Intrathecal Immune Response and Virus-Specific Immunoglobulin M Antibodies in Laboratory Diagnosis of Acute Poliomyelitis

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The intrathecal immune response in 114 patients with clinically diagnosed acute poliomyelitis was studied by measuring poliovirus-specific immunoglobulin M (IgM) antibodies in cerebrospinal fluid (CSF) by a μ-capture immunoassay and by assessing the ratio between levels of poliovirus-neutralizing antibodies in serum and CSF. Fecal specimens were used for attempts to isolate the causative agents. Eighty-five percent of CSF specimens collected during the first 15 days of disease contained virus-specific IgM antibodies. Forty-five of 48 tested children (94%) also showed virus-specific IgM responses in their sera. Later on, the antibody levels decreased, and positive results after 30 days of onset of paralytic symptoms were rare. If the presence of poliovirus-specific IgM antibodies in the CSF was considered diagnostic, more cases were confirmed by this test than by virus isolation. A relative increase in poliovirus-neutralizing antibodies in the CSF was observed in about one-third of the cases; in all but three cases the increase was observed together with the presence of virus-specific IgM antibodies. A systemic virus-specific response can be seen and poliovirus can be isolated from a subclinically infected individual suffering from a concomitant poliomyelitis-like disease, while positive results by the two methods demonstrating an intrathecal immune response are likely to indicate a true causal relationship between infection and disease. Demonstration of poliovirus-specific IgM antibodies in the CSF thus appears to be a sensitive and specific method for laboratory confirmation of clinically diagnosed poliomyelitis.

Poliomyelitis is a serious public health problem in developing countries, with more than 100,000 new paralytic cases occurring annually. In areas endemic for poliovirus, the paralyzed cases are diagnosed by clinical criteria, usually without laboratory confirmation. Because the incidence of disease is decreasing, it has become increasingly important to prove the etiology of individual cases of poliomyelitis by virological techniques. Polioviruses grow well in cell cultures, and virus isolation from stool specimens is the standard laboratory method for diagnosis of poliovirus infection in industrialized countries. The other conventional method for the laboratory diagnosis of poliovirus infection is determination of neutralizing antibodies in paired serum samples from the acute and convalescent phases to three standard laboratory serotypes of poliovirus. The conventional methods are highly specific, but they are also rather laborious and time-consuming and require the continuous use of cell cultures. A further problem with the conventional procedures is that although a positive result confirms the existence of poliovirus infection in the patient, this does not necessarily prove that it is the causative agent for the coinciding disease. The number of asymptomatic poliovirus infections exceed by hundreds-fold the number of paralytic cases.

Clearly, new, simple, and rapid methods are needed for the virological diagnosis of poliomyelitis. Unlike many other viral diseases, the kinetics of the serum immunoglobulin M (IgM) response have not been worked out, and the demonstration of the presence of IgM antibodies is not in general use in the laboratory diagnosis of acute poliomyelitis. Intrathecal production of virus-specific antibodies can also be used for the early diagnosis of viral infection in the central nervous system. Demonstration of a relative increase in total virus-specific antibody levels or the presence of virus-specific IgM-class antibodies in the cerebrospinal fluid (CSF) has been used to diagnose other viral infections in the central nervous system (2, 3, 6, 8, 12, 13). A positive result is considered to document a causal relationship between infection and disease. A relative increase in the level of neutralizing poliovirus antibodies in the CSF of five patients with poliomyelitis was previously reported from the National Public Health Institute (4), and one patient was described by others (7). No systematic analysis has been published. The diagnostic value of poliovirus-specific IgM antibodies in the CSF, if present (5), has not been assessed.

We studied the intrathecal immune responses in 114 Pakistani children with clinically diagnosed paralytic poliomyelitis using both of the approaches mentioned above and compared the results with those obtained with regular virus isolation from fecal specimens.

