Cross-reactions of immunoglobulin M and G antibodies with enterovirus-specific viral structural proteins

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SUMMARY

We analysed the reactivity of enterovirus-specific human IgM and IgG antibodies with the structural proteins of different enteroviruses by the immunoblot technique. In general, all immunoglobulin G antibodies of the tested sera reacted with capsid polypeptide VP 1 of the viruses tested (echoviruses 9 and 11, coxsackievirus B3 and poliovirus 2). In contrast, enterovirus specific immunoglobulin M antibodies of adults reacted with capsid polypeptides VP 1, VP 2, and/or VP 3 of the viruses mentioned above. The reactions with VP 2 and/or VP 3 were often stronger than with VP 1. IgM antibodies from sera of newborns infected by echovirus 11 reacted with VP 1 and VP 2/3 of echovirus 11 and also with VP 2 and VP 3 of poliovirus 2. Preabsorption experiments indicate that cross-reactive IgG antibodies react with epitopes of VP 1 not present on the surface of intact virus particles. The results from the immunoblot technique were compared to data from microneutralization tests and M-antibody capture radioimmunoassays.

INTRODUCTION

Recently developed IgM specific assays (MACRIA) (Burkhardt, Reigel & Schilt, 1983; El-Hagrassy, Banatvala & Coltart, 1980; Morgan-Capner & McSorley, 1983; Pattison, 1983) for the diagnosis of enterovirus infections and other serological test systems such as complement-fixation (Bussel *et al.* 1962; Halonen, Rosen & Huebner, 1959; Kraft & Melnick, 1952; Schmidt, Dennis & Lennette, 1962), haemagglutination-inhibition (Bussel *et al.* 1962), immunodiffusion (Schmidt, Magoffin & Lennette, 1973), counterimmunoelectrophoresis (Minor *et al.* 1979) or solid-phase immunoassays (Katze & Crowell, 1980*a*, *b*; King *et al.* 1983) showed cross-reactivities of IgM antibodies to heterologous viruses. Apparently type-specific reactions in a 'reverse IgM RIA' were described by Torfason, Frisk & Diderholm (1984) using purified virions as antigen.

We were therefore interested to know which virus structural proteins are associated with the cross-reactivity. We recently showed that IgM antibodies from sera of echovirus 11 infected persons reacted to VP 1, VP 2 and VP 3 of echovirus 11, whereas IgG antibodies reacted exclusively to VP 1 (Reigel, Burkhardt & Schilt, 1984).

Other data lead to the conclusion that cross-reactivity of IgM antibodies

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depends on the age of the patients (King *et al.* 1983). We therefore analysed the reactivity of IgM and IgG antibodies from sera of enterovirus-infected newborns to determine whether they cross-reacted with proteins of heterologous enteroviruses or whether a primary enteroviral infection of humans induces type-specific IgM antibodies.

In this study we describe the reactions of IgM and IgG antibodies of sera from echovirus 11 infected newborns and sera from adult persons with various enterovirus infections. Sera were tested for reactions with the structural proteins of echovirus 11, coxsackievirus B3, poliovirus 2 and some of those of echovirus 9.

MATERIALS AND METHODS

Sera. Sera from adults were collected from enterovirus outbreaks (echoviruses 9 and 11) or from sporadic infections caused by different enteroviruses, and sera of newborns from an echovirus 11 outbreak in an infant ward. In all cases either the infecting virus was isolated, and/or a fourfold increase of neutralizing antibodies in paired sera was shown, and/or the presence of IgM antibodies was demonstrated using M-antibody capture radioimmunoassays (MACRIA) to echoviruses (Burkhardt, Reigel & Schilt, 1983) and hepatitis A (HAV, enterovirus 72; Flehmig, 1978). Briefly, polystyrene beads coated with anti-human IgM antibodies were incubated with diluted sera. After washing the antigen was added and the bound antigen was detected by incubation with ^{125}I-labelled anti-echovirus 9, anti-echovirus 11 or anti-HAV specific antibodies. Sera were fractionated by centrifugation through 10–40 % sucrose gradients in phosphate-buffered saline (PBS) and the IgG- and IgM-containing fractions were pooled and used for immunoblot testing. Some sera from newborns and control sera taken from umbilical cord blood were used without fractionation.

[^{35}S]*methionine-labelled viral proteins.* Vero cell monolayers were infected with the different enterovirus strains at a multiplicity of infection of ⩽1 plaque forming unit (p.f.u.) per cell until the cytopathic effect was first visible. Then the medium was replaced by methionine-free minimal essential medium (MEM) without serum and the viral proteins were labelled with 5 μCi/ml [^{35}S]methionine (New England Nuclear, Boston, USA) until cytopathic effects were complete. Labelled viruses were isolated as described below.

