

Improved Detection of Virus-Specific IgM Antibodies. Elimination of Non-Specific IgM Binding

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SUMMARY

A non-specific reaction between human IgM and cytoplasmic structures of virus infected cells can often be observed if IgM antibodies to virus antigens are detected by indirect immunofluorescence or by immuno enzyme assays. Formaldehyde selectively inactivates the cytoplasmic receptors for human IgM without affecting the virus structural proteins. Alternatively, receptor-free antigens can be obtained by isolation of nuclei from virus infected cells. Due to reduced background, a more specific and more sensitive detection of IgM antibodies to Epstein-Barr virus, cytomegalovirus or central European encephalitis virus is possible.

Detection of specific IgM antibodies is of great value for the diagnosis of acute virus infections. However, falsely positive reactions due to rheumatoid factors (Fraser *et al.* 1971; Reimer *et al.* 1975), anti-nuclear antibodies belonging to the IgM immunoglobulin class, or even IgM coated bacteria (Schmitz *et al.* 1977) have been reported. These diagnostic pitfalls can be easily avoided either by pre-absorption of rheumatoid factors with IgG-coated latex particles or by using appropriate control antigens.

A problem which has been given little consideration concerns non-specific reactions apparently arising from the adsorption of human IgM molecules to cytoplasmic structures of virus infected cells, while non-specific binding of human IgG, particularly in cytomegalovirus (CMV) infected cells, has been investigated by several authors: evidence for such an IgG binding came from the observation that the IgG of practically every human being produces brilliant immunofluorescent staining of the cytoplasmic inclusions of CMV infected cells. This staining is no longer demonstrable, however, if the anti-complement immunofluorescence (ACIF) technique of Goldwasser & Rozansky (1958) is applied (Schmitz & Haas, 1972). It has also been shown that even F_c-fragments of human IgG can produce this non-specific staining (Keller *et al.* 1976; Westmoreland *et al.* 1976). We have recently studied the binding of human IgM antibodies without antiviral activity to the cytoplasm of CMV infected cells. This non-specific IgM binding strongly interfered with the specific detection of IgM antibodies to CMV in an enzyme immunoassay. It could be reduced, however, if isolated nuclei were used instead of complete cell extracts as antigen (Schmitz *et al.* 1977).

A similar background reaction can be observed if IgM antibodies to Epstein-Barr virus (EBV) are tested for by immunofluorescence (Schmitz & Scherer, 1972). For this assay we used cell smears of 'aged' P3HR1 cells which had been cultivated at 32 °C for about 2 weeks without changing the nutrition medium. The smears were either fixed in acetone or used unfixed but dried since 'aged' cells are obviously permeable for antibodies without any fixation. Immediately after drying and/or fixation, the smears were rinsed in phosphate buffered saline (PBS) and then serum dilutions (from 1:32 onwards) were incubated for 2 h at 37 °C. After 30 min washing, FITC-labelled anti-human IgM globulin (diluted 1:50; Hyland Laboratories, Costa Mesa, Calif., U.S.A.) was added for 30 min at room

