Rapid Diagnosis of Tick-borne Encephalitis by means of Enzyme Linked Immunosorbent Assay

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SUMMARY

An enzyme-linked immunosorbent assay was applied for determining separately IgM and IgG antibodies against tick-borne encephalitis virus. A micro-modification in microtitre plates proved to be at least as sensitive as the HI test. However, more precise information could be achieved by a macrotest using antigen coated polystyrene balls. False positive results in IgM antibody determinations could be caused by a rheumatoid factor. A high content of IgM antibodies in a serum could impair the determination of its IgG antibodies but not vice versa. Titres were expressed in comparison to a positive control serum.

INTRODUCTION

Considerable progress in diagnostic virology was achieved by the introduction of enzyme-linked immunosorbent assay (ELISA; Voller et al. 1976). So far this test has been worked out for several virus diseases (Voller & Bidwell, 1975, 1976; Bidwell et al. 1977; Castellano et al. 1977; Delia et al. 1977; Gilman & Docherty, 1977; Gravell et al. 1977; Leinikki & Passila, 1977; Wallen et al. 1977). This paper describes an ELISA for the detection of IgM and IgG antibodies against tick-borne encephalitis (TBE) virus and its application in diagnostic virology.

METHODS

Clinical specimens. Serum and cerebrospinal fluid (c.s.f.) were sent to our diagnostic laboratory from different hospitals in Austria during 1977.

Enzyme linked antiglobulin. The alkaline phosphatase conjugated antisera (from swine) were purchased from Orion Diagnostica, Helsinki.

Macrotest. In this test the antigen was bound to polystyrene balls 6 mm in diam. (Ziola et al. 1977) from Precision Plastic Ball Company, Chicago, by incubation at room temperature for at least 4 h (usually overnight). The adsorption of the antigen on to the surface of the balls posed no problems (Frisch-Niggemeyer et al. 1978). The preparation of the antigen was described elsewhere (Heinz & Kunz, 1977). It was diluted in 0.05 M-phosphate buffer of pH 7.8 to a protein content of 20 μg/ml measured by the method of Lowry et al. (1951). After binding of the antigen, the balls were dried in a warm stream of air and then stored in the refrigerator. They could be used for a period of several weeks. To depress non-specific adsorption of immunoglobulins, the antigen-coated balls had to be placed for at least 2 h in PBS, pH 7.5, containing 2% sheep serum. Then the balls were transferred individually into plastic tubes (diam. 9 mm and length 70 mm) and incubated with 0.2 ml of appropriate

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dilutions of the serum or c.s.f. to be tested. Dilutions were made in PBS, pH 7.4, containing 2 % Tween 20, 2 % sheep serum and 0.02 % NaN₃. After incubation for 2 h at 37 °C, the serum samples were sucked away and the balls washed three times with 4 ml PBS, pH 7.5, containing 2 % sheep serum, using a variable speed automatic dispenser (Oxford Laboratories) with a uniwash (Abbot Laboratories). Thereafter, to each tube were added 0.2 ml of the enzyme-linked anti IgM or anti IgG serum respectively and the mixtures incubated for 1 h at 37 °C. The balls were washed again three times as described, transferred to new tubes and 0.4 ml of the substrate added. As substrate, 4-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets) in diethanolamine buffer (Voller et al. 1976) was used. After an incubation for 30 min the enzyme reaction was stopped by adding 0.4 ml of 3 M-NaOH. To fit the optical cells, the vol. in the tubes had to be adjusted to 2.5 ml with distilled water. The dilution reduced the extinction to about 1/3 without decreasing the relative accuracy. It could be judged visually whether a serum was positive or negative. For accurate assay, the absorbance was measured at 395 nm using Zeiss Spektalphotometer PMQ 2 DL. In preliminary experiments incubation periods, temperatures and dilutions of antisera were varied. Thus, optimal conditions were established which correspond well to the data of Gilman & Docherty (1977) in their toxoplasmosis test.

*Microtest.* For this modification, microtitre plates (Immunolon, Flow Laboratories M-129 A) were used. The antigen, diluted as for the macrotest, was adsorbed on to the walls of the wells by adding 0.1 ml to each well and incubating for at least 9 h at room temperature. The plates were dried in a warm air stream and inactivated with PBS + 2 % sheep serum. Then 0.05 ml of a serial twofold dilution of the serum to be tested was added to each well. For serial analysis it was convenient to use only a single dilution step (1:40). After an incubation period of 2 h at 37 °C the wells were washed three times and the anti-IgM or IgG serum, diluted 1:50, was added (0.05 ml/well). The plates were incubated for 1 h at 37 °C, washed and the substrate (0.05 ml/well) was added. Enzymic splitting was allowed to occur for 30 min at room temperature. The reaction was then stopped with 0.1 ml 3 M-NaOH.