Viruses. Prototype strains Hill (echovirus 9), Gregory (echovirus 11), Nancy (coxsackievirus B3) and Lansing (poliovirus 2) were propagated in Vero cells and the viruses isolated by CsCl density-gradient centrifugation as described for echovirus 11 (Reigel, Burkhardt & Schilt, 1984). Virions banding at a density of 1·34 g/cm³ were used as antigens. This virus material was sedimented by ultra-centrifugation (130000 *g*, SW 27·0) and the virus particles disrupted by boiling for 2 min at 100 °C in sample buffer (62·5 mM Tris/HCl, pH 6·8, 2 % SDS 5 % mercaptoethanol, 10 % glycerin and bromophenolblue) and then separated by polyacrylamide gel electrophoresis.

Immunoblotting. The method has been described in detail (Reigel, Burkhardt & Schilt, 1984). Briefly, viral proteins were isolated from purified virions by polyacrylamide gel electrophoresis according to Laemmli (1970), as modified by Weintraub, Palter & van Lente (1975). The proteins were then transferred from

Table 1. *Serological data: sera of adults*

Patient	Infecting virus	Echo 9	Echo 11	CB 3	CB 5	CA 9	Polio 2	Echo 9	Echo 11	HAV	Total (i.u./ml)‡
		Neutralizing antibody titre* to						Virus-specific†			IgM antibodies
1§	Echovirus 11	<10	320	<10	—	—	40	13·7	23·0	0·9	150
2§	Echovirus 9	640	<10	<10	—	—	20	14·9	3·5	0·9	190
3	HAV	<10	80	20	—	—	10	0·6	0·7	26·7	500
4	Coxsackievirus B3	<10	<10	40	—	—	10	15·9	8·4	0·8	170
5	Coxsackievirus B5	<10	80	40	320	—	80	0·6	0·5	0·9	150
6	Coxsackievirus A9	<10	<10	160	—	80	80	8·9	10·3	0·9	225
7	Poliovirus 2	<10	<10	<10	—	—	≥640	1·4	0·6	0·9	140

* Determined in microneutralization tests against 50 $TCID_{50}$ of virus per assay.

† Figures are test-negative ratios ($\leq 2·1$ is negative).

‡ Determined using NOR-IgM plates (Bering, Marburg, Germany): 100 i.u. is equivalent to 0·89 g/l.

§ Patients 1 and 2 are representatives from persons infected during two enterovirus outbreaks (Burkhardt, Reigel & Schilt, 1983).

the gel to nitrocellulose (western blotting; Towbin, Staehelin & Gordon, 1979). Nitrocellulose strips containing the viral proteins were incubated with human sera and the immune reactions visualized by incubation with peroxidase-conjugated anti-human IgM or IgG specific antibodies followed by the dye reaction. The dye used was *o*-dianisidine. Peroxidase-conjugated anti-human IgG or anti-human IgM specific immunoglobulins were obtained from DAKO (DAKO Immunoglobulins, Denmark) and tested for specificity by F. Skvaril, Berne. Peroxidase-conjugated anti-IgG immunoglobulins were used at a 1 in 200 dilution and anti-IgM immunoglobulins at a 1 in 100 dilution. IgG- and IgM-containing fractions of human sera or unfractionated sera from newborns were diluted with 3 % FCS-PBS and used for the immunoblot reactions at final dilutions of 1 in 25 for IgM and 1 in 100 for IgG antibodies.

RESULTS

Serological data of sera. The tables summarize the results of neutralizing antibody and MACRIA activity for seven sera from the acute phase of different enterovirus infections from adult persons (Table 1) and from six newborns infected by echovirus 11 (Table 2). In one case, serum from the mother was available and the data are included in Table 2.

Sensitivity and specificity of the immunoblot reactions. To obtain optimal conditions we used a checker-board titration system on the immunoblots for echovirus 11. We analysed dilutions of serum 1 (Table 1) from 1 in 25 to 1 in 5000 and virus particles of 10^6 to 10^9 p.f.u. per slot. Optimal conditions for best visible bands were found at a dilution of IgM antibodies of ≤ 1 in 100 and of proteins from $\geq 10^8$ p.f.u. per strip. Using 10^9 p.f.u. per slot, reactions could be demonstrated up to serum dilutions of ≥ 1 in 1000, while 10^7 or less p.f.u. per slot did not lead to visible

Table 2. *Serological data: sera of newborns*

Patient	Age	Day after onset of illness	Neutralizing antibody titre to					IgM Virus specific		Total
			Echo 6	Echo 9	Echo 11	CB3	Polio 2	Echo 9	Echo 11	(i.u./m
X1180	2 weeks	0	<10	<10	<10	n.d.	n.d.	0·4	0·6	100
		12	<10	20	160	160	40	0·4	10·2	180
		49	<10	<10	80	n.d.	n.d.	0·3	2·9	140
X1181	2 weeks	0	<10	40	80	n.d.	n.d.	0·3	15·5	205
		40	<10	20	320	160	20	0·5	8·1	125
X1182	2 weeks	0	<10	10	<10	n.d.	n.d.	0·5	0·3	60
		12	<10	20	≥640	80	20	0·6	16·0	155
		31	<10	10	320	n.d.	n.d.	0·4	3·6	110
X1201	1 week	2	10	10	<10	n.d.	n.d.	1·0	0·5	50
		61	<10	<10	320	<10	20	0·3	3·1	155
X1672	1 week	0	<10	<10	10	<10	10	0·6	0·2	140
X1683	Mother of X1672		n.d.	<10	10	20	20	n.d.	0·6	n.d.
X1774	3 weeks	12	<10	10	80	n.d.	n.d.	1·1	14·0	205

Footnotes as for Table 1. X1180 and X1182 are twins. n.d., Not determined.

reactions. IgG antibodies were in some cases tested at a 1 in 10 dilution and showed no additional reactions. Sera were routinely fractionated for testing of IgM specific reactions. Unfractionated sera gave similar results.

