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Comparison of Two Different Enzyme Immunoassays for Detection of Immunoglobulin M Antibodies Against Tick-Borne Encephalitis Virus in Serum and Cerebrospinal Fluid

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Two enzyme immunoassays for the detection in immunoglobulin M (IgM) antibodies against tick-borne encephalitis virus were compared, employing a solid phase coated either with antigen or with μ-chain-specific antiserum to human IgM. The latter IgM-capturing assay system proved to be more sensitive, and its superiority was especially prominent when high titers of tick-borne encephalitis virus-specific IgG antibodies in addition to specific IgM antibodies were present in the sample. The application of this test is a valuable extension of the diagnostic tools for the rapid diagnosis of tick-borne encephalitis by IgM detection. Since specific IgG does not interfere, diagnosis can be readily established when patients have already developed high titers of IgG antibodies relatively late after the onset of the disease.

Similar to other members of the flavivirus genus of togaviruses, such as Japanese encephalitis virus, the dengue viruses, yellow fever virus or St. Louis encephalitis virus, tick-borne encephalitis (TBE) virus is the causative agent of a disease which represents an important public health problem in endemic areas. As shown by virus isolation or serological surveys, TBE virus occurs in most European countries, Russia, and probably also China. In Austria, some hundreds of hospitalized patients suffering from TBE are recorded each year and diagnosis has to depend upon serological tests, due to the lack of specific clinical symptoms. The development of solid-phase immunoassays for the selective determination of virus-specific immunoglobulin M (IgM) and IgG antibodies has been a major breakthrough for the rapid serodiagnosis of virus infections (3, 17). In 1978, a radioimmunoassay was developed for the diagnosis of TBE (6) and then an enzyme immunoassay employing the same test principle was established (9) which currently is used for the routine diagnosis of this disease at our institute. Because of the possibility of determining antibody activity in different immunoglobulin classes directly, without serum fractionation, and the very high sensitivity of these tests, they have increasingly replaced the conventional, previously used, complement fixation or hemagglutination inhibition tests. The use of enzyme-labeled, instead of radioactively labeled, tracer molecules and the possibility of using inactivated noninfectious antigens make these techniques even more feasible for the routine diagnostic laboratory. As with the above cited immunoassays for TBE virus antibodies, most frequently the antigen is coated to the solid phase and, after reaction with specific antibodies, these are detected by the use of radioactively or enzyme-labeled sera specific for either μ or γ chains of human immunoglobulins. More recently "reverse" solid-phase enzyme immunoassays or radioimmunoassays have been introduced to diagnostic virology, and this principle has been successfully applied to the detection of IgM antibodies against hepatitis A virus (1, 4, 5, 12-15), hepatitis B core antigen (7), rubella virus (10) and also TBE virus (Roggendorf et al., J. Med. Virol., in press). In these tests, μ-chain-specific antibodies are bound to the solid phase to "capture" IgM from the test serum sample. Antigen bound by virus-specific IgM is then detected by anti-viral labeled antiserum. In this communication, we compare these two test systems for the detection of IgM antibodies against TBE virus with respect to sensitivity as well as to susceptibility to different interfering factors.

MATERIALS AND METHODS

Clinical specimens. Sera and cerebrospinal fluids of patients were sent to the Institute of Virology, Vienna, and stored at −20°C.

Preparation of antigen. The antigen used in immunoassays and also for immunization of rabbits was TBE virus grown in chicken embryo cells, concentrated by ultracentrifugation, and purified by two cycles of sucrose-density gradient centrifugation as described in detail previously (8). Before being used as antigen, infectivity was destroyed by Formalin treatment (1:2,000) overnight at 37°C, and the preparation was stored at −80°C.

Preparation of immune serum. Rabbits were immunized subcutaneously in the back with purified TBE virus (200 μg/ml) in 0.05 M triethanolamine buffer–0.1 M NaCl (pH 8.0) emulsified in complete Freund adjuvant, and they received two more similar doses of virus emulsified in incomplete Freund adjuvant, each one month apart. After 2 additional weeks, blood was taken by ear vein puncture and the immune serum was stored at −20°C.

