyperimmune hamster antisera were added at a 1:500 dilution and the assay performed as described in Methods.
† Determination of equivalent amounts of p.f.u. and protein detected by ELISA at the endpoint dilution is made by dividing p.f.u. or protein by the ELISA titre.
‡ ELISA titre is defined as the highest virus dilution giving an absorbance 0.25 A units above control levels.

RESULTS

Sensitivity of the sandwich ELISA for the detection of viral antigen

A series of preliminary experiments were performed to develop a sensitive and specific sandwich ELISA for detection of the group B Coxsackieviruses. Coxsackievirus B1 and hyperimmune rabbit and hamster antisera prepared against the purified virus served in the development of the test, which is described in Methods. A representative standard curve for antigen detection is given in Fig. 1. Depiction of antigen dilution, virion protein concentration and infectivity (p.f.u.) on the abscissa permits ready determination of the
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Table 2. Heterotypic antigenic reactions among native and disrupted group B Coxsackieviruses as detected by sandwich ELISA

<table>
<thead>
<tr>
<th>γ-Globulin antibody system*</th>
<th>Virus type‡</th>
<th>Native virions</th>
<th>Disrupted virions§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox B3 – Cox B3</td>
<td>Cox B3</td>
<td>3.95</td>
<td>3.90</td>
</tr>
<tr>
<td></td>
<td>Cox B1</td>
<td>0.10</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>Cox B2</td>
<td>0.08</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Cox B4</td>
<td>0.14</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>Cox B5</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Cox B6</td>
<td>0.08</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>HeLa II</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Cox B1 – Cox B1</td>
<td>Cox B1</td>
<td>3.90</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td>Cox B5</td>
<td>0.16</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Cox B6</td>
<td>0.15</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>0.04</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Wells were coated with γ-globulin at 10 μg/ml. Captured virus antigen was then reacted with appropriate hamster antisera at a 1:100 dilution.
† All reactions were tested in duplicate and the average value reported.
‡ All virion preparations were purified and used at a dilution of 1:20 except the homologous Cox B1 and Cox B3 reactions in which the purified virus was used at a 1:100 dilution.
§ Virions were disrupted with 3 M-urea for 60 min at 37°C.
‖ Uninfected HeLa cell control antigen used at a 1:20 dilution.

sensitivity of the test in terms of the respective antigen units. The enzyme activity in absorbance (A) units is plotted on the ordinate. It can be seen that this assay measured as little as 10 to 15 ng/ml (2 to 3 ng/well) of the purified virus preparation. The specificity of the ELISA was demonstrated by the lack of reactivity with the uninfected HeLa cell antigen.

The ELISA system was also utilized to measure virus antigen in both purified and unpurified preparations of each of the six group B Coxsackieviruses and poliovirus type 2 (Table 1). The purified virion preparations were measured to high titres. Less than 10 ng virion protein per microtitre well was detected for all seven viruses tested. Furthermore, high dilutions of virus antigen in crude Coxsackievirus- and poliovirus-infected cell extracts were also detectable. In the case of Coxsackievirus B2 as little as 5 x 10⁴ p.f.u./well were detected. The results in Table 1 clearly establish the high degree of sensitivity of the sandwich ELISA for the quantification of virus antigen in both purified and crude preparations.

Heterotypic reactivity of native and disrupted virus antigens as detected by sandwich ELISA

It has been reported that the native antigenicity of the group B Coxsackieviruses is altered after virus disruption (Schmidt et al. 1963). The homotypic and heterotypic reactivity of native and urea-disrupted virions were examined in the ELISA system (Table 2). The 3 M-urea treatment (37°C; 60 min) inactivated >99.9% infectivity of all six Coxsackie B viruses (our unpublished observations). The group B Coxsackieviruses were sandwiched between the rabbit and hamster Cox B3 antibodies and Cox B1 antibodies, respectively. The homologous reactions for the Cox B1 and Cox B3 viruses resulted in the highest ELISA activity. The absorbance readings (near 4.0) for the homotypic virus–antibody reactions were close to the maximum observable levels under these assay conditions. The reactions of native heterotypic group B virions resulted in background absorbance values of 0.08 to 0.16, levels not significantly different from those of the HeLa cell control antigen.
Table 3. Comparative titrations of homotypic and heterotypic* hyperimmune hamster antisera to the group B Coxsackieviruses and poliovirus type 2 by the sandwich ELISA and virus neutralization test