Serum from umbilical cord blood did not show IgM specific reactions to structural proteins of echoviruses 9 and 11, coxsackievirus B3 and poliovirus 2. Sera of adults presented here did not react to proteins from uninfected Vero cells (control antigen) at the supposed positions of the viral structural proteins.

Reactions of IgG antibodies to the viral structural proteins. Figs. 1–5 show the reactivity of the sera from different enterovirus infections to viral structural proteins of echoviruses 9 and 11, coxsackievirus B3 and poliovirus 2.

IgG antibodies from adult sera 1–7 and from sera of six newborns all reacted to VP 1 of all viruses tested (Figs. 1 A–5 A) irrespective of the presence or absence of neutralizing antibodies to the corresponding viruses (Tables 1 and 2). In adult sera a weak reaction to VP 2 of poliovirus 2 may be detected (Fig. 4 A, lanes 1–4, 6, 7). Also sera from umbilical cord blood showed IgG reactions to capsid polypeptide VP 1 of echovirus 11, echovirus 9, coxsackievirus B3 and poliovirus 2 (data not shown).

An additional 24 sera from infections of adults caused by echoviruses 9, 11, 30, coxsackieviruses B2 to B6, polioviruses 1 and 2, coxsackievirus A9 and HAV were tested for the reactivity to the structural proteins of echovirus 11 and six additional sera for reactions to viral proteins of echovirus 9. All sera showed IgG specific reactions to VP 1 of echoviruses 9 and 11 respectively (data not shown).