*Serum fractionation.* Some sera were fractionated into IgM and IgG by sucrose gradient ultracentrifugation or by exclusion chromatography on controlled pore glass as described by Frisch-Niggemeyer (1975).

*Haemagglutination inhibition test.* This test, using 2-mercaptoethanol (2-ME) for elimination of IgM antibody, was described earlier (Kunz & Hofmann, 1971).

**RESULTS**

*IgM and IgG antibody determination in the macrotest*

Characteristic curves were achieved with sera containing antibodies against TBE virus. Sera from patients with previous infection showed no activity in the IgM assay although high values in the IgG test were observed (Fig. 1). It was noted that in the IgG antibody determination the shape of the curves depended on the content of the serum on specific IgM antibody. Sera with no, or only low, IgM antibodies showed the maximal absorbance at a serum dilution of 1:20 to 1:40, while sera with a high content of IgM antibodies showed the absorbance peak at a fourfold dilution (curves 1 and 2 in Fig. 1).

*Antibody determination in serum fractions*

A serum (2-ME-HI titre 320/80) from a recent infection was fractionated by exclusion chromatography on controlled pore glass (Frisch-Niggemeyer, 1975) and the fractions were
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Fig. 1. Determination of IgG (a, b) and IgM (c) antibodies. 1 and 2, sera from patients with very recent infections – high content of IgM antibodies and moderate content of IgG antibodies; 3 and 4, sera from patients with recent infections – low content of IgM and moderate content of IgG antibodies; 5 and 6, sera from patients with old infections – no IgM but high content of IgG antibodies; 7 and 8, negative control sera containing neither IgM nor IgG antibodies.

tested individually for IgM and IgG antibodies. As can be seen in Fig. 2, IgM and IgG antibodies were separated fairly well. There was no evidence that in the IgG assay IgM antibodies were measured and vice versa.

To test for possible interference, the fraction with the highest IgM content was mixed with the peak fraction and with elution buffer respectively. Equal vol. of IgM fraction and buffer (1), IgM fraction and IgG fraction (2) and IgG fraction and buffer (3) were mixed and tested for IgM and IgG antibodies individually. As can be seen in Fig. 3, a high content of IgM antibodies in a serum not only reduces its IgG values but shifts the absorbance maximum to a higher dilution. On the other hand, there was no interference of high levels of IgG with the measurement of IgM. Exactly the same result was observed when IgM and IgG fractions obtained by gradient ultracentrifugation were used.

Measurement of antibody content

In conventional serology the antibody content is usually expressed as the lowest serum dilution still reacting positively. Therefore some authors also express ELISA results as titres. In this case titre means the serum dilution which gives an absorbance significantly greater than the value of the negative control. Fig. 1. shows clearly that the antibody content of a serum is much better characterized by its peak absorbance than by its endpoint titre, because sera with different antibody contents show a similar titre when this is estimated by the endpoint method. Therefore we prepared solutions with known antibody content. Serial twofold dilutions were made from a highly positive reacting serum into one which was devoid of specific antibodies.

Those sera together with the negative and positive control were tested in the macro ELISA. In Fig. 4 the maximal extinction is plotted against the antibody dilution. As can be seen, a
smooth curve results. The positive serum can be accepted as a standard and given arbitrarily a titre of, say, 1:1000. Thus using this standard curve, antibody-titres of sera tested can be read from their peak absorbance.

Specificity of the test

It is known that the rheumatoid factor may interfere with IgM antibody assay for virus diseases (Reimer et al. 1975; Robertson et al. 1977). We therefore tested two sera positive for the rheumatoid factor in the latex test but without TBE antibodies in the HI test. Four of them gave a false positive result in the IgM ELISA. However, the IgG antibody assay was negative.

Microtest

For the microtest the antigen was bound to microtitre plastic plates. A positive or negative result could be read by eye. However, titres could be not determined accurately because the colour diminished gradually in consecutive wells and no clear end point could be determined. It was found convenient to test only a single serum dilution (1:40 or 1:80) to see whether or not the serum contained antibodies. Again the differentiation could be made readily between IgM and IgG antibodies. The sensitivity of the microtest was slightly higher than that of the HI test. All sera considered positive in the HI test, i.e. having a titre higher than 1:10,
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Fig. 4. Standard curves for IgM (a) and IgG (b) antibody determination. A serum from a patient with recent TBE was diluted with a negative control serum and these ‘artificial’ sera were assayed by macro ELISA. Absorbance was plotted against serum dilutions. (neg = absorbance of the negative serum.) Each point represents the mean of three determinations.

were also found to contain antibodies in the micro ELISA. On the other hand a few sera negative in the HI test, but positive in macro ELISA, also proved to be positive in micro ELISA.