MATERIALS AND METHODS

Patients. All consecutive children with acute poliomyelitis of up to 3 months of onset coming to the Department of Paediatrics, Civil Hospital, Karachi, Pakistan, from November 1989 to August 1991 were enrolled in the study. As recommended by the World Health Organization, only patients with paralytic symptoms persisting for at least 60 days were included. Most patients were in the acute phase of the disease, but some patients were enrolled in the study only after 1 month or more had elapsed from the time of onset of the disease. A lower limb was the body part affected in 94% of the children. Clinical and epidemiological features of the

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patients and observations during the follow-up period will be reported separately. The median age of the patients was 14 months, with a range of between 2 and 60 months. Eighty-three children had no history of immunization against polio, while 18 children had received a complete series of at least three doses of oral poliovirus vaccine and 13 children had received one or two doses. Four milliliters of blood was collected from each patient by using aseptic precautions, and the blood was centrifuged at room temperature to separate the serum. Lumbar puncture was done, with the consent of the child's parents, by using aseptic precautions under local anesthesia, and CSF was collected. Fecal specimens of patients examined during the first weeks of disease were collected in clean containers. For some patients, additional samples of blood and CSF were collected during later visits to the hospital. The serum and CSF samples were designed to be collected on the same day, and for only five patients was there a 1- to 5-day interval between the times of collection of the samples. Specimens were kept frozen at −20°C in Karachi and were eventually sent in dry ice to Helsinki for analysis. Of the 154 patients initially enrolled, 40 were excluded from the present evaluation because CSF was not available for the IgM tests.

Reference specimens. CSF specimens from 20 Finnish children with nonparalytic neurological disease, kindly provided by M.-L. Koskiniemi from the Department of Virology, University of Helsinki, and 58 CSF specimens from patients without polio from the collections of the KTL National Public Health Institute were used as negative controls in the μ-capture test. A human serum specimen which was known to have a high-titer IgM class rheumatoid factor (RF) activity was a generous gift from T. Palosuo of the KTL National Public Health Institute.

Virus isolation. Viruses were isolated from stool specimens by standard techniques by using monkey kidney-derived Vero and GMK cells, a human lung carcinoma cell line (A-549), and human embryonic fibroblast cells. Growth of virus was observed by microscopy, and the isolated strains were identified by neutralization with type-specific antisera. Specimens were considered negative if no cytopathic effect was seen after one blind passage and a total incubation time of at least 4 weeks. For intratypic differentiation, we used a sandwich-type enzyme immunoassay with cross-absorbed rabbit antisera (14) to the attenuated Sabin viruses and the corresponding wild-type polioviruses. The antisera were a gift from A. van Loon, Bilthoven, The Netherlands.

Neutralization assay for antibodies. Neutralizing antibodies in CSF and heat-inactivated sera were measured by a standard microneutralization test in Vero cells (1a). The lowest dilutions of serum and CSF that scored positive were 1:4 and 1:2, respectively. Reciprocals of the titers obtained were used to calculate the serum/CSF ratio.

μ-Capture assay for IgM antibodies. Flat-bottom micro-well plates were coated overnight at room temperature with human μ-chain-specific immunoglobulin (from Sigma; diluted in carbonate buffer [pH 9.4]) and were then blocked with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. After washing three times with 0.1% Tween 20 in PBS (PBS-Tween), CSF samples diluted 1:10 in enzyme immunoassay buffer (PBS with 1% BSA, 0.1% Tween 20, and 1% fetal calf serum) were added and allowed to react with the solid-phase-bound antibody for 2 h at 37°C. Serum samples were diluted in the same buffer and were tested at dilutions of 1:100, 1:1,000, and 1:10,000. After three washes with PBS-

Tween, 30 μl of metabolically [^{35}S]methionine-radiolabelled purified (9) polioviruses of serotypes 1, 2, and 3, corresponding to about 20,000 cpm per well, was added to duplicate wells. After 1 h at 37°C, unbound virus was removed, the wells were washed three times with PBS-Tween, and the bound material was solubilized in 50 μl of 0.3 N sodium hydroxide and assayed for radioactivity by using a Wallac MicroBeta scintillation counter. A titer of IgM antibodies was calculated by using the following formula: titer = (cpm test − cpm blank)/input cpm × 100 × dilution factor. The means for two test wells and at least six blanks were used in the calculations. The dilution factor was the reciprocal of the dilution. Pilot tests on series of dilutions suggested that the formula applied to an individual dilution gives an estimate of the titer that is at least as accurate as that which can be obtained graphically. At high IgM levels, however, falsely low titers were obtained if only low test dilutions were used. Since the level of IgM varied considerably between individual serum specimens, the use of three serum dilutions throughout the study turned out to be practical. CSF specimens from the first 30 patients without polio gave radioactivity values close to those of the buffer blanks. We considered calculated CSF titers of 5 or more to be positive in the present study. Later on, we studied CSF from an additional 48 patients without polio. In two cases, a calculated titer of 5 was found. While occasional serum specimens from healthy persons with old immunity bound slightly more radioactivity than the buffer blanks, the calculated titer always remained below 100. Acute-phase serum specimens from patients with polio usually showed titers in the range of thousands. A titer of 100 was taken as the cutoff level for serum specimens.