Conjugation with horseradish peroxidase. IgG from rabbit immune serum was isolated by ammonium sulfate precipitation followed by diethylaminoethyl Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) column chromatography. Conjugation with horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) was done essentially as described by Wilson and Nakane (21).

Enzyme immunoassays. To obtain maximal sensitivity, the assay conditions were optimized in previous tests. According to the results of those tests, the appropriate concentrations for the antigens and the enzyme conjugates were selected as follows.

Antigen coated to the solid phase (test A). Polystyrene U-shaped microtiter plates (Nunc, Kamstrup, Denmark) were coated with purified, inactivated TBE virus (2 μg/ml in phosphate-buffered saline [PBS], pH 8.0) by incubation with 50 μl per well overnight at 4°C. The coating solution was then removed, and the plates were dried by the use of a ventilator without heating. Before the beginning of the test, each well was incubated for 1 h at 37°C with 100 μl of PBS (pH 7.4) containing 2% sheep serum. Then the wells were emptied, and 50 μl of test serum diluted in PBS (pH 7.4) containing 2% Tween 20, 2% sheep serum, and 0.02% NaN_3 (PBS-enzyme-linked immunosorbent assay [ELISA]) was added. After an incubation period of 2 h at 37°C, the plates were washed three times with PBS (pH 7.4) containing 0.2% sheep serum. Fifty microliters of alkaline phosphatase-conjugated heavy chain-specific swine antibodies against human IgG or IgM (Orion Diagnostica, Helsinki, Finland) diluted 1 in 50 in PBS-ELISA were added to each well and further incubated at 37°C for 1 h. These conjugates did not show cross-reactions between human IgM and IgG. The plates were again washed three times as above, and then 0.1 ml of substrate (*p*-nitrophenylphosphate [Sigma 104 phosphatase substrate tablets]; 1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added. Color development was allowed to proceed for 30 min at room temperature, and the reaction was stopped by the addition of 100 μl of 3 N NaOH. Absorbance at 405 nm was measured by the use of a multichannel photometer (Multiskan; Flow Laboratories, Bonn, West Germany).

Anti-human μ coated to the solid phase (test B). Fifty microliters of rabbit immunoglobulin against human μ chains diluted 1:1,000 (DAKO, Copenhagen, Denmark) in carbonate buffer (pH 9.6; 0.159% Na_2CO_3 and 0.293% $NaHCO_3$) was added to U-shaped microtiter polystyrene plates (Nunc) for coating overnight at 4°C. At the beginning of the test, the plates were washed three times with PBS (pH 7.4) containing 0.05% Tween 20 (washing buffer), and then 50 μl of test serum diluted in PBS (pH 7.4) containing 3% Tween and 2% sheep serum was added to each well and incubated at 37°C for 2 h. The plates were washed three times with washing buffer, and 50 μl of purified inactivated TBE virus (2 μg/ml) diluted in PBS (pH 7.4) containing 2% sheep serum was added. The plates were incubated overnight at 4°C, and then washed three times with washing buffer before the addition of 50 μl of horseradish peroxidase conjugated rabbit IgG against TBE virus and incubation for 2 h at 37°C. The plates were again washed four times as above, and 50 μl of substrate (*o*-phenylenediamine; 1 mg/ml in 0.1 M phosphate buffer, pH 6.0, containing 1 μl of Perhydrol per ml) was added. The enzyme was allowed to react for 30 min at room temperature in the dark, and then was stopped by the addition of 100 μl of 2N H_2SO_4. Absorbance was determined by the use of the Multiskan set at 492 nm.

Determination of RF. The presence of rheumatoid factor (RF) in sera was analyzed by using a test kit containing latex particles coated with human IgG (Hyland Diagnostics, Costa Mesa, Calif.).

