Coxsackie B virus-specific IgM antibody and myocardial infarction

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Summary. The ELISA technique was shown to be group-specific for the detection of IgM antibodies against coxsackie B viruses, and probably against a wider range of enteroviruses. No evidence was obtained that recent coxsackie B-virus infection predisposes to myocardial infarction.

Introduction

The coxsackie B viruses undoubtedly possess cardiotropic activity, and are important causes of myopericarditis in children and adults (Grist and Bell, 1974). It has been proposed that infection may also predispose to myocardial infarction but findings have been contradictory (Woods *et al.*, 1975; Grist and Bell, 1977; Nicholls and Thomas, 1977; Griffiths *et al.*, 1980; Nikoskelainen *et al.*, 1983). A major problem is that cardiac complications tend to present relatively late, when virus excretion has ceased and rising titres of antibody are no longer demonstrable. A raised level of neutralising antibody can persist for very long periods and this is an unreliable indicator of recent infection (Grist and Bell, 1974).

The use of the sensitive, indirect enzyme-linked immunosorbent assay (ELISA) technique for detecting coxsackie B virus-specific IgG antibodies has not proved helpful for the diagnosis of recent infection, largely because it recognises group-enteroviral antibody, which masks the type-specific antibody response (Hannington *et al.*, 1983). More recently, ELISA has been shown to be group-reactive for enterovirus IgM antibodies (Dorries and Ter Meulen, 1983; King *et al.*, 1983; Pugh, 1984; Morgan-Capner and McSorley, 1983). We have, therefore, re-examined 208 sera from patients with myocardial infarction and control patients, by the ELISA-IgM technique and by the type-specific IgM-neutralisation test, for evidence of recent coxsackie B-virus infection.

Materials and methods

Viruses and antigens

Coxsackie virus types B1–B5 were obtained from Dr G.

Cambridge, Department of Virology, Hammersmith Hospital, London. Stocks were prepared in continuous African green monkey kidney (VERO) cells and stored at -70°C . Purified viruses for use as antigens in ELISA, and the control antigen, were prepared as described previously (Hannington *et al.*, 1983).

Indirect ELISA for virus IgM

The ELISA technique was performed as described by Hannington *et al.* (1983). To achieve equal sensitivity in detecting antibody against all five viruses, the antigens were chessboard titrated against a human serum pool containing cross-reactive coxsackie B IgG antibody. The working dilution for each of the five virus antigens was that giving an absorbance reading of 1.0 with the serum pool; the dilutions were within the range 1 in 10 to 1 in 80, and the control antigen was used at a dilution of 1 in 10. The anti-human IgM conjugate was titrated in plates optimally coated with antigen and already incubated with selected sera, positive and negative for coxsackie B-virus IgM.

For routine screening, 0.3 ml of each serum specimen, diluted 1 in 100 in PBS containing Tween 20 0.05%, was incubated at 4°C overnight, in duplicate wells, in a microtitration plate, with each of the virus antigens and the control antigen. After washing, alkaline phosphatase-labelled anti-human IgM was added and incubated for 2 h at 37°C . This was followed by further washing and incubation with *p*-nitrophenyl phosphate for 30 min at 37°C . Each test included known positive and negative IgM serum controls, as well as serum-free controls. The mean reading for the serum-free control was subtracted from test readings to give the ELISA value for each serum specimen. The cut-off value for differentiating 'positive' from 'negative' results was calculated, in each test run, from the values obtained for all the test sera against the control antigen; it was defined as the mean absorbance value plus four standard deviations.

Sera that gave positive results for coxsackie B IgM were retested after absorption with heat-aggregated human IgG to remove possible IgM-rheumatoid factor antibody.

Sucrose gradient fractionation of serum

One ml of a 1 in 4 dilution of serum in PBS was layered on to a continuous 12.5–37.5% (w/v in PBS, pH 7.2) sucrose gradient and centrifuged for 24 h at 25 000 rpm in the Beckman SW41 rotor. Eleven 1-ml fractions were collected from each sample.

Neutralisation test

This was done as described previously (Griffiths *et al.*, 1980). Sucrose gradient fractions were tested in two-fold dilutions from 2 to 256.

Removal of rheumatoid factor

A 20- μ l sample of each serum was mixed thoroughly with 200 μ l of a 10% v/v suspension of heat-aggregated human IgG (Leinikki *et al.*, 1978) and incubated at room temperature for 1 h with frequent shaking. After centrifugation for 1 min in a Beckman microfuge, the supernate (1 in 10 dilution of the original serum) was removed for testing.