The virus strains used for type 1 and type 2 assays were the regular laboratory strains PV1/Mahoney and PV2/MEF-1, respectively. In the pilot phase of the study, we observed that the standard PV3/Saukett strain missed cases of type 3 poliovirus infection that could be detected with a local isolate PV3/Pakistan/KTL080/90. The results presented here are based on tests with the latter strain only. In a large majority of the tested specimens, a positive reaction was recorded against one serotype only. However, with 6 CSF and 11 serum specimens, low or moderate amounts of radioactivity were bound from one or both of the other serotypes as well. See the Discussion for the putative backgrounds of these cross-reactions. For simplification, these specimens are listed in the Results according to the serotype showing the highest titer. This practice was, in most cases, supported by the other diagnostic tests used.

RF is a well-known source of falsely positive results in solid-phase antigen IgM assays when μ-chain-specific labelled antibodies are used for detection (indirect assays). While RF is theoretically much less likely to interfere with μ-capture-based assays, we tested its effects on our μ-capture radioimmunoassay. A serum specimen with high RF activity was found to be highly positive in both indirect IgG and indirect IgM assays for poliovirus type 1 antibodies (11) but was clearly negative in our μ-capture radioimmunoassay. A serum specimen from a patient with polio with a low IgM titer in our μ-capture test bound the same amounts of radioactivity in the presence and absence of the RF-containing serum (data not shown). These results indicate that RF does not significantly affect the results of our μ-capture IgM assay.
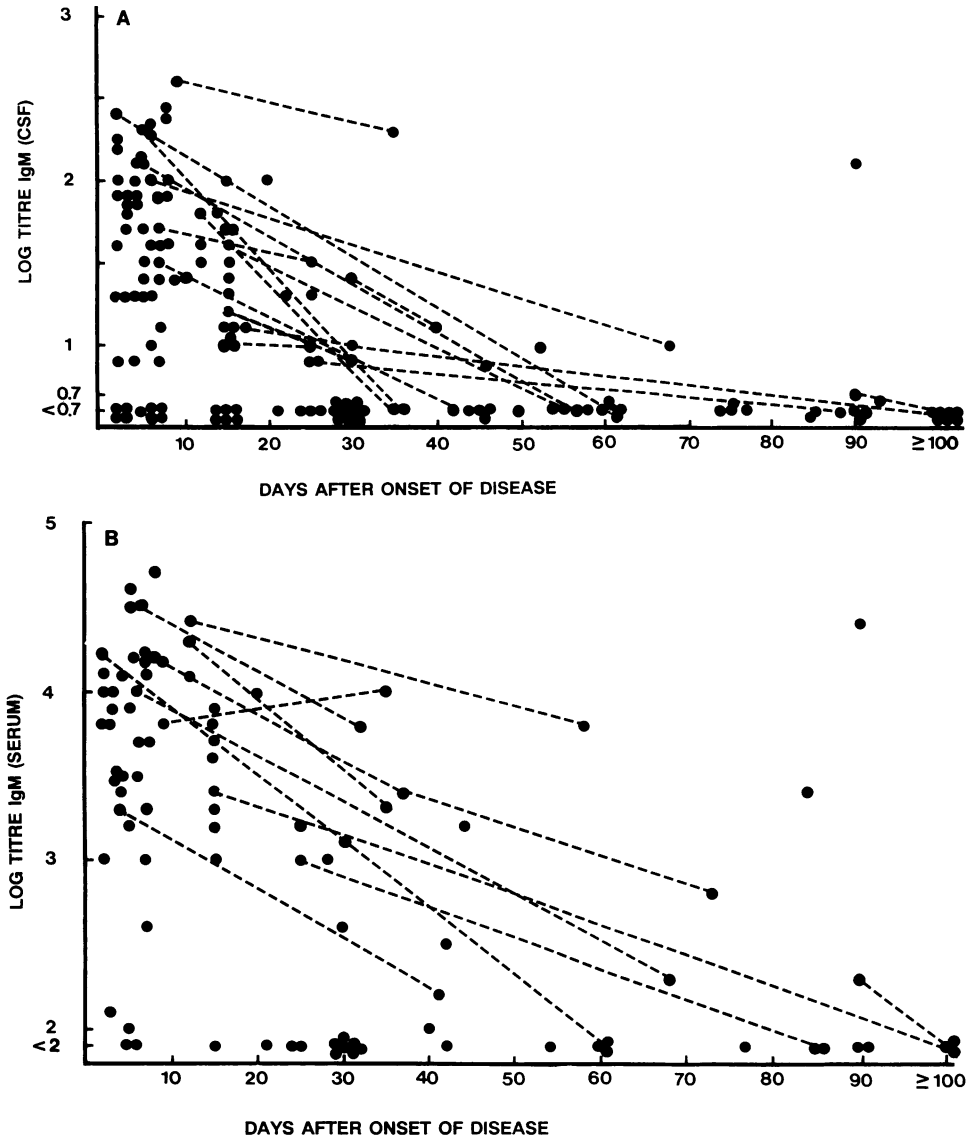
FIG. 1. Presence and decay of poliovirus-specific IgM antibodies in CSF (A) and serum (B) of patients with poliomyelitis. Specimens collected from 114 children at various times after the onset of paralytic symptoms were analyzed as described in the text. Readings of successive specimens are connected with dashed lines if the first specimen was scored positive.