RESULTS

Due to the completely different test principles employed, different factors can be expected to interfere with the sensitivity of IgM detection when either antigen or anti-human μ is coated to the solid phase. To analyze these factors, the following experiment was performed. A human serum containing antibodies to TBE virus of both the IgM and IgG class was adjusted to give an absorbance value of 0.7 to 0.9 units under the conditions used in the experiment, which represented a final serum dilution of 1:200 in test A and 1:1000 in test B. This serum was mixed with an equal volume of a second (interfering) serum in decreasing concentration (i.e., final dilutions of 1:125 to 1:4,000) (Fig. 1) to test the influence of this second serum on the IgM determination in the first serum. The following sera were used as second sera: (i) human serum containing IgG but no IgM antibodies against TBE virus, (ii) human serum without antibodies to TBE virus but IgM and IgG antibodies to rubella virus, and (iii) rabbit immune serum against TBE virus. The influence of these sera in both types of enzyme immunoassay is shown in Fig. 1A and B. When antigen was coated to the solid phase (Fig. 1A), the absorbance value of the test serum was strongly depressed by the addition of a serum containing TBE virus-specific IgG of hu-

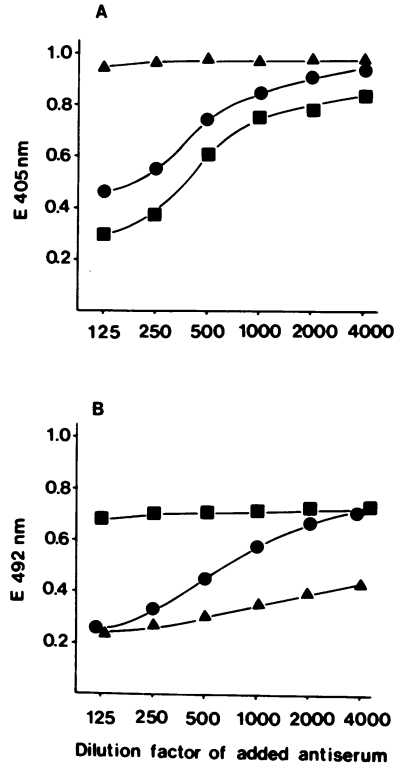

FIG. 1. *Demonstration of different mechanisms of interference in enzyme immunoassays for IgM detection employing solid phase coated with antigen (A) and with anti-human μ (B). A fixed dilution of a TBE IgM-positive serum was mixed with decreasing amounts of interfering sera and their effect on the color yield obtained was measured. Interfering sera used were as follows: human TBE IgG positive, IgM negative (●); human TBE negative, rubella IgM positive (▲); rabbit TBE IgG positive (■).*

man serum and also by antiserum of rabbit origin, due to competitive blocking of binding sites for specific IgM on the solid phase. No interference, however, was observed by the addition of TBE antibody-negative serum. In the test with anti-human μ on the solid phase (Fig. 1B) no interference could be shown with the TBE-positive rabbit serum. However, irrespective of its specificity, human IgM present in TBE IgG-positive or completely TBE-negative sera competed for binding sites with the specific IgM against TBE virus in the test serum and decreased the color yield obtained. The specific IgM titer of each individual serum is, therefore, strongly dependent on the test principle employed and influenced by the quantitative ratios of specific IgG to specific IgM in test A and by the ratio of specific IgM to total IgM in test B.

