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Immunoglobulin M Capture Immunoassay in Investigation of Coxsackievirus B5 and B6 Outbreaks in South Australia

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An immunoglobulin M (IgM) capture enzyme immunoassay was used to detect major overlapping outbreaks of disease in South Australia caused by coxsackieviruses B5 (CBV-5) and B6 (CBV-6). CBV-5-specific IgM was detected in patients presenting in spring 1992 with acute febrile illnesses, rash, severe acute respiratory disease, meningitis, myocarditis and/or pericarditis, while tests for other viruses were negative. CBV-5 was isolated from an early case. In December 1992 it was noted that CBV-6 had replaced CBV-5 as the major cause of disease. The CBV-6 epidemic continued until April 1993. Serum samples from 495 patients (276 inpatients) were submitted for testing. CBV-6 infection was associated with lower respiratory tract infection and persistent cough. This study demonstrated success of the IgM enzyme immunoassay and the need for diagnostic virology laboratories to look for CBV-6 infection in addition to the other five CBVs.

Nonpoliovirus enteroviruses are a major cause of acute symptomatic illness. These viruses are responsible for an estimated 5 to 10 million cases annually in the United States. Most of these infections occur in the summer months (11). Reports from the Australian Federal Infectious Diseases Surveillance Centre (Communicable Disease Intelligence) utilize data based on isolations of viruses in the states and territories of Australia. The low numbers of coxsackie B virus (CBV) isolations reported presumably do not reflect the true epidemiology of these viruses. The diagnosis of nonpolio enteroviral illness is difficult on clinical grounds owing to the variability of severity of illness and the spectrum of presentations (7, 8). Furthermore, laboratory diagnosis by virus isolation or increasing neutralizing antibody titers demands timely collection of specimens. These tests do not lend themselves to large numbers of specimens; are labor-intensive, time-consuming, and costly; and do not provide timely results (2). Detection of CBV-specific immunoglobulin M (IgM) antibody, by contrast, is relatively rapid, and the timing of blood collection is not critical because IgM antibody is detectable, in most cases, from the time of presentation and persists for several months (1). This article describes the use of an IgM capture immunoassay to detect CBV infection during overlapping CBV B5 (CBV-5) and B6 (CBV-6) epidemics. The CBV-5 epidemic was reported previously (3).

MATERIALS AND METHODS

The IgM immunoassay was based on that of King et al. (5) with the same reagents except for the substitution of lamb serum by human serum albumin. CBV-1, CBV-2, CBV-3, CBV-4, CBV-5, and CBV-6 antigens (complement fixation test antigens; Whittaker Bioproducts, Walkersville, Md.) were diluted 1 in 20 in phosphate-buffered saline with 0.5% Tween 20 and 25% human serum albumin. Sera were screened with pooled CBV-6 antigens. A specific test (performed on screen-positive sera) utilized 50 μl of a 1 in 20 dilution of each antigen. The method described by Muir (9) was used to set the lower (negative) cutoff at 4 standard deviations above the mean value of sera negative for CBV IgM. The upper (positive) cutoff was set at 8 standard deviations above the same mean. Positive-control sera heterotypically and homotypically reactive for IgM were obtained from cases of CBV-1, CBV-4, and CBV-5 infection proven by virus isolation. These and negative sera were divided into aliquots and frozen to avoid changes in IgM reactivity that may occur on freeze-thawing. IgM responses to CBV infection were divided into two groups: homotypic, defined as a monotypic IgM response to a specific CBV, and heterotypic, defined as an IgM response to two or more CBVs. In some instances of heterotypic response, the optical density was higher for one CBV than the others and allowed a presumptive diagnosis of that virus infection. Sensitivity was assessed by testing sera in 10-fold dilutions (up to 1 in 100,000), and specificity was assessed by testing a variety of sera positive for IgM to other agents including Epstein-Barr virus, the viruses causing measles, mumps, and hepatitis A, and *Mycoplasma pneumoniae* and sera positive for rheumatoid factor. The IgM assay was used to diagnose infection in adults and children in South Australia and adjacent states who became unwell with an unusually broad range of clinical illnesses manifest by involvement of several different organ systems including the respiratory tract, gastrointestinal tract, central nervous system, heart, skeletal muscle, and skin (rashes). Patient information was obtained from laboratory test request forms and from case notes of pediatric patients (<18 years old) admitted to the Women's and Children's Hospital (WCH). Sera were received from patients at the WCH and other hospitals in the Adelaide metropolitan area (population, 1,049,800) and other areas of South Australia (population, 409,822) as well as adjacent states (Victoria, New South Wales, and Northern Territory) between 1 August 1992 and 30 April 1993. In addition, serum samples were sent by general practitioners in all of these areas (through private laboratories). In many cases, the onset date was not provided. Virus isolation was performed by the Division of Virology, Institute of Medical and Veterinary Science, on specimens of cerebrospinal fluid (CSF) (11 specimens), feces (14 specimens), and nasopharyngeal aspirates and/or throat swabs (15 specimens) from 11 patients admitted to the WCH. Only A549 cells (a human lung carcinoma cell line) were used (without blind passage).