RESULTS

Poliovirus-specific IgM antibodies in CSF of patients with polio. One or more CSF specimens collected from 114 Pakistani children with clinically diagnosed paralytic poliomyelitis were tested in the μ-capture radioimmunoassay for IgM antibodies against the three serotypes of poliovirus. A positive result was obtained for 76 patients. Forty-six patients had type 1 poliovirus-specific IgM antibodies in their CSF, 21 had type 2-specific IgM antibodies, and 9 had type 3-specific IgM antibodies.

When the calculated IgM titers in CSF were plotted against time after the onset of paralytic symptoms, it was found that the highest titers were seen during the first 15 days. Eighty-five percent of specimens tested within this period were positive (Fig. 1A). Thereafter, the antibody levels declined rapidly; in most children the test was negative for specimens collected 30 days or later after the onset

of disease. However, exceptionally high titers were still seen in two children at 1 and 3 months, respectively (Fig. 1A).

Poliovirus-specific IgM response in serum. One or more serum specimens from 77 patients were also tested for IgM antibodies. The test was positive for 59 patients. Thirty-six of these patients had a response against type 1 poliovirus, 21 had a response against type 2 poliovirus, and 7 had a response against type 3 poliovirus. Like in the case of CSF IgM results, the titers were high immediately after the onset of disease and usually declined during the subsequent few weeks (Fig. 1B). Forty-five of 48 serum specimens (94%) collected during the first 15 days of disease contained poliovirus-specific IgM class antibodies.

Neutralizing poliovirus antibodies in CSF. For some children, two or three successive sets of serum and CSF specimens collected up to 4 months after the onset of disease were tested for neutralizing poliovirus antibodies to assess

TABLE 1. Correlation between results obtained by the virus isolation test and CSF IgM test for patients from whom samples were obtained within 15 days after the onset of disease

CSF IgM test result	No. of tests with the following virus isolation test result[a]:		
	+	−	Total
+	34	23	57
−	6	138	144
Total	40	161	201

[a] Note that separate tests for the three serotypes on a given specimen are recorded cumulatively.

TABLE 3. Correlation between results obtained by serum IgM and CSF IgM tests for patients from whom samples were obtained within 15 days after the onset of disease

CSF IgM test result	No. of tests with the following serum IgM test result[a]:		
	+	−	Total
+	42	2	44
−	8	107	115
Total	50	109	159

[a] Note that separate tests for the three serotypes on a given specimen are recorded cumulatively.

the putative relative increase in antibody levels in CSF. Altogether, CSF samples from 84 of 105 children tested had neutralizing antibodies against one or more serotypes of poliovirus. We considered a specimen pair positive if the reciprocals of the serum and CSF titers had a ratio of 64 or less. In this way, 39 of 105 tested children fulfilled this diagnostic criterion. Twenty-six of these children showed a decreased ratio for type 1 poliovirus antibodies, 8 children showed a decreased ratio for type 2 poliovirus antibodies, and 5 children showed a decreased ratio for type 3 poliovirus antibodies. Unlike the IgM tests, positive results for this test did not show a correlation with time after the onset of disease. Rather, several pairs of specimens collected 1 or more months after the onset of disease showed the decreased ratio (data not shown).