Preabsorption of sera from echovirus 11 infected adults with intact echovirus 11 led to the disappearance of IgG reactions with VP 1 of echovirus 11 and poliovirus 2. This reaction was again observed after disruption of the antigen–antibody
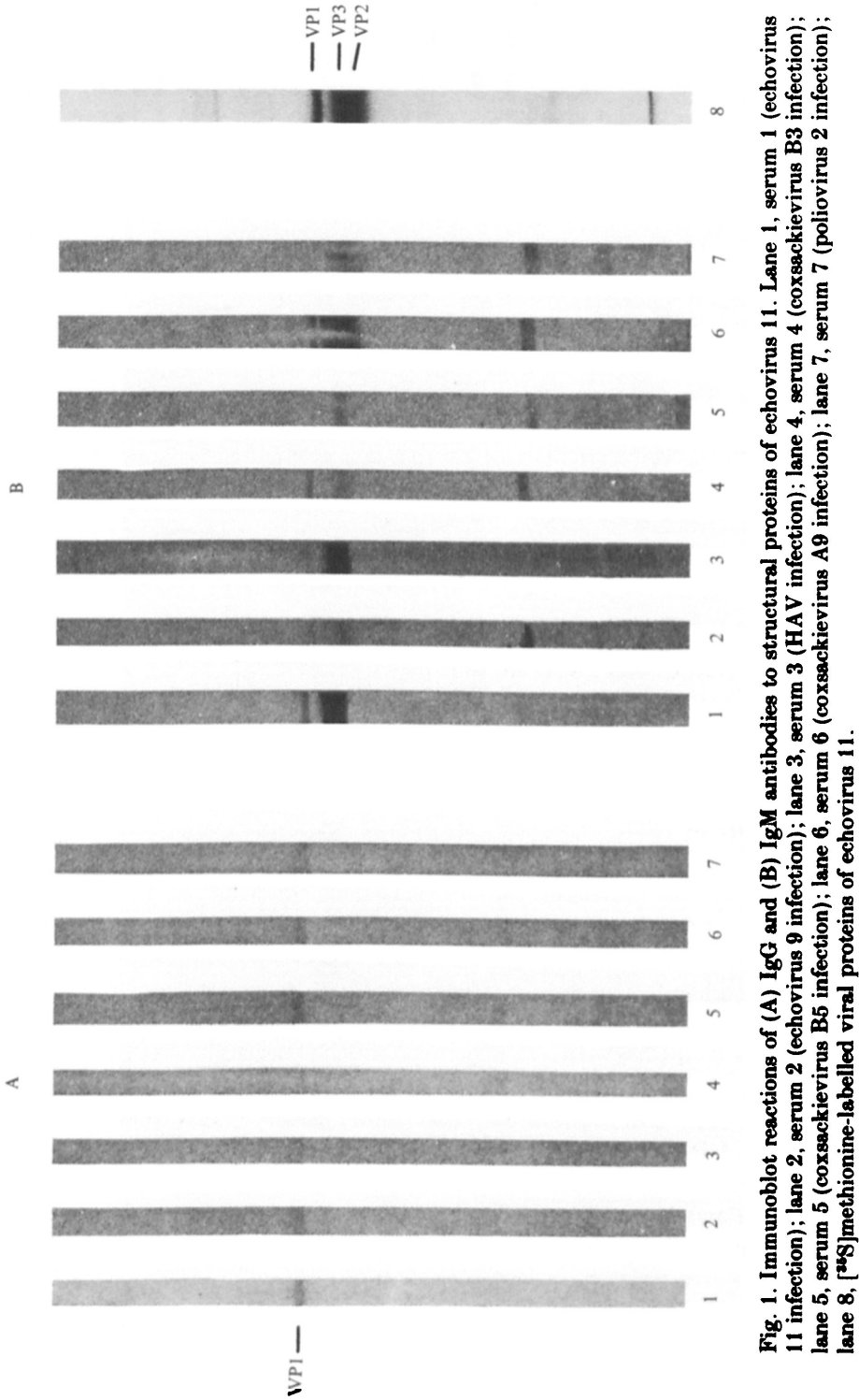
Fig. 1. Immunoblot reactions of (A) IgG and (B) IgM antibodies to structural proteins of echovirus 11. **Lane 1**, serum 1 (echovirus 11 infection); lane 2, serum 2 (echovirus 9 infection); lane 3, serum 3 (HAV infection); lane 4, serum 4 (coxsackievirus B3 infection); lane 5, serum 5 (coxsackievirus B5 infection); lane 6, serum 6 (coxsackievirus A9 infection); lane 7, serum 7 (poliovirus 2 infection); lane 8, [^{35}S]methionine-labelled viral proteins of echovirus 11.