<table>
<thead>
<tr>
<th>Virus</th>
<th>Reciprocal of homotypic antiserum titre (x 10^9)</th>
<th>ELISA†</th>
<th>Neutralization (PR₅₀)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox B₁</td>
<td>25</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cox B₂</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cox B₃</td>
<td>100</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Cox B₄</td>
<td>25</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Cox B₅</td>
<td>60</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Cox B₆</td>
<td>25</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Polio type 2</td>
<td>40</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* Heterotypic titres in the neutralization test were all < 1:20 and in the ELISA were < 1:100 except for the Cox B₁ (1:200) and Cox B₅ (1:1000) antisera reactions with Coxsackievirus B₆.
† Microtitre wells were coated initially with rabbit γ-globulin (10 μg/ml). A constant amount of homologous purified virus antigen (1:200 dilution) was added, followed by addition of serial dilutions of the appropriate hamster antisera.
‡ PR₅₀: plaque reduction, 50% endpoint.

However, when the virions were disrupted by urea before being used as antigen between the heterotypic antibodies, the absorbance values increased up to 20-fold over control values. This rise in A was consistently found in heterologous reactions among disrupted group B viruses. In contrast, neither native nor disrupted Coxsackievirus A₂, poliovirus type 2, echovirus 6, nor human rhinovirus 2 were reactive when sandwiched between the group B Coxsackievirus antibodies in the ELISA test. Thus, disrupted group B viruses share common antigens which are detectable by the sandwich ELISA system. However, these antigens are probably internal since they are not detected in purified native virion preparations.

**Titration of hyperimmune hamster antisera to the group B Coxsackieviruses by the sandwich ELISA**

The ELISA system also was found to be highly sensitive for the detection and measurement of antiviral antibodies. By using a constant dilution of purified virions as antigen, the homotypic and heterotypic reactivities of the group B viral antisera were tested. Results of the titrations of the respective antisera are shown in Table 3. Homotypic antibody titres obtained by the sandwich ELISA technique were found to be comparable and, in some cases, five- to tenfold higher than titres obtained by the virus neutralization test. The reactions with the native virions and antibodies were primarily type-specific as noted by the lack of cross reactivity detectable among the group B viruses. As an additional control, poliovirus type 2 and hyperimmune antisera to the purified virus were included in the titration and found to be non-reactive with the Coxsackieviruses.

Antibody titrations were also performed using urea-disrupted virions, rather than native virions, as antigen (Fig. 2). As expected, the reactions of Cox B₂, B₃ and B₄ hamster antibodies were negative when tested against native Cox B₁ virus. However, when the Cox B₁ virions were disrupted with urea before assay, the antiviral antibodies recognized the common group B Coxsackievirus antigens and were reactive in the ELISA system. From these results, it was concluded that the sandwich ELISA permitted a type-specific titration of antibodies with a sensitivity equal to or greater than the neutralization test.
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Fig. 2. Virus group-reactivity of Coxsackievirus B2, B3, B4 hamster antisera tested against urea-disrupted Cox B1 virions in the sandwich ELISA. Wells were coated with γ-globulin (10 μg/ml) from rabbit antiserum prepared against Coxsackievirus B1. A 1:200 dilution of urea-disrupted B1 virions was reacted with serial dilutions of hamster antiserum prepared against the following viruses: Cox B2 (○); Cox B3 (□); Cox B4 (■). The reaction of each antiserum against native B1 virions was negative (●). The reaction of native Cox B1 virions (1:200) with dilutions of Cox B1 hamster antiserum (△) served as a positive control.