To compare sensitivity of the tests for the

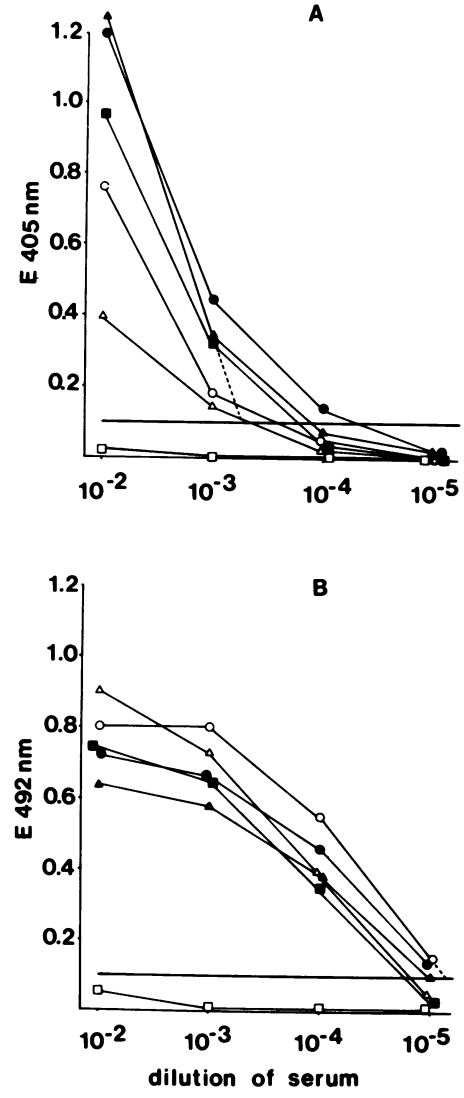

FIG. 2. *Titration curves of five individual TBE IgM-positive sera in enzyme immunoassays employing antigen-coated solid phase (A) and anti-human μ-coated solid phase (B). Serum numbers shown are 1 (●), 2 (▲), 3 (■), 4 (○), 5 (△); negative control (□).*

detection of IgM antibodies, titration curves of five individual sera were determined in each test (Fig. 2). These sera were previously shown to be IgM-positive by our routine ELISA procedure (9) as well as by the hemagglutination inhibition test in combination with 2-mercaptoethanol treatment (11). For measuring absorbance values, the buffer control was taken as zero and the cutoff line for titer determinations was arbitrarily set at 0.1 absorbance unit in each test. The titer of each serum was determined as the recip-

rocal value of that dilution at which the extension of the linear part of the titration curve crossed the cutoff line. It must, however, be kept in mind that these values are strongly dependent on the slopes of the curves, which may vary from one serum to another due to different avidities for the antigen, and, therefore, only the same individual sera can be compared. Table 1 shows the results of titer determination of the titrations shown in Fig. 2. It is evident that the test using anti-human μ-coated solid phase had a much higher sensitivity than test A. However, titers were not parallel in both tests, and their ratios varied markedly between 28 and 223 in the examples shown in Table 1. This is a reflection of the different combinations of specific IgM, specific IgG, and nonspecific IgM in individual sera which had different influences on the titers in these tests. Thus, the superiority of the IgM-capturing test was most prominent when large amounts of specific IgG were present, as in serum sample no. 5 (Table 1) which was taken from a patient who had developed clinical symptoms of TBE 4 weeks earlier.

A sensitive test would also be especially valuable for the determination of specific IgM antibodies in cerebrospinal fluid (CSF). Five samples of CSF from patients with TBE were analyzed in both tests, and the results of titration are shown in Fig. 3. Due to the lower sensitivity of test A, the linear part of the titration curve of all except one sample did not show up between the lowest dilution of 10^{-1} to 10^{-2}. Titers were, therefore, determined under the assumption that the slope of the curve of these CSF samples at a dilution between 1 and 10^{-1} would be equal to that of CSF samples no. 2 (▲——▲) between 10^{-1} and 10^{-2}. Similar to the results found with sera, the IgM-capturing test showed higher sensitivity, and the quantitative evaluation of the results is presented in Table 2. Again, the ratios

TABLE 1. *Titers of IgM antibodies to TBE virus in sera obtained with enzyme immunoassay with either antigen-coated or anti-human μ-coated solid phase*

Serum no.	Titer of IgM solid phase coated with:		Ratio b/a	Titer of IgG[a]
	TBE virus	Anti-human μ		
1	2,800	106,000	37.9	ND[b]
2	1,900	100,000	52.6	650
3	2,100	60,000	28.6	300
4	1,400	108,000	77.1	400
5	300	67,000	223	10,000

[a] Determined in ELISA with antigen-coated solid phase using swine anti-human IgG, as described in the text.

