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EDITORIAL

Tests for coxsackie B virus-specific IgM

During the last 10–15 years there has been a relative decline in virus isolation procedures and an increasing emphasis on both direct antigen detection by immunological methods and serological tests for the diagnosis of recent virus infections. This relative decline has been due to a number of factors. There have been increasing difficulties in maintaining a suitably wide range of cell culture systems for routine diagnostic purposes. There has been increasing dissatisfaction with the time taken to isolate and identify a virus in cell culture and, finally, cell culture systems for the isolation of many of the more recently recognized human pathogens have not yet been developed.

The change in emphasis to serology is even present for the diagnosis of infection due to enteroviruses, a group for which virus isolation has been the cornerstone of diagnosis for many years. Virus isolation has proved satisfactory in many instances for the diagnosis of acute enteroviral infection including meningitis and severe neonatal disease. Serological tests, notably neutralizing antibody studies on the isolated virus, had their place. They could be used to add weight to the proposed aetiological role of a virus, particularly when the virus was isolated from a system such as the gastro-intestinal tract which was not involved in the main clinical symptoms and signs of the patient. However, there were other syndromes of disease (e.g. myocarditis and pericarditis) which came to be regarded as relatively late manifestations of enteroviral infection. Thus patients would present for investigation at a time when virus was no longer being shed and therefore diagnosis by virus isolation was impossible. Furthermore, diagnosis by demonstrating rising titres was not possible since the first specimen of serum would be taken at a time when antibody levels were already elevated. Thus a virus–disease association was based upon finding elevated amounts of neutralizing antibody or amounts which declined over the succeeding months. This was plainly unsatisfactory, so attention was turned to the possibility of detecting enterovirus-specific IgM in order to diagnose recent infection. Work has centred round the group B coxsackie viruses (CBVs) since there are only a limited number of serotypes and, with the decline of poliovirus infection in developed countries, it is these enteroviruses which have been associated with more serious diseases of man, e.g. myocarditis and pericarditis. There also remains the interesting question of the role of CBVs in juvenile-onset diabetes. In this issue of the *Journal* there is a further contribution to the field (Morgan-Capner & McSorley, 1983) which again illustrates the difficulties of devising tests for specific IgM for individual coxsackie B viruses which are sensitive, specific and suitable for widespread use.

The first test for CBV-specific IgM to be extensively evaluated was based on an immunodiffusion technique (Schmidt & Lennette, 1962; Schmidt, Magoffin & Lennette, 1973). Using high-titre antigen preparations containing a mixture of

intact virions and empty capsids, two distinct lines of precipitation were obtained, one being IgM antibody precipitating intact virions and the other IgG antibody reacting with empty capsids. Initial studies (Schmidt & Lennette, 1962), showed that this technique could be used for the detection of specific IgM to all CBVs except CBV-2. Specific IgM was present in the sera of 77 of 79 patients from whom CBV-1, 3, 4, 5 or 6 had been isolated. It was noted that not all specific IgM antibody was homotypic for the virus isolated but that heterotypic reactions occurred in 22% of patients. There was no strict control group in this study but CBV-specific IgM was found in 25% of 86 patients with CNS disease from whom CBVs had not been isolated. Subsequently, the test was used to detect the presence of CBV-specific IgM in a group of 259 patients with pericarditis (148 cases), myocarditis (92 cases) and pleurodynia (19 cases) and a group of 259 control patients with a diagnosis of viral or mycoplasma pneumonia (Schmidt *et al.* 1973). Virus-specific IgM antibody was detected in 27% of patients with pericarditis, 25% of patients with myocarditis and 37% of those with pleurodynia. Virus-specific IgM was also found in 21 (8%) of the 259 controls, but on closer scrutiny of the clinical records it was noted that six of these had evidence of cardiac or central nervous system disease. Specific IgM antibody was not detected in any specimen from a patient in the test group that was taken more than 42 days after the onset of illness. Thus the test appeared to have a sensitivity and specificity that would make it useful for the diagnosis of recent coxsackie B virus infection. Again the authors noted that some sera contained IgM antibody that reacted with more than one CBV. Of the 70 IgM-positive sera in the test group 56 (80%) gave a positive result with only one CBV; 11 (16%) gave a positive result with two virus types; one serum gave a positive result with three, another with four and another with five types. Even allowing for this cross-reactivity, the requirement to produce high-titre antigens and the fact that a different method had to be used for IgM antibody to coxsackie B2 virus, it is surprising that the immunodiffusion test for coxsackie B virus antibody was not more widely used.