RESULTS

CBV-5 was isolated from the CSF of a 1.8-year-old girl with meningitis in September 1992. Other enteroviruses were isolated occasionally. These enteroviruses included coxsackievirus A9 from the feces of a 5-week-old girl with meningitis in August 1992, echovirus 9 from the CSF of a 7-year-old girl with meningoencephalitis in December 1992, and CBV-1 from the feces of a 2.7-year-old girl with meningitis in March 1993.

Serum samples were received from a total of 495 patients of whom 276 were hospital inpatients. Diagnosis of CBV infection was based on the results of CBV-specific IgM enzyme immunoassay (EIA) testing of sera. In the two cases from which a CBV was isolated, the corresponding virus-specific IgM was also detected as homotypic responses. In the case of the coxsackievirus A9 infection, a heterotypic IgM response was observed. CBV-specific IgM was not detected in the case of echovirus 9 meningoencephalitis, diagnosed by isolation of

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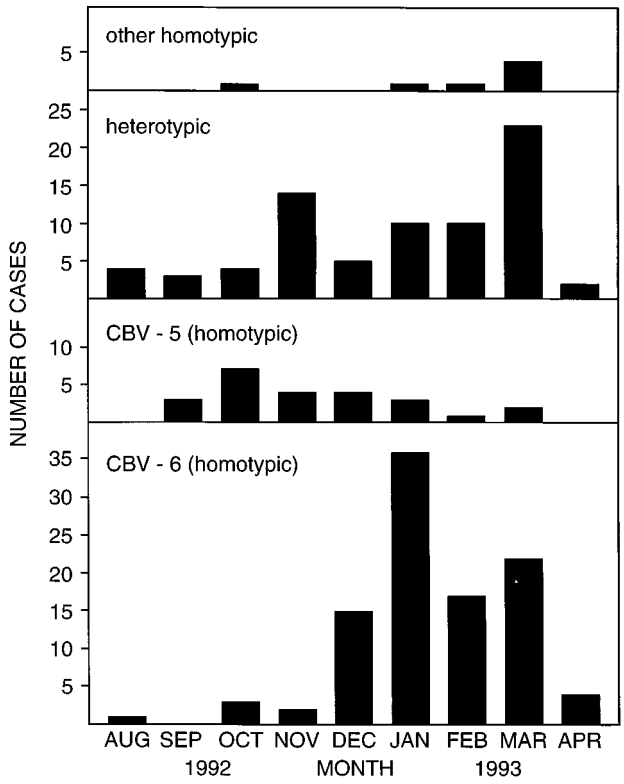

FIG. 1. Epidemic pattern of virological diagnoses based on homotypic and heterotypic coxsackie B virus IgM responses according to month of presentation in 1992 and 1993.

the virus from the CSF. There were 100 cases of CBV-6 and 24 cases of CBV-5 infection based on homotypic IgM antibody responses. Heterotypic responses (positive CBV-specific IgM to two or more of the six CBV subtypes) were observed in 74 patients. Homotypic responses to CBV-1 (one case), CBV-2 (two cases), CBV-3 (one case), and CBV-4 (three cases) were also recorded. Of the 205 pediatric and adult patients diagnosed as having CBV infection on the basis of homotypic or heterotypic IgM responses, 131 (63.9%) were admitted to a hospital as a result of their illness. For patients with negative CBV IgM responses, 145 of 290 (50%) were admitted to a hospital, 68 pediatric patients to the WCH. Serum samples positive for IgM to viruses including Epstein-Barr virus (5 samples), herpes simplex virus (1 sample), and the viruses causing measles (10 samples), mumps (1 sample), and hepatitis A (1 sample) were negative by the CBV IgM EIA. There were 20 serum samples positive in the CBV IgM EIA that were weakly positive for *M. pneumoniae* IgM. In addition, 23 serum samples positive for CBV IgM were negative for *M. pneumoniae* IgM EIA.