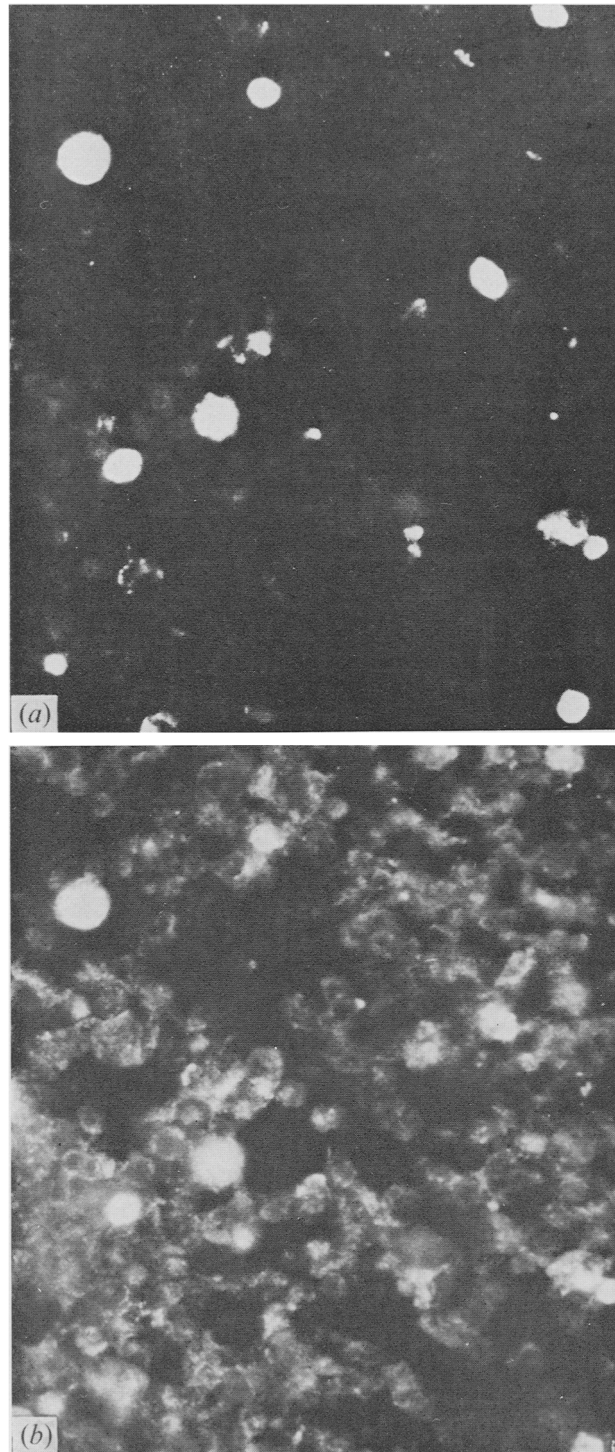


Fig. 1. Detection of IgM antibodies to EBV in a human IgM antibody fraction (0.05 mg/ml protein) isolated by sucrose gradient centrifugation (4). (a) ACIF technique, using human complement (EBV antibody negative human serum, diluted 1:80) and rhodamine-labelled anti-human C_{37} globulin. (b) Additional staining with FITC-anti IgM globulin (diluted 1:80, u-chain specific).

temperature and the tests were read after a final 30 min wash. Especially with serum at low dilutions or with concentrated, purified human IgM fractions an intense fluorescent staining of the cytoplasmic areas of most lymphoblastoid cells can be seen and a reliable reading of the smears is difficult. On the other hand, this cytoplasmic staining no longer interferes when the ACIF technique is used to detect IgM antibodies (Fig. 1*a*). For this technique we employed human serum without EBV antibodies (diluted 1:80) as source of complement and tetramethylrhodamine-labelled anti-human C₃-globulin (diluted 1:20) for the final staining step. Thus specific fluorescence can be differentiated from background staining if purified IgM antibody fractions are tested both by the indirect and by the ACIF method, the latter detecting only antibodies which have reacted with an antigen.

Since non-specifically binding IgM antibodies in the cytoplasm of the lymphoblastoid cells do not fix complement, they probably react with their F_c-region. We therefore looked for a chemical substance which might inactivate possible F_c-receptors without affecting the antigenicity of the virus structural antigens. After testing several fixatives or enzymes it became apparent that the application of 3.7% formaldehyde (37% solution diluted 1:10 in PBS; Ross *et al.* 1973; Knez *et al.* 1976) fulfilled the above conditions. After a 10 min incubation of the air-dried EBV containing lymphoblastoid cells, followed by a short washing, the antigenicity of the virus capsid antigens seemed to be unchanged, while, even with low dilutions, the non-specific background staining was no longer demonstrable. After an overnight wash at 4 °C the cell smears could be stored frozen at -20 °C in 10% glycerol-PBS without loss of antigenicity. As with any intracellular detection of IgM antibodies, drying of the fixed cell smears must be avoided (Schmitz & Scherer, 1972) as this probably inhibits the penetration of the large IgM molecules through the cellular membranes. Due to reduced background activity after formaldehyde treatment, serum dilutions of less than 1:16 can be applied in the immunofluorescence detection of EBV IgM antibodies with a marked increase in the sensitivity of the method.