Intrathecal and systemic IgM responses as diagnostic tests. To assess mutual correlations of the results of the two IgM assays and in order to compare them with virus isolation, we took into consideration only patients from whom specimens were collected during the first 15 days of disease (cf. Fig. 1). Overall sensitivities of 94, 85, and 60% were recorded for the serum IgM test, CSF IgM test, and virus isolation, respectively, if detection of any of the serotypes was taken into account. The mutual concordances of the results obtained by the three methods are shown in Tables 1 to 3. We acknowledge the fact that every specimen was independently tested for signs of infection by each of the three poliovirus serotypes. Hence, the number of tests is three times the number of tested specimens. For simplification, the data for the three serotypes are combined, despite potential masking of putative serotype-specific differences (see Discussion).

Twenty-three of 57 children showed a positive CSF IgM result while being negative by virus isolation. On the other hand, six patients excreted a poliovirus but did not show an intrathecal IgM response (Table 1). With regard to serum

IgM tests, 11 of 45 positive patients did not excrete poliovirus, but on the other hand, two poliovirus excretors did not show an IgM response (Table 2). When the two IgM tests were compared, it was found that in 94% of the test pairs the results for serum and CSF IgM agreed. Slightly more patients had serum IgM than CSF IgM (Table 3). The overall performance of each of the novel assays and virus isolation in confirming poliovirus infection in the patient material used in the present study is given in Table 4. To calculate the total numbers of cases of illness caused by each serotype, we first added all cases in which any of the three immunological tests described above or poliovirus isolation was positive. Of the remaining 20 patients, 3 showed a diagnostic increase in serum neutralizing antibodies and 1 had a fourfold decrease according to an expected schedule. In 12 additional patients (10 with poliovirus type 1 infections and 2 with poliovirus type 3 infections) we considered a high serum level of neutralizing antibodies against one serotype with no antibodies or a very low level against the two other serotypes "suggestively diagnostic." For eight of these patients, the first specimens were collected 1 month or more after the onset of disease; for four patients, earlier specimens were

TABLE 4. Overall performance of different diagnostic tests for specimens from 110 children with virologically confirmed paralytic poliomyelitis[a]

Test	No. of children with confirmed infection/no. of children tested for the following serotype[b]:			
	PV1	PV2	PV3	Total
Virus isolation				
Identical serotype	23/48	16/21	10/17	49/86
Other virus	18/48[c]	2/21[d]	4/17[e]	
Ratio of neutralizing antibodies (serum/CSF)	26/61	8/28	5/16	39/105
CSF IgM	46/63	21/28	9/18	76/109
Serum IgM	31/36	21/25	7/11	59/72

[a] Infection was considered to be due to the indicated poliovirus serotype if any of the four tests was positive (see text). In addition, in 11 type 1, 1 type 2, and 4 type 3 infections, none of the tests was positive, but neutralizing serum antibody levels supported the diagnosis (see text).

[b] PV1, PV2, and PV3, poliovirus serotypes 1, 2, and 3, respectively.

[c] Two poliovirus type 2/Sabin; two poliovirus type 3/Sabin; one coxsackievirus type A9, one echovirus type 7; one echovirus type 12; one echovirus type 19; one echovirus type 19 and adenovirus; one echovirus type 32; three adenovirus; five untyped viruses.

[d] One coxsackievirus type B4, one echovirus type 4.

[e] One coxsackievirus type B4; one echovirus type 1; one echovirus type 21 plus adenovirus; one adenovirus.

TABLE 2. Correlation between results obtained by the virus isolation test and serum IgM test for patients from whom samples were obtained within 15 days after the onset of disease

Serum IgM test result	No. of tests with the following virus isolation test result[a]:		
	+	−	Total
+	34	11	45
−	2	97	99
Total	36	108	144

[a] Note that separate tests for the three serotypes on a given specimen are recorded cumulatively.

available but the serum could not be tested for poliovirus-specific IgM antibodies. In this way, we confirmed 63 type 1, 28 type 2, and 18 type 3 poliovirus infections in 114 patients.

