ETIOLOGY OF CHILDHOOD INFECTIOUS MONONUCLEOSIS SYNDROME IN SINGAPORE

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SYNOPSIS

In a study of 40 consecutive children with Infectious Mononucleosis Syndrome (IMS) in a major hospital in Singapore over a period of 13 months, it was found that 33 (82.5%) was due to Epstein Barr Virus (EBV), 2 (5.0%) to cytomegalovirus and 5 (12.5%) to unidentified agents. Primary infection to EBV was established by an IgM specific anti-EBV viral capsid antigen (VCA) response during the acute phase of the disease. Other methods of laboratory diagnosis like a four-fold rise in antibody titre between acute and convalescent sera, establishment of continuous cell lines from peripheral blood lymphocytes and the heterophile antibody response, were found to be less useful. The least useful of all was the heterophile antibody test. The IgM anti EBV-VCA should replace the heterophile antibody for the definition of IM caused by EBV.

Infectious mononucleosis (IM) in Western countries has been shown to be caused by Epstein Barr virus (EBV). The usual criteria for diagnosis of IM are

1. Clinical manifestation of fever, pharyngitis, cervical lymphadenopathy and hepatosplenomegaly.
2. A differential peripheral blood leukocyte count of greater than 50% mononuclear cells including 10% or more of atypical monocytes.
3. A positive heterophile antibody (Paul-Burnell-Davidsohn) test.

In Singapore, IM as defined by the above triad is extremely rare. However patients fulfilling the clinical and haematological criteria (1 and 2) with a negative heterophile antibody (criteria 3) are not uncommon especially in children. Infectious Mononucleosis Syndrome (IMS) was the term used to describe this condition (1), the aetiology of which is unknown. Heterophile antibody negative IM can be caused by EBV especially in children. Even though EBV is commonly implicated in heterophile negative IM (2, 3), other infectious agents like cytomegalovirus (4, 5), adenovirus (4), rubella virus (2), toxoplasma gondii (2, 6, 7) and non-infectious agents like drug reactions (8) may be implicated.

The classical laboratory methods for diagnosis of acute viral infections are viral isolation, viral antigen detection and serology. However there are deficiencies in the use of such methods in the diagnosis of primary EBV infection. In IM caused by EBV (IM - EBV) virus isolation or detection of viral antigen is possible from peripheral blood B lymphocytes and from saliva of acute-phase patients. Peripheral blood B lymphocytes from acute IM patients can spontaneously proliferate to form continuous cell lines (transformation) with detectable surface and intracellular EBV specific antigens and infective viruses. However not all peripheral blood lymphocytes (especially unfractionated) from acute IM - EBV patients will give rise to cell lines and B-lymphocytes

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from some seropositive normals can also give rise to cell lines. Similarly infective EBV may not be detectable in the saliva of late acute phase patients and some normal individuals may continue to excrete EBV. Antibody appearance is usually early in IM and by the time the patient is seen, EBV specific antibody is usually present sometimes in high titres. Therefore to depend on a classical 4 fold rise of antibody titre for the diagnosis of IM - EBV will result in a high proportion of false negatives. Recently an EBV specific IgM assay has been employed for the diagnosis of IM - EBV (2, 9, 10). This assay is sensitive and positive in all heterophile positive IM patients. If rheumatoid factors could be excluded, then this assay is generally negative in non IM patients. We have attempted to study the aetiology of 40 consecutive children with IMS in a major hospital in Singapore using this assay and reported here are the results.

MATERIALS AND METHODS

Patients

Consecutive IMS patients from Paediatric Units East and West, Singapore General Hospital during a period between February 1978 and February 1979 were included in the study. These patients fulfilled the clinical and haematological criteria of fever, pharyngitis with whitish-grey membrane over the fauces, cervical lymphadenopathy, hepatosplenomegaly and the presence of 10% or greater of atypical mononuclear cells in the peripheral blood. There were 40 IMS patients (mean age 4.2 ± 2.1, range 1 - 8 years; male:female = 1:4) and 48 controls (mean age 6.9 ± 2.9, range 1 - 11 years; male:female = 1:8). Control patients were either normals or were admitted following accidents or for minor procedures.

Clotted and heparinized blood samples were obtained from all IMS patients during the acute phase of their illness and in 29 also during the convalescent phase (11 - 200 days between acute and convalescent blood samples). Clotted and heparinized blood samples were also obtained from controls. The sera were separated immediately, aliquoted and stored at −20°C until tested. Tests performed on the serum samples were IgM and IgG antibodies to EBV - viral capsid antigens (EBV - VCA), antibodies to heterophile antigen and antibodies to cytomegalovirus (CMV).