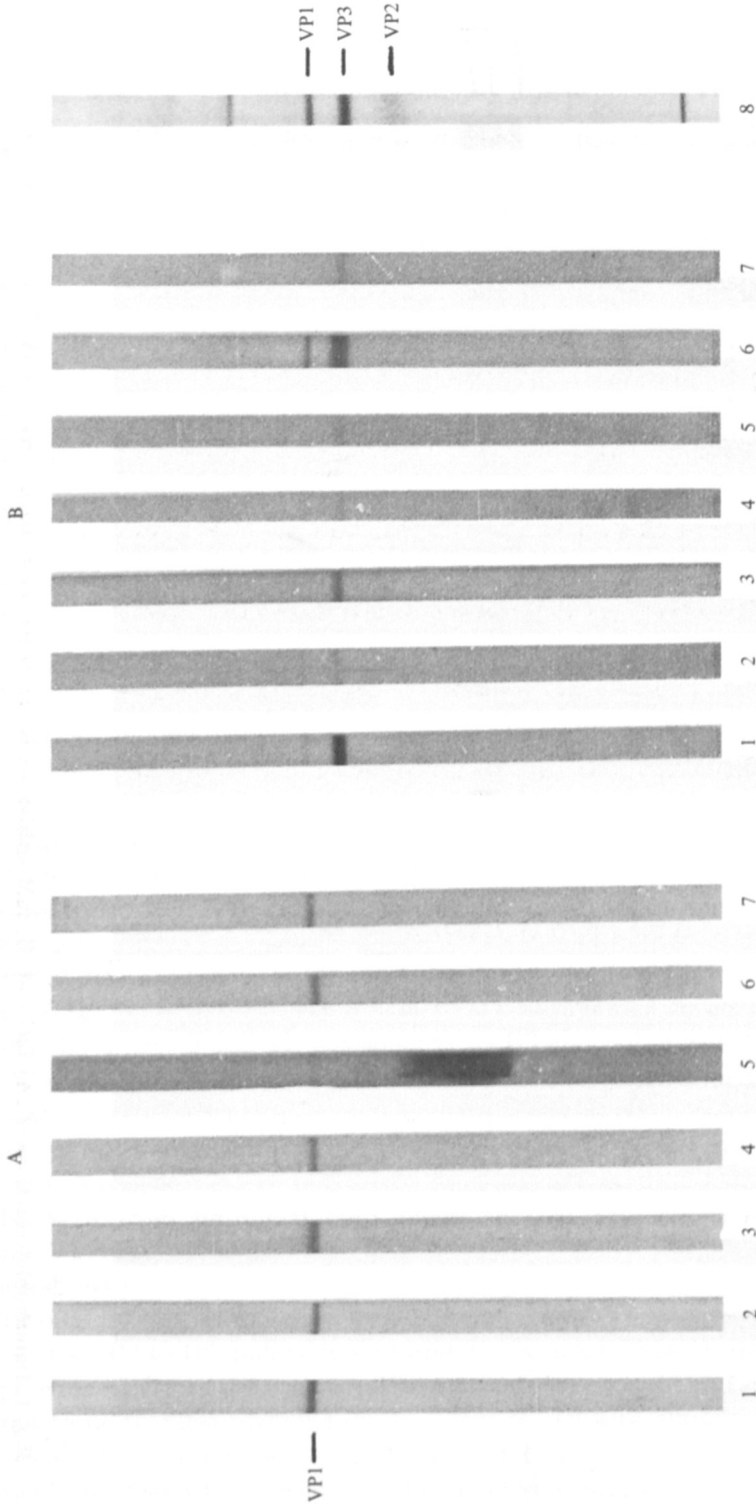
Fig. 2. Immunoblot reactions of (A) IgG and (B) IgM antibodies to structural proteins of echovirus 9. Lanes 1–7 as in Fig. 1. Lane 8, [^{35}S]methionine-labelled viral proteins of echovirus 9. Nomenclature of echovirus-9 proteins as described by Rosenworth & Eggers (1983).
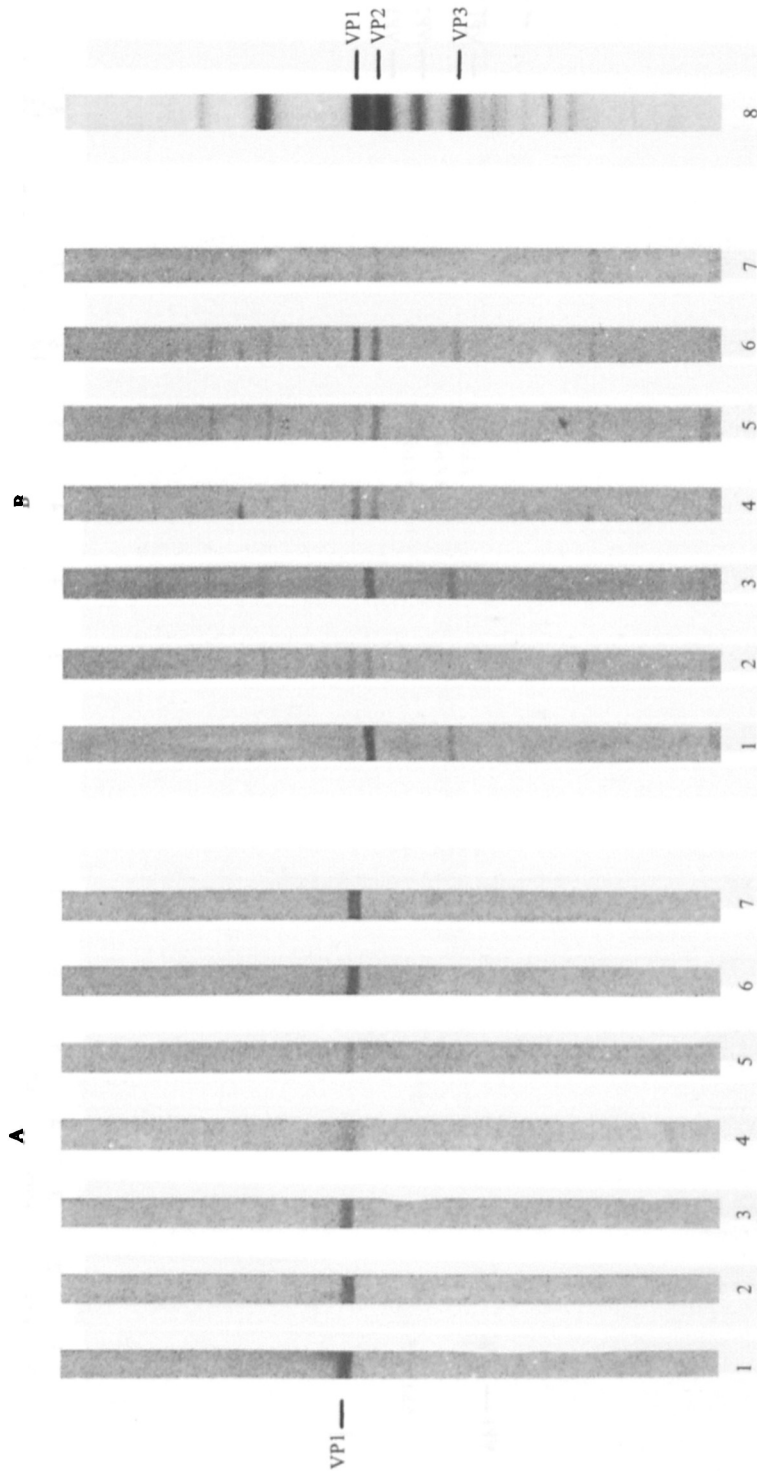
Fig. 3. Immunoblot reactions of (A) IgG and (B) IgM antibodies to structural proteins of coxsackievirus B3. Lanes 1–7 as in Fig. 1. Lane 8, [^{35}S]methionine-labelled viral proteins of coxsackievirus B3.
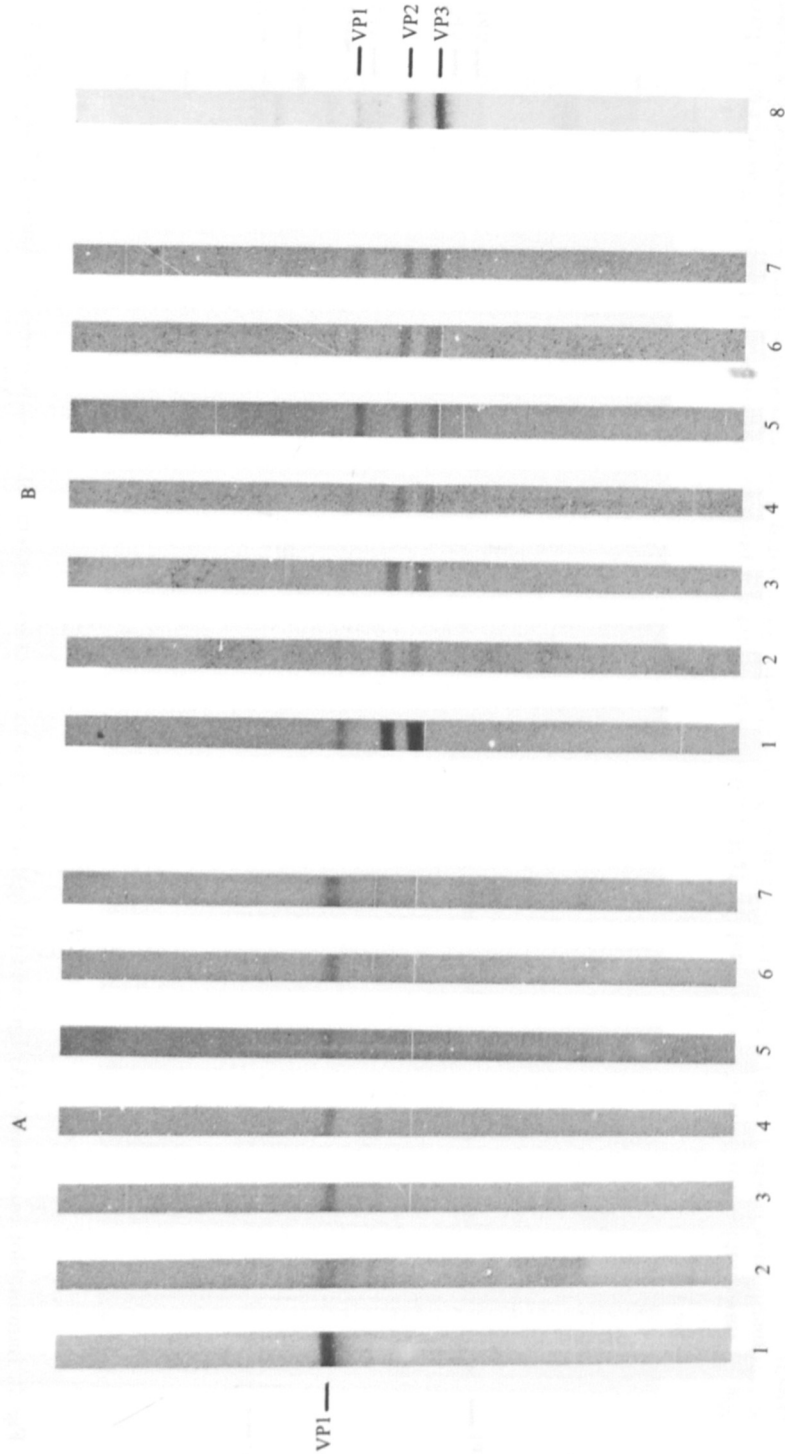