Subsequently Minor *et al.* (1979) utilized the same basic principle of two distinct precipitin lines in a counter-immunoelectrophoresis test with a view to devising a more sensitive and more rapid technique. Again concentrated antigen preparations were required and it was found necessary to use recent coxsackie B virus isolates rather than prototype strains which gave inconsistent results. The test group consisted of 29 patients from whom CBVs had been isolated and who exhibited an $\geqslant$ 4-fold rise in neutralizing antibody titre to the isolated virus. Twenty-two of these patients were infected with CBV 1, 3, 4 or 5, and 20 (91%) of these were positive for IgM antibody; 19 had IgM antibody to the infecting serotype and one patient infected with coxsackie B5 virus had heterologous IgM antibody only. Again problems of detecting IgM antibody to coxsackie B2 virus were demonstrated; only three of seven patients infected with this serotype were positive and one of the three exhibited a heterologous reaction only. Overall of the 23 patients with detectable IgM antibody 14 (61%) were positive for IgM antibody to a CBV other than the one with which they had recently been infected. The control group investigated by Minor and colleagues consisted of 22 patients from whom coxsackie A or echoviruses had been isolated and 50 patients from whom a non-enterovirus had been isolated. Two (9%) of the former (both echovirus type

6 infections) and one (2 %) of the latter (an influenza A infection) had IgM antibody to a single CBV serotype.

The two studies described above suggested that detection of CBV-specific IgM was a useful diagnostic test. Over 90 % of patients with a recent CBV infection had detectable CBV-specific IgM, although the occurrence of heterologous reactions made it difficult to predict accurately the infecting serotype. By contrast, less than 10 % of sera from patients with no evidence of recent infection with CBV had detectable specific IgM antibody.

The detection of coxsackie B virus IgM next enters the era of solid-phase immunoassays. El-Hagrassy, Banatvala & Coltart (1980) described such an assay based on the antibody-capture principle with the enzyme-labelled final indicator antibody. The antigen used in the assay was a pool of coxsackie B 1–5 antigens and each sample was also tested against a control antigen prepared from uninfected vero cell cultures. The reading for each sample was taken as the difference between the readings obtained with the test and control antigens. Three positive and three negative control sera were included in each test and any test sample with an absorbance of more than twice the mean negative control value was considered positive. Sera from 12 patients in whom a recent CBV infection had been confirmed by virus isolation and/or an $\geqslant$ 4-fold rise in neutralizing antibody titre were tested and 10 (83 %) of these patients had detectable CBV-specific IgM antibody. A further 10 sera shown to contain CBV-specific IgM by neutralization tests on IgM fractions obtained by sucrose density-gradient centrifugation were all positive by the ELISA test. As a control group 46 patients with recent infections other than CBV (*M. pneumoniae* 12, influenza A4, influenza B8, *Chlamydia psittaci* 5, adenovirus 4, parainfluenza viruses 3, cytomegalovirus 3, poliovirus 2, herpes simplex virus 3 and varicella-zoster virus 2) were tested. None of them gave a reaction in the ELISA test for CBV-specific IgM. In addition to the above results on positive and negative control groups the authors found positive results in approximately 37 % of patients with cardiologically confirmed acute myocarditis (64 patients) and acute pericarditis (38 patients) and in patients with a clinical diagnosis of Bornholm disease (57) or aseptic meningitis (8). Lower rates of positivity were found in acute ischaemic heart disease (13 % of 30 patients), congestive cardiomyopathy (7 % of 28 patients) and chronic heart valve disease (none of 39 patients). Although only four patients with juvenile-onset diabetes were tested, two were positive. The authors elect to use a polyvalent antigen in order to diagnose recent CBV infection rather than infection with a specific serotype. Bearing this in mind their results are very much in accord with those of Schmidt, Magoffin & Lennette (1973) and Minor *et al.* (1979) with respect to patients with confirmed CBV infection and those with myocarditis, pericarditis and pleurodynia. The test seems highly specific in that no positive result was found with sera from patients with infections other than those due to CBV, although it may be significant that only two of the 46 were due to enteroviruses (both poliovirus infections). CBV-specific IgM was found to persist for 6–8 weeks after the onset of symptoms in those 10 patients from whom sequential samples were available. Nevertheless, the sensitivity appears only moderate since the screening dilution was 1 in 100 and 70 % of positive sera had a titre of 400 or less.