Application of ELISA in diagnostic virology

In our laboratory, TBE is diagnosed routinely by means of the HI test. Differentiation between IgM and IgG antibodies is achieved by the 2-mercaptoethanol modification (Kunz & Hofmann, 1971). Two hundred and thirty-eight cases of TBE were diagnosed in 1977; from these, 27 sera were retested by macro ELISA and IgM anti-TBE antibodies were also detected by this test. However, 26 additional cases could not be diagnosed by 2-ME-HI test because the serum sample was obtained too late, thus giving no significant reduction of HI titre after reaction with 2-ME. Those cases were proven by complement fixation test (CFT) and/or IgM-specific radioimmunoassay (RIA; Frisch et al. 1978). In all 26 cases diagnosis could also be established by IgM-specific macro ELISA. On the other hand, in 22 sera from persons with a previous infection of TBE, IgM antibodies could not be found by the 2-ME-HI test and could also not be detected with the more sensitive macro ELISA.

Cerebrospinal fluids from patients suspected to have TBE were tested against antibodies for TBE by macro ELISA. From 26 proven TBE cases, IgM and IgG antibodies could be detected in 22 c.s.f. samples, IgG antibodies alone in two and no antibodies at all in the remaining two cases. Five c.s.f. specimens were available from persons with past TBE,
now suffering from aseptic meningitis caused by a different agent. Of these, four had IgG type antibodies. Eight more patients without antibodies in serum also had none in their c.s.f.

Sera from patients vaccinated against TBE several years ago in which HI antibodies against TBE could not be detected any longer were tested in IgG macro ELISA. Out of 64 such sera, IgG antibodies were traced in 44 samples, indicating the greater sensitivity of this method.

**DISCUSSION**

Our study shows that TBE can be diagnosed easily and rapidly by ELISA. For diagnosis we propose two steps. Initially, sera should be tested using the 2-ME-HI test (Kunz & Hofmann, 1971) or the micro ELISA test. About 90% of the TBE cases can be diagnosed with these methods within one day. Cases negative in these tests should be investigated next day by macro ELISA before a final decision is made. Compared with the HI test, ELISA has a higher sensitivity and the advantage of handling a non-infectious antigen and an untreated serum. In many aspects, the macro ELISA is similar to the RIA developed for assay of TBE IgM and IgG antibodies in our laboratory by Frisch-Niggemeyer et al. (1978), the RIA being more sensitive for IgM antibodies but having the disadvantage of requiring radioactive substances.

The ELISA was highly specific and suitable for the classification of the antibody as IgM or IgG. However, sera containing rheumatoid factor (RF) may occasionally give a false positive result. This hardly causes doubtful diagnosis of recent TBE, because RF influences only the IgM, but not the IgG assay. Sera from recent cases always contain IgM as well as IgG antibodies. Thus, a serum positive only in the IgM assay but not in the IgG assay is probably falsely positive due to RF. On the other hand, a serum reacting positively for IgM and IgG could be derived either from a patient with recent TBE or from a person with previous TBE and RF. Regarding the frequency of RF in the population, which is about 3%, and the frequency of previous TBE infections in the sera sent to our laboratory, which is 2.7%, it follows that only one out of about 1200 sera has this combination.

Measurement of antibodies by ELISA poses a minor problem. Some authors determine titres as in classical serology. However, as mentioned earlier, this leads to incorrect results when applied in the ELISA technique. Other authors determine the ratio of the absorbance of the positive serum to that of a negative ("signal to noise ratio"; Saunders et al. 1977). We regard it as best to compare the absorbance of a positive and a negative standard serum as proposed by Leinikki & Passila (1977). This method too has some problems, because the antibody content is calculated from the highest absorbance which, in the IgG test, is influenced by the IgM content of the serum. Thus, a serum with a high content of IgM antibodies yields IgG antibody titres that are too low. However, this is not misleading where diagnosis of recent TBE is concerned.

As was shown, macro ELISA can be used to examine some other medically important questions. An example is the detection of low levels of antibodies in sera from persons vaccinated against TBE a long time previously, which could not be detected by the HI test. Also in cerebrospinal fluid IgM and IgG antibodies could be detected by ELISA. We feel that antibody assay in c.s.f. may help to answer not only diagnostic but also some pathogenetic and prognostic questions of TBE. Such a study is in progress.

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REFERENCES


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