Fig. 4. Immunoblot reactions of (A) IgG and (B) IgM antibodies to structural proteins of poliovirus 2. Lanes 1–7 as in Fig. 1. Lane 8, [^{35}S]methionine-labelled viral proteins of poliovirus 2.
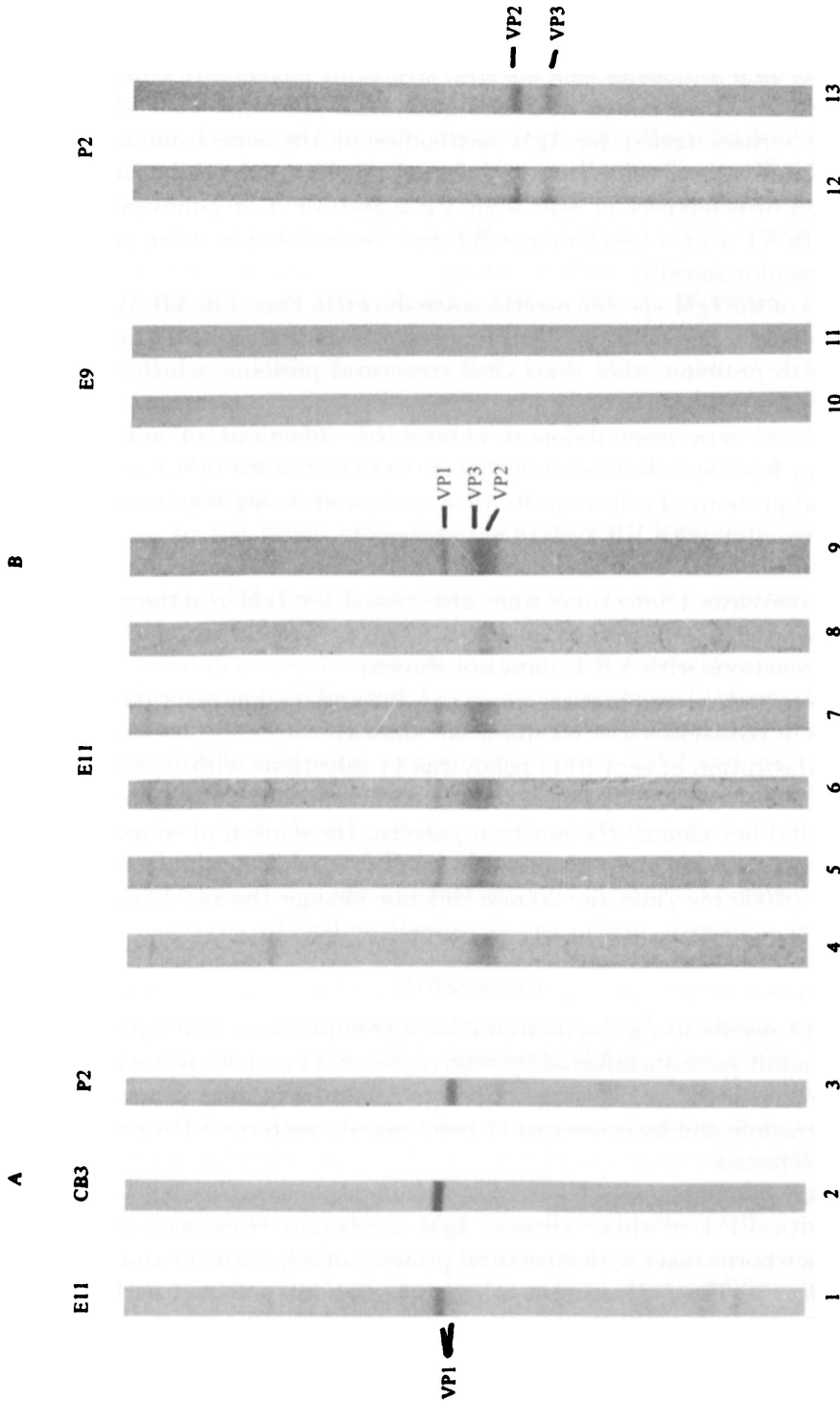
Fig. 5. Immunoblot reactions of (A) IgG and (B) IgM antibodies from sera of newborns to enteroviral structural proteins. Lanes 1–3, serum X1180 with echovirus 11 proteins (lane 1), coxsackievirus B3 proteins (lane 2), poliovirus 2 proteins (lane 3). Lanes 4–9, echovirus 11 proteins; lanes 10–11, echovirus 9 proteins; lanes 12–13, poliovirus 2 proteins. The strips are from different gel runs. Lane 4, serum X1180, day 0; lanes 5, 10, 12, serum X1180, day 12; lane 6, serum X1181, day 0; lane 7, serum X1180, day 40; lane 8, serum X1182, day 0; lanes 9, 11, 13, serum X1182, day 12.