[b] ND, Not done.

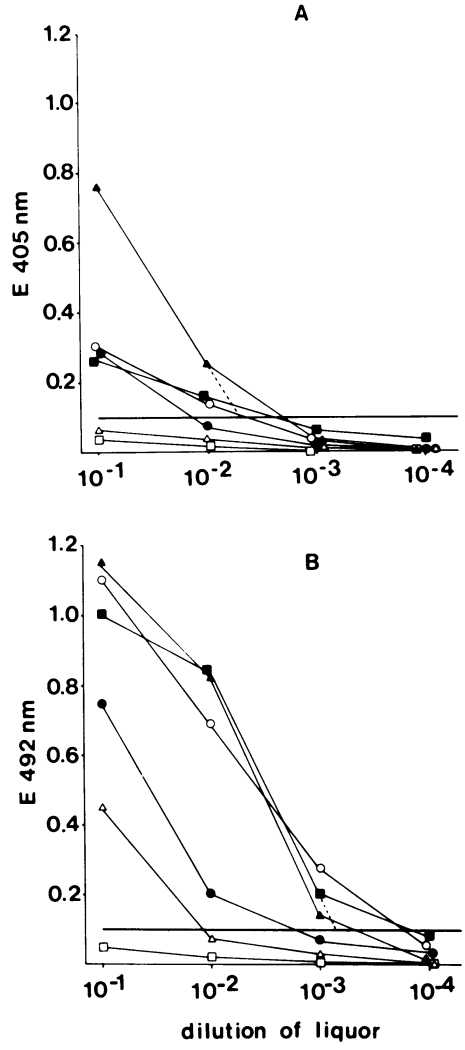

FIG. 3. *Titration curves of five individual TBE IgM-positive CSFs in enzyme immunoassays employing antigen coated solid phase (A) and anti-human μ-coated solid phase (B). CSF numbers shown are 1 (●), 2 (▲), 3 (■), 4 (○), 5 (△); negative control (□).*

of the titers obtained in individual CSF samples showed strong variations, between about 5 and 104, and the highest ratios were obtained in CSF samples containing high titers of specific IgG (CSF sample no. 3 and 4).

Application in routine diagnosis. In 1980, 272 cases of TBE were diagnosed at the Institute of Virology (Vienna) by the demonstration of TBE virus-specific IgM antibodies by using the previously described ELISA system (9) with antigen coated to the solid phase, which was performed identically to the procedure described for test A.

TABLE 2. *Titers of IgM antibodies to TBE virus in CSF obtained with enzyme immunoassay with either antigen-coated solid phase or anti-human μ-coated solid phase*

CSF	Titer of IgM solid phase coated with:		Ratio b/a	Titer of IgG[a]
	TBE virus	Anti-human μ		
1	23	110	4.8	200
2	200	1,100	5.5	65
3	20	1,400	70	>1,000
4	25	2,600	104	>1,000
5	<10	80		35

[a] Determined in ELISA with antigen-coated solid phase by using swine anti-human IgG, as described in the text.

In addition to these cases, there were some uncertain cases of meningitis or encephalitis which, according to their history of tick-bite in an endemic area and the presence of high titers of IgG antibodies, also were suspect as being TBE. However, IgM antibodies could be demonstrated reliably neither by the above-mentioned ELISA technique nor by the hemagglutination inhibition test in combination with 2-mercaptoethanol treatment of sera (11). By the use of the new IgM-capturing assay technique, IgM antibodies could be unequivocally demonstrated in 19 additional cases and, thus, the diagnosis was confirmed despite a high level of specific IgG antibodies.

Due to the high sensitivity of the test (Fig. 2) it might be sufficient for routine diagnosis of acute cases of TBE to test serum samples at a single dilution of 1:1,000.