An indirect solid-phase immunoassay has been described by Dörries & ter Meulen

(1980, 1983). Viral antigen is adsorbed to the wells of microtitre plates and reacted with test serum dilutions. Binding of specific IgM is then determined by reaction with a labelled anti-human IgM. In their initial description of the detection of enterovirus-specific IgM using this technique Dörries & ter Meulen (1980) used a radiolabelled indicator antibody but changed to an enzyme label when investigating the specificity of the IgM antibodies in acute coxsackie B virus infections (Dörries & ter Meulen, 1983). In the latter test dilutions of serum are reacted with viral and control antigen and a net absorbance value calculated. A positive result is indicated by a net absorbance value of $\geqslant 0{\cdot}25$, this being the mean plus three standard deviations (SDs) of a 1 in 100 dilution of 40 sera from healthy newborn children. To investigate the specificity of the IgM response Dörries & ter Meulen (1983) tested sera from patients with meningoencephalitis in whom a recent CBV infection had been confirmed by virus isolation and a significant change in neutralizing antibody titre. By testing these sera for IgM antibody to each of the CBVs 1–5 the authors were able to define three patterns of response, homotypic, type-predominant and heterotypic. In the homotypic pattern sera gave a positive reaction with only one of the five viral antigens and this was of the same serotype as the virus isolated from the patient. In the type-predominant pattern there was a strongly positive reaction with antigen equivalent to the isolated virus with weakly positive reactions to the other antigens. In the heterotypic pattern moderate to strongly positive reactions were found with all five antigens and the most strongly positive reaction was not necessarily with the antigen equivalent to the virus isolated from the patient. The authors further investigated the problem of homotypic and heterotypic reactions by analysing the virus polypeptides present in their antigen preparations. The antigens were found to consist of intact infectious virus and incomplete provirions on the basis of the presence of large amounts of VP0, VP1 and VP3 and small amounts of VP2 and VP4 after sucrose density-gradient centrifugation and SDS–polyacrylamide gel electrophoresis. Reaction of these separated polypeptides with sera exhibiting a heterotypic or a homotypic response indicated that both the type- and group-specific antigenic determinants were located on VP1. These results are very much in agreement with those of Katze & Crowell (1980*a*, *b*), who investigated the homologous and heterologous antigenic reactivity of CBV using IgG antibody in an ELISA-based immunosorbent assay. They found that a type-specific antigen/antibody reaction was dependent upon the use of native, purified virions. Disruption by urea or even absorption of virus on microtitre wells revealed antigen determinants with group reactivity. This latter phenomenon necessitates the use of a sandwich immunoassay to ensure type specificity of the reaction. Interestingly this group reactivity was confined within the CBVs and did not extend to coxsackie A2, poliovirus type 2, echovirus type 6 or human rhinovirus 2. By contrast, the heterotypic response described by Dörries & ter Meulen (1983) was demonstrated with the VP1 of all five CBVs and the serum from a patient with an acute cosackie A-9 infection. This together with the two CBV IgM-reactive sera from cases of echovirus 6 infection described by Minor *et al.* (1979) are the only previously documented cross-reactions in CBV IgM tests involving infections among the enterovirus genus as a whole. However, in this issue Morgan-Capner & McSorley (1983) produce more extensive evidence for this.

The basis of their test is again the antibody-capture principle with, in this instance, a radiolabelled final indicator antibody. The authors (Morgan-Capner & McSorley, 1983) describe assays for CBV 4 and CBV 5 IgM antibody and set strict criteria for a positive reaction. Only sera giving a reaction greater than the mean plus three SDs of 100 adult sera are considered positive. This value is equivalent to a T:N ratio of 2·5 for the CBV 4 assay and 2·9 for the CBV 5 assay. In spite of this high threshold value all sera taken during the early convalescent period from patients from whom CBV 4 (4 cases) or CBV 5 (6 cases) was isolated were positive in the homotypic assay. As expected, heterotypic responses were also found in early convalescent sera from cases of CBV infection. Sera from 10 of 20 cases of CBV 1, 2, 3, 5 or 6 infection reacted in the CBV 4 IgM assay and sera from seven (39 %) of 18 CBV 1, 2, 3, 4 and 6 infections reacted in the CBV 5 assay. Moreover, early convalescent sera from 9 (69 %) of 13 cases from whom coxsackie A or echoviruses had been isolated gave a positive reaction in both the CBV 4 and CBV 5 IgM assays. However, positive reactions were not found with sera from 41 cases of infection due to viruses other than enteroviruses or five cases of *Mycoplasma pneumoniae* infection.