We further examined the non-specific binding of human myeloma IgM to different commercially obtainable antigens. Both complement-fixation reagents, the 'central European encephalitis' (CEE) virus antigen and the CMV antigen (Behringwerke, Marburg, Germany) are prepared by hypotonic rupture of virus-infected human fibroblasts. The binding of human IgM was detected by a solid-phase enzyme immunoassay (Schmitz *et al.* 1977). In brief, the antigens (diluted 1:4) were air-dried to glass plates and then fixed either in acetone or formalin (1:10 in PBS) for 10 min at 4 °C. After washing, the sera were incubated for 1.5 h on the antigen-coated glass plates and finally the human IgM molecules were detected by horseradish peroxidase-labelled anti-IgM globulin (diluted 1:200; Dacopatts, Copenhagen).

As can be seen in Table 1, the specific IgM antibody titre to the formalin as well as to the acetone treated CEE virus antigen are identical, while the non-specific binding of human myeloma IgM is clearly reduced after formalin as compared to acetone fixation. On the other hand an identical formalin treatment of the CMV antigen did not completely reduce the non-specific IgM binding. Here a nuclear antigen prepared from the nuclei of CMV-infected cells gives more satisfactory results. In principle, nuclei can be obtained from pre-swollen and homogenized CMV infected cells by low speed centrifugation on 0.25 M-sucrose, as described in detail elsewhere (Schmitz *et al.* 1977). Such a nuclear antigen will no longer contain immunoglobulin receptors, which are only located in the cytoplasm of CMV infected cells (Schmitz & Haas, 1972, Keller *et al.* 1976; Westmoreland *et al.* 1976).

The role of formalin as a fixative requires further comment. As can easily be seen from immunofluorescence testing, formalin in the low concentrations applied here does not by

Table 1. *Specific and non-specific binding of human IgM to two different antigens after formaldehyde or acetone fixation**

	CEE virus antigen, fixation in:		CMV antigen, fixation in:	
	Formaldehyde	Acetone	Formaldehyde	Acetone
Serum containing specific IgM antibody	640	640	320	640
Human myeloma IgM (5 mg/ml)	40	160	80	160

* The IgM antibody titres were determined by an enzyme-immunoassay.

itself produce a considerable background fluorescence. Neither does it markedly reduce the antigenicity of structural proteins, since antibody titres to EBV, CMV or CEE virus structural antigens are virtually unchanged after formalin as compared to acetone treatment. It is well known that formalin does not open the cell membranes for the access of antibodies but merely stabilizes the membrane without altering its permeability. Therefore, in contrast to the EBV-producing P3HR1 cells, which are permeable for antibodies even in an unfixed state, formalin may not be used with other cells without rendering them permeable prior to formalin treatment. Penetration problems do not, of course, occur if soluble antigens are applied in an immuno enzyme assay. The differential reduction of non-specific IgM binding after formalin treatment, as shown with either CEE or CMV antigens, might possibly be explained by the quality or quantity of immunoglobulin receptors in different antigen preparations.

Virus-specific IgM antibodies are usually detected by FITC-, iodine- or enzyme-labelled anti-human-IgM globulin, which does not differentiate between specifically or non-specifically bound IgM antibodies. Thus non-specific reactions will probably occur with low serum dilutions in many IgM antibody assays, if cytoplasmic structures are present in the antigens used. Serum specimens containing high concentrations of human IgM without specific antibody activity (i.e. myeloma sera) should therefore be included as negative controls. The non-specific binding of IgM to cytoplasmic structures can be reduced either by employing nuclear antigens, by formalin treatment or eventually by purification of the virus antigens.

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