Epstein-Barr virus serology

Sera were examined for heterophile agglutinins by the Monospot slide test (Ortho Diagnostic Systems). Tests for specific IgM antibody were by indirect immunofluorescence, with acetone-fixed preparations of infected human lymphoblastoid cells (HR1K) (Gerber, 1974).

Results

Specificity of the indirect ELISA test for IgM

Serial serum specimens from two patients with recent coxsackie B virus infection, confirmed by virus isolation (coxsackievirus types B4 and B5, respectively), were generously supplied by Dr P. Morgan-Capner, King's College Hospital Medical School, London. In both patients seroconversion for IgM antibody was demonstrated by ELISA (table I) and the early serum specimens possessed IgM antibodies against all five coxsackie B virus antigens (types 1–5). Subsequently, antibody levels declined, at different rates for the different antigens. However, IgM against one or more of the virus antigens was detectable for 1 month in one patient and for 4 months in the other.

Coxsackie IgM antibody was not demonstrable in serum specimens known to contain IgM specific for rubella virus (six sera), cytomegalovirus (six sera), herpes simplex virus (six sera) or herpes zoster virus (three sera). On the other hand, three out of seven sera from cases of Paul Bunnell-positive infectious mononucleosis did give strong positive

Table I. Serial IgM-antibody responses detected by ELISA in two patients with recent coxsackie B-virus infections

Patient no.	Weeks after onset of illness	Serum ELISA-IgM absorbance readings* against coxsackie type					control antigen
		B1	B2	B3	B4	B5	
1†	1–2	0.15	0.17	0.15	0.15	0.18	0.06
	3	0.52	0.43	0.48	0.52	0.61	0.08
	4	0.45	0.31	0.38	0.36	0.49	0.07
	6	0.37	0.24	0.28	0.27	0.28	0.07
	9	0.31	0.24	0.24	0.28	0.27	0.06
	13	0.19	0.23	0.23	0.22	0.26	0.09
	16	0.19	0.20	0.20	0.23	0.27	0.07
2‡	1–2	0.10	0.13	0.15	0.12	0.17	0.07
	2	0.25	0.32	0.21	0.35	0.28	0.06
	3	0.22	0.28	0.20	0.30	0.28	0.08
	4	0.18	0.19	0.19	0.20	0.29	0.05
	6	0.16	0.18	0.17	0.15	0.20	0.06
	8	0.10	0.20	0.11	0.18	0.17	0.10
	14	0.10	0.20	0.13	0.12	0.17	0.08
	16	0.10	0.20	0.11	0.11	0.15	0.09
	28	0.10	0.15	0.17	0.09	0.13	0.08

* Positive readings, greater than 0.2, are in **bold** type.

† Coxsackievirus B4 isolated.

‡ Coxsackievirus B5 isolated.

reactions with all five coxsackie antigens, despite the fact that these sera had been processed to remove rheumatoid factor.

Sera from cases of myocardial infarction

Serum specimens had been collected from 105 patients with a clinical diagnosis of myocardial infarction and from 99 age- and sex-matched controls; the patients were described in a previous report (Griffiths *et al.*, 1980). The sera were stored at -20°C .

Twenty eight of the 204 sera (13.7%) gave positive results for IgM in the indirect-ELISA test (table II); none of the positive sera contained either heterophile antibody or EB virus-specific IgM antibody. Fourteen of the 28 sera were positive for IgM against all five coxsackie B-virus antigens, two were positive against four antigens, three against three antigens, one against two antigens and eight against only one antigen. The 14 sera that reacted with all five antigens gave much higher absorbance readings in the ELISA test than those that reacted with fewer. There was no obvious bias towards any particular coxsackie virus serotypes; 18 sera were positive against B1, 17 against B2, 18 against B3, 24 against B4 and 20 against B5.