We had four patients with clinically diagnosed paralytic poliomyelitis with no evidence of concomitant poliovirus infection by any of the criteria used. One of these patients excreted echovirus type 19, but we do not know whether this virus had a role in the etiology of the disease. Eleven strains of nonpolio enteroviruses, six adenoviruses, and five unidentified cytopathogenic agents were also isolated from 20 patients with the designated poliovirus etiology of disease. In addition, in three patients with an intrathecal immune response against type 1 poliovirus (listed in Table 4 according to their immune responses), virus isolation yielded a Sabin-like type 2 or type 3 poliovirus strain. One patient excreted wild-type type 1 and Sabin-like type 3 polioviruses. For one of the patients who excreted type 2 Sabin-like poliovirus, another set of specimens collected 1 month later was available. The second serum specimen showed a systemic immune response, including an IgM response, against type 2 poliovirus, while the CSF still had a low level of IgM antibodies to type 1 poliovirus but none to type 2 poliovirus.

DISCUSSION

We showed in this report that both systemic and intrathecal IgM responses can be reliably demonstrated in patients with acute paralytic poliomyelitis and that the regularity and the transient nature of the responses enables their use in the virological diagnosis of poliomyelitis with an accuracy that appears to exceed that of the old "gold standard," virus isolation. Because of the hundreds-fold excess of subclinical poliovirus infections, the use of the systemic IgM response for laboratory confirmation of paralytic poliomyelitis faces the same relative bias as the conventional techniques: subclinical wild-type poliovirus infections and primary vaccine responses cannot be distinguished from those associated with paralytic disease. In contrast, an intrathecal virus-specific immune response, whether based on demonstration of IgM antibodies or on the relative increase in the level of neutralizing antibodies, is likely to reveal the causative agent of the disease. In the study described here we concentrated on assessing the usefulness of the latter assays as diagnostic tests.

The performance of a new diagnostic test is usually evaluated by comparing the results with those obtained with a gold standard reference method and calculating the sensitivity and specificity of the new test. In the case of virological diagnosis of paralytic poliomyelitis, this is difficult since conventional methods, virus isolation, and an expected increase of neutralizing antibodies are known to be not only relatively insensitive but are also known to yield false-positive results. In the present study, we tried to avoid false-positive results by using strict criteria in patient selection. Although some nonpolio enteroviruses are known to cause rare cases of paralytic disease, it is generally agreed that in a large majority of these cases the paralytic symptoms do not persist for 8 weeks, which we used as a selection criterion for the material used in the present study.

Leakage of poliovirus-specific IgM antibodies through a damaged blood-brain barrier to the CSF or accidental contamination with blood during specimen collection could compromise the selectivity of the CSF IgM tests described above and are difficult to exclude unequivocally. Fixed limit proportions, which are used as diagnostic criteria in the approach that assesses the putative relative increase in IgG
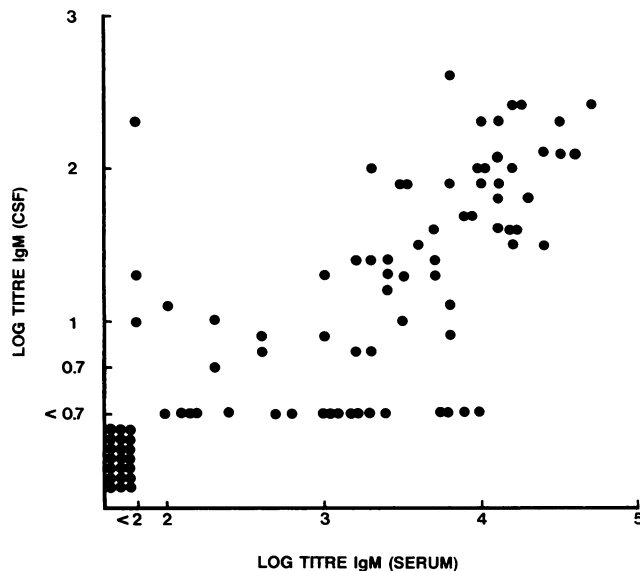

FIG. 2. Correlation of IgM titers in CSF and serum.