It was also shown that the virus type specificity was dependent on the use of native virions as antigen. The group reactivity of the antisera was expressed only when disrupted virions were used as antigen.

Immunoprecipitation SDS-PAGE analysis of native and disrupted Coxsackievirus B3

Immunoprecipitation studies with 35S-methionine-labelled Cox B3 virus and group B virus antisera were performed to establish which virion polypeptide(s) were responsible for the virus group-reactivity detected by the ELISA. Initial studies were done with native virions to test the specificity of the precipitation reaction (Fig. 3a). Purified Coxsackievirus B3 virions were reacted with homotypic and heterotypic viral antisera, precipitated with S. aureus and electrophoresed as described in Methods. Native virions, consisting of VP1 to 4, were precipitated only by homologous antiserum (row D). Detection of the labelled VP4 band was quite difficult but a darker band was produced when fluorographs were exposed for longer periods. Pre-immune serum (row B) as well as antisera to Cox B1 (row C) or to Cox B6 (row E) or to Cox B2, B4 or B5 (data not shown) failed to precipitate labelled virions. As an additional control of virus specificity, antisera to poliovirus type 2 and human rhinovirus 2 were found to be non-reactive with Coxsackievirus B3.

The reactivity of the heterologous antisera with disrupted virions was tested. Purified Coxsackievirus B3 was disrupted with 1.5% SDS and centrifuged at approx. 150 000 g for 3 h to remove any undegraded virus particles. The supernatant fluid, containing the dissociated virus polypeptides, was reacted with the appropriate antisera in a manner similar to that used for native virions. The results of the immunoprecipitation, shown in Fig. 3(b), revealed that pre-immune serum failed to precipitate any labelled virion polypeptides (row F), whereas antiserum to Coxsackievirus B3 precipitated the major polypeptides, VP1, 2 and 3 (row C). The low mol. wt. VP4 was not precipitated in the disrupted preparations and may be both a weak immunogen and antigen as discussed later. When the disrupted Cox B3 preparation was reacted with heterotypic antisera to
Fig. 3. Fluorographs of immunoprecipitation and SDS-PAGE analysis of native and disrupted Coxsackievirus B3 by homotypic and heterotypic rabbit antisera. (a) Immunoprecipitation of native Coxsackievirus B3 by antiserum to Cox B3 (D); pre-immune serum (B); antiserum to Cox B1 (C); antiserum to Cox B6 (E). Cox B3 virions were run as marker (A, F). The position of virion polypeptides VP1 to 4 is indicated. Exposure time was 2 days. (b) Immunoprecipitation of SDS-disrupted B3 virus by pre-immune serum (F); antiserum to Cox B3 (C); antiserum to Cox B1 (B); antiserum to Cox B5 (D); antiserum to Cox B6 (E). Cox B3 virions were run as marker (A, G). Exposure time was 2 days.
the group B viruses only the VP1 polypeptide was precipitated. Antiserum to Coxsackievirus B1, B5 and B6 reacted only with VP1 of Coxsackievirus B3 (rows B, D and E respectively). Antiserum to Coxsackievirus B4 also precipitated VP1 specifically but only when higher concentrations of virus and antibody were used (data not shown). Rabbit antisera to Coxsackievirus B2 failed to precipitate VP1 probably because it contained fewer group-reactive antibodies. These results have confirmed those of the ELISA in showing the serological reactivity of native virions to be virus type-specific. In addition, it was found that VP1 is the virion polypeptide containing the common sub-surface antigenic determinants recognized by the heterotypic group B viral antisera.

DISCUSSION

Adaptation of the sandwich ELISA system to assay group B Coxsackieviruses along with their homotypic and heterotypic antisera has provided a rapid and sensitive method to explore further the antigenic structures of these viruses. The use of native purified virions and hyperimmune antisera, made against the purified viruses, was found to be essential for the type-specific identification of members of the group B Coxsackieviruses. In a recent report on the development of an enterovirus ELISA, Herrmann et al. (1979) encountered difficulty using partially purified virus and commercially prepared antisera. These investigators identified the Coxsackie B viruses only as a group. The extensive cross-reactivity detected in their studies precluded any virus type-specific identification.