Table II. Cross-reactions within 28 serum specimens positive for coxsackie B virus-specific ELISA-IgM

Number of serotype cross-reactions	Number of IgM positive sera	ELISA-IgM absorbance readings	
		mean	range
Five (B1-5)	14	0.60	(0.30-1.46)
Four	2	0.36	(0.30-0.46)
Three	3	0.34	(0.30-0.41)
Two	1	0.34	(0.31-0.34)
One	8	0.35	(0.31-0.41)

The 28 positive sera were then fractionated by sucrose gradient centrifugation, and the various fractions of each serum were tested for neutralising antibody against the five viruses (types B1-5); each fraction was also examined for the presence of virus-specific IgG and IgM by ELISA to confirm proper separation of the IgG and IgM antibody fractions. The 28 sera were, additionally, titrated whole for total neutralising antibody activity against each of the coxsackie virus serotypes. Table III illustrates the results obtained with 15 of the sera, including the 14 sera that give cross-reactions in the ELISA-IgM test against all five coxsackie

viruses. Only eight sera possessed detectable IgM neutralising-antibody activity and seven of these were among the 14 ELISA group-specific sera; the remaining serum (no. 54) showed IgM cross-reactions by ELISA only against three virus serotypes—B1, B3 and B5. Unlike the group-specific ELISA-IgM results, the IgM neutralising-antibody test proved to be type-specific.

The neutralisation test performed with whole serum does not, of course, differentiate between IgG and IgM antibody activity, but, in each of the eight IgM neutralising-antibody-positive sera, the highest titre of total neutralising antibody reflected the IgM neutralising-antibody specificity of the serum fractions. However, a high titre of total neutralising antibody did not necessarily specify the presence of IgM neutralising antibody. Thus, sera nos. 112, 307, 117, 329 and 350 had neutralising-antibody titres of 256-1024 but had no detectable IgM neutralising-antibody activity; several other sera, not shown in the table, also had neutralising antibody titres of 1024 or greater but were without demonstrable IgM neutralising-antibody activity.

Similarly, some sera with high levels of cross-reactive activity in the ELISA-IgM test (e.g., sera nos. 170 and 307) had no detectable IgM neutralising antibody. The possibility that this was due to broader reactivity of the ELISA-IgM test within the enterovirus group was examined by testing sera

Table III. Comparison of specific IgM neutralising- and total neutralising-antibody titres in 15 sera possessing coxsackie B-virus group-reactive ELISA-IgM antibodies

Serum no.	ELISA-IgM absorbance reading* against antigen type						Neutralising antibody titres†									
							specific IgM against antigen type					total antibody against antigen type				
	B1	B2	B3	B4	B5	control	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
134	1.04	0.78	1.46	0.86	1.29	0.19	—	32	—	—	—	32	> 1024	512	256	128
140	0.47	0.31	0.79	0.38	0.64	0.11	—	—	—	2	—	256	<8	<8	512	32
282	0.45	0.71	0.73	0.74	0.73	0.13	—	—	—	16	—	16	<8	<8	512	<8
221	0.50	0.56	0.53	0.53	0.67	0.13	—	—	—	16	—	16	<8	<8	> 1024	<8
303	0.46	0.63	0.63	0.64	0.67	0.06	—	—	—	64	—	128	<8	256	256	<8
136	0.48	0.44	0.30	0.57	0.45	0.16	—	2	—	—	—	16	512	64	128	<8
343	0.35	0.34	0.30	0.39	0.43	0.06	—	—	—	8	—	<8	32	64	512	<8
112	0.33	0.34	0.33	0.39	0.57	0.14	—	—	—	—	—	64	256	<8	<8	<8
170	0.70	0.65	0.81	0.81	1.05	0.17	—	—	—	—	—	128	32	<8	8	<8
307	0.90	0.88	1.27	1.23	1.28	0.15	—	—	—	—	—	<8	256	<8	> 1024	<8
117	0.30	0.33	0.36	0.42	0.30	0.11	—	—	—	—	—	512	32	8	128	256
329	0.37	0.41	0.35	0.58	0.37	0.09	—	—	—	—	—	16	256	<8	64	<8
350	0.40	0.55	0.48	0.53	0.61	0.09	—	—	—	—	—	8	<8	<8	512	<8
341	0.47	0.42	0.84	0.39	0.81	0.06	—	—	—	—	—	<8	<8	<8	16	<8
54	0.32	0.28	0.35	0.22	0.35	0.06	32	—	—	—	—	> 1024	32	<8	128	<8

* Positive readings are > 0.29.

† — = Reading of < 2.

from five patients with recent echovirus infections, confirmed by virus isolation (echovirus types 7, 7, 14, 17 and 25); four patients were positive for coxsackie B virus ELISA-IgM.