antibody levels, cannot be used in a study such as the one described here because of the transient nature of the IgM responses. To assess possible leakage of IgM antibodies in the material used in the present study, we plotted individual poliovirus-specific IgM titers in CSF against the corresponding titers in the serum specimens collected at the same time. While a significant part of the titers of linked specimen pairs appeared to be proportional, a large number of them showed no mutual correlation (Fig. 2). This suggests that the titers measured in CSF are not due to leakage of or contamination by the systemic IgM antibodies but, rather, represent an independently regulated local immune response. The independence of the two IgM responses is also likely to explain the mismatched cases, in which only either a CSF or a serum specimen was positive. A long incubation time and/or delayed sampling of specimens may have been the background in three patients whose CSF was positive and whose serum was negative for IgM antibodies. The opposite situation was found in four patients, whose specimens were collected 2 to 7 days after the onset of disease. The antibody response in the CSF might perhaps have been evident in later specimens if they had been available.

The results of our μ-capture radioimmunoassay were highly serotype specific, with a few exceptions. In 5% of patients the CSF and in 15% of patients the serum contained IgM antibodies that bound radioactivity from more than one serotype. However, the extent of these reactions remained limited and in most cases did not confuse judgment of which serotype caused the infection. Other methods were used to select the correct serotype in those four patients whose sera contained highly cross-reactive IgM antibodies. While neutralizing antibodies to poliovirus show cross-reactivity between the three serotypes only rarely and to a low degree, the viruses are known to share antigenic regions that are more readily detectable by other assays. We have previously identified an antigenic region with almost complete sequence identity at amino acids 37 to 53 of the VP1 capsid protein (10), and in the present study we found that serum and CSF specimens that react with more than one serotype usually also had antibodies to synthetic peptides derived from this sequence (data not shown).

When the data in Tables 1 to 3 were broken down by serotype, there appeared to be less concordance between the virus isolation and CSF IgM results in the case of poliovirus type 3 infections than in those for the two other serotypes. One explanation for this would be that the type 3 strains that caused the infections differed from the strains used in the IgM tests. Failure of the laboratory strain PV3/Saukett to detect type 3 infections was observed in the pilot phase of the present study, and so a local isolate was used in the tests instead. However, we do not know whether more than one antigenic variant of type 3 poliovirus contributed to the cases of disease described here.

Nonpolio enteroviruses and Sabin-like oral poliovirus vaccine-derived polioviruses are frequently detected in fecal specimens of healthy children in Karachi (1), as they are in developing countries in general, and may confuse attempts to document wild-type poliovirus etiology of a paralytic disease. In one of the patients examined in the present study, we found an intrathecal IgM response against type 1 poliovirus, while a Sabin-like type 2 virus was isolated from the patient's feces. We do not know whether the type 1 virus, which appeared to have been the causative agent of the disease, also was vaccine derived. Better knowledge of specific antigenic properties of the wild-type poliovirus strains circulating in countries endemic for poliovirus might help to develop strain-specific IgM assays, which could be used to distinguish IgM responses against wild-type- and vaccine-derived polioviruses.

We found that about one-third of the patients in the present study showed a decreased ratio between neutralizing poliovirus antibody levels in serum and CSF. Almost all of these children also had poliovirus-specific IgM antibodies in their CSF, indicating a high degree of specificity of a positive result in the calculated ratio approach. On the other hand, the sensitivity of the test as a single diagnostic procedure for acute poliomyelitis appeared to be relatively low. However, the test may be useful for patients coming for their first visit several weeks after the onset of the paralytic symptoms. At this time, the sensitivities of both the CSF IgM and the virus isolation tests have decreased to relatively low levels.

In conclusion, the µ-capture-based demonstration of poliovirus-specific IgM antibodies in CSF appears to have a great potential as a diagnostic test for laboratory confirmation of paralytic poliomyelitis. The lumbar puncture required for collection of the CSF specimen is a routine diagnostic procedure for patients with paralytic symptoms. Apart from the apparently high degree of sensitivity and specificity of this novel test, it has a great advantage over the conventional diagnostic methods in that it demonstrates a local immune response to the causative agent of the disease. Both virus isolation and tests that measure a systemic immune response can also yield positive results for patients with subclinical infections, which occur frequently. The technique used in the present study involved radioactively labelled purified polioviruses as probes for anti-µ-captured IgM antibodies. Studies are in progress to develop a nonradioactive modification of the test for which the reagents would be more readily available in different laboratories.

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