complexes at pH 2·2. Preabsorption with intact poliovirus 2 did not change the reaction pattern (data not shown).

Reactions of IgM antibodies with the viral structural proteins. In contrast to the IgG reactions described above we found additional reactions with VP 2 and/or VP 3 of the viruses tested for IgM antibodies of the sera from adults. IgM antibodies from sera of echovirus 11 infected newborns showed reactions with VP 1, VP 2/3 of echovirus 11 and with VP 2 and VP 3 of poliovirus 2. Weak reactions with VP 1 of coxsackievirus B3 may be detected in some cases on the original strips (not shown).

The results of the IgM-specific reactions are shown in Figs. 1 B–5 B. We classified the reactions as strong and weak according to a visual reading of the colour chart. Reactions with proteins other than viral structural proteins, which are seen on some blots, have not been analysed.

Six additional sera from different enterovirus infections of adult persons, including sera from an echovirus 9 outbreak, were tested for IgM reactivity with the structural proteins of echovirus 9. All sera showed strong reactions with VP 3 and a weak reaction with VP 1 (data not shown).

Sera from echoviruses 9 and 30, coxsackievirus A9, coxsackieviruses B4 and B6, HAV, and poliovirus 1 infections were also tested for IgM reactions with viral proteins of poliovirus 2. In general, good reactions were found with VP 2 and VP 3 and weaker reactions with VP 1 (data not shown).

Other sera tested showed best reactions to VP 2 and weaker reactions with VP 1 and/or VP 3 of coxsackievirus B3 (data not shown).