Test for antibodies to EBV - VCA

a. Antigen preparation

VCA from the Jijoye cell line was used as the source of antigen. This line was maintained in RPMI 1640 media (Flow Laboratory) supplemented with 10% foetal calf serum (FCS), Hepes buffer and Garamycin (100 ug/ml). The cells used in this study were prepared from one pooled culture containing approximately 4 - 5% VCA positive cells. For preparation of smears, the cells were washed 3 times with phosphate buffered saline and resuspended at a concentration of 1.5 x 10^6 cells per ml. One drop of this suspension was put on each hole on a "Teflon" coated glass slide. The smears were dried at room temperature, fixed with acetone (4°C for 10 minutes) and stored at -70°C until used.

b. Antibody titration and testing

The methods used were slightly modified versions of Henles (11) and Schmitz and Scherer (12). Serum samples were diluted in PBS just before tested. For IgG, the serum was tested in two-fold dilution from 1:10 to 1:1280 and for IgM, in 2-fold dilution from 1:5 to 1:80. For IgG - VCA, the antigen smears were overlaid with the different serum dilutions incubated at 37°/45 minutes in a moist chamber. The slides were washed for 15 minutes on a magnetic stirrer with three changes of PBS. Each cell smear was then overlaid with a 1:40 dilution of fluorescein-conjugated goat anti-human IgG (Behringwerke A.G., W. Germany), incubated at 37°C/30 minutes. The slides were then washed for 10 minutes, dried and mounted with a drop of mounting media and read with a Tiyoda fluorescent microscope within 24 hours. For detection of IgM-VCA, the serum dilutions were incubated with the cell smears for three hours instead of 45 minutes and fluorescein-labelled sheep anti-human IgM (Wellcome Reagents Ltd; diluted 1:20) was incubated at 37°C for 1 hour. Positive and negative serum controls (for IgG or IgM) as well as PBS controls were included in each run. The titre of each serum was given as the reciprocal of the highest dilution at which at least 2 - 5 brightly immunofluorescent cells were observed in most fields.

Test for heterophile antibodies

The quantitive heterophile antibody assay was performed according to the procedures of Paul-Bunnel-Davidsohn differential absorption test, using sheep red blood cells (SRBC) as indicator. The sera were inactivated at 56°C for 30 minutes and absorbed in 0.2 ml amounts with 0.8 ml of a suspension of guinea pig kidney. The resulting mixtures were allowed to stand for three minutes and centrifuged at 1,500 rpm (MSE Super Minor Centrifuge) for 10 minutes. Serial dilutions of the supernatant were made in 0.85% NaCl solution in a row of 10 tubes (10 x 75 mm), in quantities of 0.25 ml each. To each tube, 0.1 ml of 2% of suspension of washed sheep red blood cells was added. The final serum dilutions in each tube were 1:7, 1:14, 1:28, 1:56, 1:112 and so on. The tubes were shaken mechanically and incubated for two hours at room temperature. The final reading was made after two hours. If no clumping was visible to the naked eye, the tube was observed under the low power objective of the microscope. The titer of each serum was given as the highest dilution showing agglutination.

Test for cytomegalovirus antibodies

Sera that were found to be negative for the test for IgM antibodies to EBV-VCA were sent to the Virology Section, Department of Pathology, Ministry of Health, whereby the determination of antibodies against cytomegalovirus (CMV), using the techniques of com-

plement fixation, was carried out.

Lymphoblastoid cell lines

Lymphocytes were immediately separated from heparinized blood of IMS patients and controls by the Ficoll-Isopaque density method (13). Non specific and EBV specific cell mediated immunity assays were performed and will be reported elsewhere. Establishment of cell lines from the peripheral blood lymphocytes of patients and controls was attempted. Approximately 5×10^6 to 10^7 lymphocytes in 5 ml of RPMI 1640 media supplemented with FCS, hepes buffer and garamycin were allowed to incubate at 37°C in a humidified atmosphere of continuously flowing 5% CO_2/95% air. The cultures were observed daily and refed with fresh medium as indicated by changes of pH. When the cells transformed and divided to form clumps, the cultures were subdivided and maintained as lymphoblastoid cell lines. The cultures were observed for at least 40 days before discarded.

RESULTS

IgM - anti EBV-VCA

All the 48 control subjects had negative IgM-anti EBV-VCA titre (i.e. < 5). We therefore define a positive titre conservatively as one greater or equal to 10. Thirty-three (82.5%) of the 40 IMS patients had a positive IgM-anti EBV-VCA titre in the acute sera, indicating a primary EBV infection.