Figure 1 shows the virological diagnoses (homotypic and heterotypic IgM responses) according to the date of serum collection (the month of presentation). Table 1 shows the major clinical manifestations according to virus diagnosis. Because no significant differences in age and sex could be demonstrated between CBV-5 and CBV-6 infections and specific clinical presentations, these data are not presented. Table 2 provides an age-specific breakdown of homotypic and heterotypic IgM responses to CBV infection.

The clinical picture of pediatric patients admitted to the WCH with CBV-5 infection was either acute meningitis (associated with fever) or sometimes a morbilliform rash. On the other hand, CBV-6 infection more often presented as a severe febrile illness with cough and morbilliform rash. Diffuse or patchy pneumonia was frequently seen. A persistent cough was a frequent manifestation of CBV-6 infection. CBV-6-associated pneumonia or pneumonitis based on homotypic responses was observed in 27 of 100 (27%) of patients, and a similar value (6 of 24 [25%]) was observed for CBV-5. Meningitis occurred in 7 of 24 (29.2%) of CBV-5 infections but was seen in only 2 of 100 CBV-6 infections. This difference was significant ($P = 0.005$, Fisher's exact test). Of the 13 cases of meningitis observed, only 2 were attributable to CBV-6 and 7 were attributable to CBV-5 on the basis of homotypic IgM responses. Myocardial involvement was more common in CBV-6 infection (six cases) than CBV-5 infection (one case). A persistent cough lasting several weeks was a characteristic of this CBV-6 epidemic. Data on the duration from onset until the time of collection of serum were available on 75 pediatric and adult patients positive for CBV IgM and 68 patients negative for CBV IgM, and the durations were 19.6 and 27.8 days, respectively ($P = 0.28$). Of the 131 pediatric and adult patients hospitalized because of CBV disease, data on the length of stay were available for 66 children; the mean duration of hospitalization was 6.9 days (±7.2; median, 4 days; range, 1 to 44 days), and there was no difference between the length of stay for CBV-5 and CBV-6 infection. By contrast, the mean length of hospital stay of the 68 pediatric patients admitted to hospital with CBV IgM-negative illness was 5.6 days (±8.3; median, 2 days; range, 1 to 47 days).

TABLE 1. Major clinical manifestations according to virus diagnosis

Clinical manifestation	No. of patients with IgM response to the following virus or with the following result:								
	CBV-5	CBV-6	Heterotypic	CBV-1	CBV-2	CBV-3	CBV-4	Total positive	Negative IgM
Major signs									
Pneumonia	6	27	7		1			41	30
Rash	8	20	12	1			1	42	45
In cardiac system	1	6[a]	11					18	33
In central nervous system	8[b]	2[b]	4					14	13
In joints	1	8	4		1			14	22
Major symptoms									
Cough	2	22	14				1	39	39
No clinical information	1	4	5					10	16

[a] Five of the six were female.
[b] $P = 0.005$ by Fisher's exact test.

TABLE 2. Homotypic and heterotypic CBV IgM responses according to age

Age (yr)	No. of patients with IgM response to:			Total no. (%) of patients with IgM response		Total no. in group
	CBV-5	CBV-6	Other CBVs	Homotypic	Heterotypic	
<1	3	7	1	11 (84.6)	2 (15.4)	13
1–4	8	23	1	32 (66.6)	16 (33.3)	48
5–9	4	20	0	24 (68.5)	11 (31.4)	35
10–19	4	19	2	25 (71.4)	10 (28.5)	35
20+	5	28	3	36 (50.7)	35 (49.3)	71
Total	24	97[a]	7	128 (63.4)	74 (36.6)	202[a]

[a] Age unknown in three cases of CBV-6 infection, thus there were a total of 100 CBV-6-positive responses and a total of 205 CBV-positive responses.

DISCUSSION

The low virus isolation rate observed during the epidemics reported in this article can be explained by a combination of low rates of specimen submission and the difficulty in growing these viruses without the use of primary monkey kidney cells utilizing blind passage. Only one cell line (A549) was used. Some enteroviruses do not replicate in cultured cells or do not produce a cytopathic effect. Noninfectious virus-antibody complexes and cessation of virus shedding by the patient may also account for failure in virus isolation. The difficulties in virus isolation emphasize the importance of other alternative diagnostic tools such as virus-specific IgM and PCR-based viral nucleic acid detection, both being relatively rapid and sensitive. Without timely collection of material (e.g., nasopharyngeal swab or feces) from patients, the latter option is not viable.