Association of coxsackie B virus IgM-antibody with myocardial infarction

IgM antibody, detected by ELISA, was present in 13 (12.3%) of the 105 patients with myocardial infarction and in 15 (15.1%) of the 99 matched controls; this difference is not significant.

Among the 14 sera previously found to be positive for ELISA-IgM against all five virus antigens, six were from myocardial infarction patients (5.7% of the 105 patients), and eight were from controls (8.1% of the 99 controls); the difference is not significant.

By the IgM neutralisation test for coxsackie B virus IgM, there were eight positive sera, two from infarction patients (1.9% of the 105 patients) and six from controls (6% of the 99 controls); the difference is not significant ($\chi^2 = 1.36$; $p > 0.1$).

Discussion

Solid-phase immunoassays for coxsackie B-virus IgM have generally proved unreliable for the detection of type-specific antibody. Monotypic responses may be detected in young children recently infected, but in older children and adults the response is broadly cross-reactive (Dörries and Ter Meulen, 1983; King *et al.*, 1983). In the present study on myocardial infarction, all the patients were over 31 years old (Griffiths *et al.*, 1980), and two thirds of the IgM-positive sera exhibited cross-reactions against two or more of the virus serotypes; 14 sera reacted with all five serotypes and also gave considerably stronger readings in the ELISA test, which probably indicates more recent infection. This conclusion is supported by the results obtained with the serial serum specimens from two patients in whom recent infection was confirmed by virus isolation. In both patients the early ELISA-IgM response was group-reactive against all five coxsackie B-virus serotypes but, subsequently, became progressively less so, until after some 16 weeks and 4 weeks, respectively, their sera reacted with only two serotypes and one serotype, respectively; the loss of broad group-reactivity was also associated with weaker absorbance readings in the ELISA test.

Information on the duration of the IgM response to coxsackie B-virus infection is still limited. Morgan-Capner and McSorley (1983) examined serial serum specimens from the same two recently

infected patients described above by IgM-capture radioimmunoassay, and detected specific IgM for at least 4 weeks. El-Hagrassy *et al.* (1980) and King *et al.* (1983) recorded positive results by ELISA-antibody capture for as long as 6–8 weeks and 12–24 weeks in a small number of patients.

There is now firm evidence that solid-phase ELISA and radioimmunoassays with coxsackie B-virus antigens detect IgM antibodies that are group-reactive within the enterovirus family, including echoviruses and coxsackie A viruses (King *et al.*, 1983; Morgan-Capner and McSorley, 1983; Pugh, 1984) and the present study provides additional confirmation. Thus five patients with confirmed echovirus infection had ELISA-IgM responses against the coxsackie B viruses. The quality of the antigen preparation appears to have little bearing on the specificity of those tests in which the antigen is allowed to adsorb directly on to a solid phase. Even purified virus prepared by treatment with sodium lauryl sulphate to remove provirions (Brown *et al.*, 1976) shows heterotypic activity when used under such conditions (Hannington *et al.*, 1983). On the other hand, Katze and Crowell (1980) have shown that the type-specificity of purified coxsackie B viruses is preserved when they attach to a solid phase previously coated with specific 'capture' antibody. That the IgM-antibody-capture technique can be made type-specific was confirmed by Torfason *et al.* (1984) with radiolabelled purified enteroviruses as antigens. However, the antigen dose was critical to avoid cross-reactions. For laboratory diagnostic purposes, a test for broadly cross-reactive enteroviral IgM would be of more practical value, to recognise recent infection with any of the very large number of enteroviruses. The actual infecting virus could then be identified by a more specific immunoassay, such as that described by Torfason *et al.* (1984), or, as in the present study, by fractionating the serum and testing for type-specific neutralising IgM.

An earlier study of the same patients with the standard neutralisation test failed to recognise any significant association between recent coxsackie B-virus infection and myocardial infarction (Griffiths *et al.*, 1980). This was so, also, in the present investigation, which used the transitory nature of the IgM response to diagnose recent infection. The IgM response is not, of course, an entirely reliable indicator of recent infection. El Hagrassy *et al.* (1980) and King *et al.* (1983) detected IgM in only 83–97.5% of cases of recent coxsackie B-virus infection that had been confirmed by virus isolation or a fourfold or greater rise in neutralising antibody levels. In the present study, five patients showed a

fourfold or greater increase in neutralising antibody titre, but only one of them was positive for virus-specific IgM by ELISA. However, even including the four IgM-negative individuals in the analysis made no difference to our findings.

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