The titre of the 33 positive acute sera ranged from 20 to > 80. Of these 33 patients, convalescent sera were available in 26. A decrease in IgM anti EBV-VCA titres in the convalescent serum was observed in 24 (92.3%) of the 26 paired sera; 20 of these 24 showed a four-fold or greater decrease. In 10 (30.3%) convalescent sera the IgM anti EBV-VCA titre became undetectable, confirming that the IgM response to EBV-VCA was transient and peaked during the acute phase of disease. The geometric mean titre (GMT) of the acute IMS serum samples was 63, 20 in the convalescent samples and negative in the controls.

IgG anti EBV-VCA

A positive IgG anti EBV-VCA (titre $\geqslant 10$) was detected in all 39 acute IMS serum samples (1 sample was exhausted) and in all 29 available convalescent samples. It was also positive in 42 (91.3%) of the 46 control serum samples tested. The GMT of the acute IMS, convalescent IMS and controls serum samples were 110, 138 and 68 respectively. The differences in GMT between acute and control sera ($p < 0.05$) and between convalescent and control sera ($p < 0.02$) were significant. However the difference in GMT between acute and convalescent sera was not significant.

Paired acute and convalescent sera were available in 29 patients (26 IgM anti EBV-VCA positive and 3 negative patients). A significant (four-fold) rise in IgG anti EBV-VCA titre was only observed in 7 (24.1%) and all 7 were from the IgM positive group.

Heterophile antibodies

Only one (2.5%) of the 40 IMS acute sera showed a positive heterophile antibody. This patient had a titre of 224 and belonged to the IgM anti EBV-VCA positive group.

Establishment of cell lines

Establishment of cell lines from peripheral blood lymphocytes was attempted in 32 acute IMS patients and 14 controls. Lymphoblastoid cell lines were successfully cultured from 13 (40.6%) of the 32 blood samples of acute IMS patients but from none of the 14 control blood samples ($p = 0.003$). All the successful cell lines came from IgM anti EBV-VCA positive patients. Of the remaining 19 unsuccessfully cultures, one was contaminated and 6 were from IgM anti EBV-VCA negative patients. Therefore the successful cell line culture rate was 13/25 (52%).

Non EBV associated IMS patients

The sera of the 7 IgM anti EBV-VCA negative IMS patients were tested for antibodies to CMV and of these, two had positive complement fixing antibody titre to CMV of $\geqslant 64$.

DISCUSSION

The present study showed that of the 40 consecutive IMS patients studied in a major hospital in Singapore over a period of 13 months, 33 (82.5%) was due to EBV, 2 (5.0%) to CMV and 5 (12.5%) to unidentified agents. The aetiology of a primary EBV infection was established by a positive IgM anti EBV specific viral capsid antigen response in the acute phase of the disease. The convalescent serum samples from these EBV-IM patients showed a decrease of this IgM response compared to the acute serum and in 30% of the patients this response returned to negativity, indicating the transient nature of the specific IgM response. This finding confirmed a primary immune response to EBV in these patients.

Other methods of laboratory diagnosis, like a four-fold rise in IgG antibody titre between acute and convalescent serum samples, establishment of continuous cell lines from peripheral blood-lymphocytes and the heterophile (Paul-Burnell-Davidsohn) test were found to be less useful. The least useful of all the tests was the heterophile antibody test. Only 3% of the EBV-IM patients showed a positive heterophile antibody response. This confirmed the lack of positive heterophile antibody response in IMS in Singapore (1). The reason for heterophile antibody response in EBV-IM is unknown. This heterophile response is generally seen in teenagers and young adults with EBV-IM and is seldom seen in paediatric patients (10, 14, 15). In Singapore EBV infection occurs early in life and is usually subclinical. In a seroepidemiological survey of a Singaporean normal population in a housing estate Toa Payoh, a total of 1,923 sera were tested for IgG anti EBV-VCA and it was found that 80 - 90% of the population by the age of 10 years have already been seroconverted (16). This was also reflected in the present study in which 91% of the 46 control children were IgG anti EBV-VCA positive. By the time our population reaches young adulthood they are already immune to EBV infection and this is

reflected in the absences of heterophile antibody positive EBV-IM in Singapore. Therefore in the diagnosis of IM in this part of the world, IgM anti EBV-VCA should replace the heterophile antibody test and a heterophile antibody positivity should no longer be a criteria for the definition of IM.

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