The specificity of the IgM assay in detecting CBV infections is supported by the fact that only five homotypic responses to CBV-1 to CBV-4 during the epidemic period were detected, and in nonepidemic years over 600 consecutive serum samples were negative for CBV IgM (data not shown). In addition, sera positive for IgM to unrelated viruses including Epstein-Barr virus, herpes simplex virus, and viruses causing measles, mumps, and hepatitis A were negative by the CBV IgM EIA. The observed weak cross-reactivity of some CBV IgM-positive sera in *M. pneumoniae* IgM EIAs has been previously reported (12).

The IgM capture EIA used showed some cross-reactivity with other enteroviruses in that it detected IgM antibody produced in a coxsackievirus A9 infection, but it failed to detect an echovirus 9 infection. Such cross-reactivity is well-known (9, 10) and may be a manifestation of a heterotypic response to enterovirus group antigens which may, in some cases, be an anamnestic response directed against such antigens encountered previously (9). It is conceivable that some positive CBV IgM responses may relate to infections acquired some months previously, but clinical presentations were entirely compatible with an enteroviral infection and tests for other agents were generally negative.

It is possible that a proportion of the patients negative for CBV IgM may have been tested too early in their illness or that the test was falsely negative for other reasons. It was apparent that there were some differences between the CBV IgM-positive and -negative patient groups (e.g., age distribution and length of hospitalization) which could reflect a true difference in the causes of their illnesses. In particular, a smaller number of children aged 1 to <2 years were seen in the CBV IgM-negative group (data not shown). Moreover, the final diagnoses of CBV IgM-negative children admitted to hospital were defined in 51 of 68 cases. A nonspecific fever and respiratory tract infection with or without rash was seen in the remainder. It is possible that the increasing awareness among medical practitioners of the availability of the CBV IgM EIA at the WCH could have led to a broadening of the indications for the test.

It is well-known that enteroviruses show tissue tropism and this may be seen as an association between a specific virus and a particular disease entity (6). CBV-5 is known to be associated with rash, pneumonia, and hepatitis as well as cardiac disease. On the other hand, CBV-6 is more often associated with undifferentiated febrile illness and meningitis (4). Differences in clinical manifestations were seen to occur during the Adelaide outbreaks caused by these two CBVs. It was noteworthy that diagnosis of CBV-6-associated pneumonia or pneumonitis based on homotypic responses was observed in 27% of patients and that a similar value (25%) was observed for CBV-5. Meningitis occurred in 29.2% of CBV-5 infections but was seen in only 2% of CBV-6 infections. This is a significant difference. Comparison of signs and symptoms is subject to bias in terms of the passively acquired data offered by physicians requesting the CBV IgM assay. Nevertheless, the observed preponderance of lung involvement caused by CBV-6 (confirmed by review of case notes of hospitalized patients) suggests a widening in the clinical spectrum of disease caused by this virus. No previous report of this degree of pulmonary involvement with this virus can be found. A persistent cough was a frequent manifestation of CBV-6 infection. Meningitis was less commonly associated with CBV-6 than CBV-5 infection, as measured by homotypic IgM response. It was possible that the four heterotypic IgM responses were caused by either of these viruses. Overall, most heterotypic responses corresponded more or less with the peak of the CBV-6 epidemic, and it is reasonable to assume that most were true CBV-6 infections. Indeed, examination of the individual subtype heterotypic responses showed that most occurred in 1993 during the CBV-6 epidemic. On the other hand, most (86.4%) of the heterotypic responses contained IgM antibody to both CBV-5 and CBV-6. Further separation of the responses is possible by comparison of absorbance ratios, but these data have not been presented because they add little to the overall findings. In keeping with other studies (5), homotypic IgM responses predominated in those under 20 years (and especially infants) and those in this age group represented 82.2% of all patients.

The mean hospital length of stay for children admitted with CBV disease was 1 week, and by comparison, that for patients with CBV IgM-negative disease was 5.8 days. These values provide some indication of the severity of illness caused by CBV-5 and CBV-6.

In conclusion, the CBV IgM EIA was shown to be useful in enabling detection of two large outbreaks of disease that would otherwise have passed without attributing causes. In addition, the inclusion of CBV-6 antigen in the assay provided additional valuable information. Diagnostic virology laboratories would be encouraged to follow suit.

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