The indirect ELISA (Katze & Crowell, 1980) was not as type-specific as the assay described here, probably because of partial disruption of virions following adsorption to microtitre wells in the former test. In the sandwich ELISA, urea disruption of Coxsackievirions exposed common antigens and produced an alteration from type-specificity to group-reactivity. This alteration in native antigenicity has also been reported for other picornaviruses including the polioviruses (Hummeler & Hamparian, 1958), the human rhinoviruses (Lonberg-Holm & Yin, 1973) and foot-and-mouth disease virus (Rowlands et al. 1969). The heterotypic group B antibodies were demonstrable in both hyperimmune rabbit and hamster antisera because of the high sensitivity of the ELISA system. This is in contrast to some early reports which found virus group antibodies only in human sera or sera from monkeys orally infected with these viruses (Schmidt & Lennette, 1962; Schmidt et al. 1965). The heterologous reactivity of antigens and antibodies, found within the group B Coxsackieviruses, did not extend to the other human viruses tested (Chaudry et al. 1971; Hughes et al. 1977).

The immunoprecipitation data revealed that VP1 contains the major common antigenic determinants shared by the group B Coxsackieviruses. However, this does not exclude the presence of minor virus group-reactive determinants on the polypeptides of the other virions, especially VP3, which in some experiments was precipitated in small amounts. Even though iodination studies (M. L. McGeady and R. L. Crowell, unpublished data) have shown that VP1 is present on the surface of native virions, the heterotypic antigens of this polypeptide are in a cryptic location since native virions were not precipitated by heterologous antisera. Together these results suggest that there are sites on VP1 that occupy both an external and internal location. The VP2 of Coxsackievirus B3 also resides on the surface of the virion and is the polypeptide responsible for the induction of type-specific neutralizing antibodies to this virus (Beatrice et al. 1980). Other picornaviruses seem to have a different polypeptide for the immunogen which gives rise to neutralizing antibodies. For example, Breindl (1971) reported that VP4 of poliovirus was the neutralizing immunogen, although direct evidence for this assignment remains to be provided. The VP1 of both mengovirus (Lund et al. 1977) and FMDV (Meloen et al. 1979) has
been shown to be the protein that induced the formation of neutralizing antibodies in animals.

Katagiri et al. (1971) have shown that the VP1, 3 sub-structure of poliovirus exhibited 'H' or group-reactive antigenicity as did the VP1 of Coxsackievirus B3 as reported here. Immunological studies with FMDV have demonstrated that the VP4 polypeptide, shown to be an internal protein, was responsible for the group-reactivity within that virus group (Talbot et al. 1973). Our results do not exclude VP4 of the group B Coxsackieviruses from possessing a common antigen. As discussed previously (Beatrice et al. 1980), VP4 of Coxsackievirus B3 appears to be both a weak immunogen and antigen. The VP4 in disrupted preparations was not precipitated by antiserum to native virus and antibodies to this polypeptide were undetected even when fluorographs were highly overexposed. In addition, Meloen et al. (1979) reported that purified VP4 of both poliovirus and FMDV failed to induce significant levels of precipitating antibody as detected by radio-immunoassay. Continued studies are in progress to characterize better the immunogenicity and antigenicity of the VP4 polypeptide.

Finally, the sandwich ELISA has potential to serve as a rapid and sensitive diagnostic test in the clinical laboratory. By substituting human sera for the hamster sera in the assay, group B virus antibodies could also be titrated. In addition, high dilutions of virus antigen corresponding to $10^4$ to $10^5$ p.f.u., were detectable in crude infected cell extracts. The specific identification of virus antigen, either directly from a clinical specimen or from a tissue culture extract infected with an isolate may prove useful. Current studies are in progress to investigate these possible diagnostic applications.

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