Thus it is clear that further work is required to develop IgM antibody tests which are specific for a single CBV or even the group as a whole. At present there is general agreement that positive reactions in CBV IgM tests do not occur as a consequence of virus infections other than those due to enteroviruses. Of the combined total of 142 sera from such infections examined by Minor *et al.* (1979), El-Hagrassy *et al.* (1980) and Morgan-Capner & McSorley (1983), only one (from a case of influenza A infection, Minor *et al.* 1979) was found to be positive. Sera from six other cases of influenza A were tested by the other workers and none was positive. The more likely explanation of the case of Minor *et al.* (1979) is that the individual had a CBV infection shortly before the confirmed influenza A infection rather than that CBV-reactive IgM is produced as a consequence of some influenza A infections.

Conclusions about the occurrence of IgM antibody which reacts with CBVs after infection with other enteroviruses are more difficult to reach on the basis of present data. The experiments of Dörries & ter Meulen (1983) show that IgM antibody which reacts with the VP1 of CBV 1–5 may be present in sera from patients who have had a recent confirmed coxsackie A infection. The data of Morgan-Capner & McSorley (1983) suggests that such antibody is found in nearly 70 % (9 of 13) of patients with recent coxsackie A or echovirus infection. By contrast Minor *et al.* (1979) find the frequency to by 9 % (2 of 22) in a similar group.

There are two possible explanations for this difference. In both the immunodiffusion and counterimmunoelectrophoresis tests two distinct lines of precipitate are formed, implying a degree of antigen separation during passage through the gels at least with respect to the point at which optimum concentrations occur for precipitation with IgM or IgG antibody. IgM antibody reacts with intact virus particles, and group-specific antigens are less likely to be present on such intact particles than incomplete or disrupted particles. Thus a greater specificity of IgM antibody reaction may be anticipated in precipitation-in-gel tests. However, heterotypic reactions within the CBV group are found with these tests, and this implies that group-reactive antigens are exposed. Moreover the work of Katze & Crowell (1980*b*) on the reactivity of IgG antibody with enterovirus antigens

indicates that the group reactivity of urea-disrupted viruses does not extend to coxsackie A, echo- or rhinoviruses. It appears therefore that IgM produced after enterovirus infection in man has a broader cross-reactivity than the group-specific reactions defined by Katze & Crowell (1980*b*). The alternative explanation of the differences between the results of Minor *et al.* (1969) and Morgan-Capner & McSorley (1983) may lie in the difference in sensitivity of the two tests. Minor *et al.* (1969) do not define the sensitivity of their counterimmunoelectrophoresis test, but Schmidt & Lennette (1962) state that specific IgM antibody can rarely be detected by the immunodiffusion test in sera diluted beyond 1 in 2. The positive control serum diluted in 1 in 20 in negative serum still gives a positive reaction in the MACRIA of Morgan-Capner & McSorley (1983). Thus it is probable that there is a tenfold difference in sensitivity between the tests of Minor *et al.* (1979) and Morgan-Capner & McSorley (1983) and this may explain the higher frequency of the cross-reactivity in the latter test.

It is important to resolve the problem of the extent of heterotypic reactions in CBV IgM tests. If a test detects heterotypic reactions which are confined to the CBV group then the test still remains a useful diagnostic and research tool. However, if a positive result merely indicates recent infection with one of the 67 human enteroviruses then the usefulness of such a test is severely limited. To pursue the current fashion for solid-phase immunoassays it seems essential to use the highly purified virion antigens of Katze & Crowell (1980*a*) and an antibody-capture principle. If extensive heterotypic reactions are still found then this would appear to be due to an inherent property of the human IgM response to enterovirus infection, and this will be difficult to overcome.

J. R. PATTISON
Editor

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