After preabsorption of sera from echovirus 11 infections with intact echovirus 11, IgM reactions with VP 2/3 of echovirus 11 disappeared. Preabsorption with poliovirus 2 did not change the reaction pattern. Incubation of serum at pH 2·2 before testing strongly reduced the reactivity with VP 2/VP 3, while centrifugation of serum at 16000 rev./min for 20 min did not change the reactions (data not shown).

DISCUSSION

The present results using the immunoblot technique show that IgG antibodies from sera of adult patients infected by echoviruses 9, 11 and 30, polioviruses 1 and 2, coxsackieviruses B2 to B6, coxsackievirus A9 and hepatitis A as well as sera from newborns infected by echovirus 11 react mainly with the VP 1 protein of the enteroviruses tested.

In contrast, IgM antibodies from sera of adult persons react with VP 2 and/or VP 3 and with VP 1 of these viruses; IgM antibodies from sera of echovirus 11 infected newborns react with structural proteins of echovirus 11 and cross-react with poliovirus 2. The IgM content in sera of newborns infected with echovirus 11 (Table 2) was substantially increased compared to the amounts in normal newborn sera and reached the values found in sera of adults. In all sera tested, IgG-specific reactions with VP 1 of the viruses mentioned were found. This reactivity is explained by persisting IgG antibodies from earlier enterovirus infections or poliomyelitis vaccination. Reactions with VP 1 proteins found for IgG antibodies in sera, where we did not detect neutralizing activity to the corresponding virus, must be explained by cross-reactivity of the antibodies with epitopes of the

VP 1 proteins of the different enteroviruses. As may be expected, umbilical cord blood from healthy newborns also showed these reactions as a consequence of trans-placental transport of maternal enterovirus-specific IgG antibodies to the fetus. Early studies (Kraft & Melnick, 1952; Bussell *et al.* 1962; Schmidt *et al.* 1962*a*; Schmidt, Dennis & Lennette, 1962*b*; Schmidt, Magoffin & Lennette, 1973; and other investigators) showed type-specific as well as group-reactive determinants among enteroviruses. Recent studies show that important epitopes leading to neutralizing antibodies are located on VP 1, provided that the virus particles are intact. Heated or denatured virus particles show different degrees of cross-reactivities (Bittle *et al.* 1982; Emini, Ostapchuk & Wimmer, 1983; Emini, Jameson & Wimmer, 1983; Evans *et al.* 1983; Haresnape & MaCahon, 1983; Hasegawa & Inouye, 1983; Robertson *et al.* 1983; Wychowski *et al.* 1983; Thorpe *et al.* 1983). Our results indicate that group-reactive determinants detected by IgG antibodies are located on the VP 1 proteins of the tested viruses. Preabsorption of the sera with intact virus indicates that these epitopes are not located on the surface of the virus particles.

In addition to the broad cross-reactivity with VP 1 proteins shown for IgG antibodies in this study, we observed that IgM antibodies reacted on the immunoblot with VP 1, and/or VP 2 and/or VP 3. This is in contrast to published results (Dörries & ter Meulen, 1983; Mertens, Pika & Eggers, 1983). Moreover, IgM antibodies from different enterovirus infections cross-reacted to structural proteins of other enteroviruses. This may be in accordance with recent findings that enteroviruses contain a highly conserved region in the nucleotide sequence between bases 220 and 1809, where the entire polypeptide VP 2 and part of VP 3 are encoded (Rotbart, Levin & Villareal, 1984). Preabsorption experiments with intact virus and the lability of these reactions in sera pretreated by incubation at pH 2·2 indicate that IgM reactions to VP 2/VP 3 may be elicited by immune complexes in the sera. This, however, must be substantiated by more extensive experimental data. Cross-reactivity of IgM antibodies among enteroviruses has been described previously in other serological tests, namely complement-fixation, haemagglutination-inhibition, immunodiffusion, MACRIA, counterimmunoelectrophoresis or indirect solid-phase immunoassays. Type-specific IgM reactions were found by Schmidt, Magoffin & Lennette (1973) in their gel-diffusion technique in 80 % of all positive cases. Schilt (1977) showed for a few sera that IgM antibody-containing serum fractions gave type-specific results in neutralization tests and Torfason, Frisk & Diderholm (1984) reported type-specific reactions in a reverse IgM RIA. The degree of cross-reactivity may be explained by the particular presentation of the antigens in the different systems and, therefore, the results may not be directly comparable.

Studies of King *et al.* (1983) showed that sera from young children behaved most type-specifically in ELISA and that heterotypic responses increased with age. Our earlier (Burkhardt, Reigel & Schilt, 1983) and the present studies using sera from adult persons infected by different enteroviruses, from newborns (1–2 weeks old) infected by echovirus 11 and our control sera from umbilical cord blood support these findings.

The immunoblot technique used to detect enterovirus-specific IgM and IgG antibodies could be extended to give quantitative measurements of IgG and IgM

antibodies during infections and convalescence. It would also be interesting to see whether small peptides derived from the viral structural proteins, which were shown to induce neutralizing antibodies, also behave type-specifically on the immunoblot.

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