RF. When using indirect immunoassays (antigen-coated solid phase), RF may cause false-positive IgM results in the presence of specific IgG antibodies (13). As has been reported previously (9), such a reaction occurred only in 1 of about 1,200 sera sent to our laboratory, and, therefore, we had only one such serum available which gave a false-positive reaction in the indirect ELISA due to the presence of RF in addition to specific IgG. No such interference was seen in the IgM-capturing test in which the same serum was clearly shown to contain no IgM antibodies against TBE virus.

When anti-human μ is coated to the solid phase, IgM containing RF will also be bound, and this could yield false-positive results when the enzyme-labeled antiserum reacts nonspecifically with the bound RF (15). We therefore tested 24 RF-positive sera at a dilution of 1:1,000, which would still be suitable for the detection of specific IgM in the diagnosis of TBE (Fig. 2). Only one of these showed a low false-positive reaction with an absorbance value of 0.22.

DISCUSSION

We have compared the two different test systems used in enzyme immunoassays for the assay of IgM antibodies against TBE virus; with one we employed a solid phase coated with antigen (test A), and with the other, we employed a solid phase coated with antibodies against μ chains of human immunoglobulins (test B). When antigen was coated to the solid phase, the specific IgM titer was strongly depressed in the presence of large amounts of specific IgG antibodies due to competition for antibody-binding sites. No such interference could be shown in the IgM-capturing system, and this is extremely valuable for the diagnosis to be established reliably in critical cases when the sera are drawn late after onset of illness and the patients have developed already high titers of IgG antibodies against TBE virus. In 1980, 272 cases were verified as TBE positive by test A. By the application of test B, 19 additional cases of TBE were diagnosed which would have been missed by the assay employing antigen-coated solid phase or hemagglutination inhibition in combination with 2-mercaptoethanol treatment (11). Overall, the IgM capturing assay was more sensitive, resulting in 5- to 200-fold higher titers; its superiority, however, was most prominent when the sample contained high titers of anti-TBE virus IgG. The high sensitivity and lack of interference by specific IgG should also permit this test to be used to investigate local synthesis of IgM antibodies in the brain during TBE and to measure the long term persistence of IgM antibodies in serum and CSF. Persistence of specific IgM for more than 1 year has been reported for severe Japanese encephalitis virus infections (2), and a long term study to investigate the persistence of IgM antibodies after TBE virus infections has been initiated.

A major drawback of the test with antigen coated to the solid phase is the danger of false-positive IgM results when RF is present in addition to specific IgG (13). In the diagnosis of TBE, the frequency of the combination RF plus TBE virus-specific IgG in sera sent to our laboratory has been estimated to be relatively low, about 1 case in 1,200. However, in the last years, thousands of people have been vaccinated against TBE (10a) and, therefore, this problem has increased. False-positive results due to RF may be even more prominent when similar tests are applied for the rapid diagnosis of other flavivirus infections, such as Japanese encephalitis in the Far East where millions of people are

vaccinated against this disease, or dengue fever in which IgG antibodies cross-reacting between the different subtypes are present in most of the people living in endemic areas. The assay system employing anti-human μ coated to the solid phase seems to be much less susceptible to interference by RF, and any observed interference may be caused by a different mechanism, i.e., nonspecific binding of the enzyme-labeled antiserum with RF-IgM bound to the solid phase (16). Roggendorf et al. (15) and Møller and Mathiesen (14) have applied the same test for IgM antibodies to hepatitis A virus and did not find any interference with RF-positive sera, whereas 75% of the same sera gave false-positive results when examined for rubella IgM antibodies in an ELISA test in which rubella antigen was coated to the solid phase (14). We have tested 24 anti-TBE-immunoglobulin-negative, RF-positive sera in test B, and only one showed a low positive reaction which, however, would be suspect by itself if no specific IgG antibodies were present. One serum containing both RF and TBE virus specific IgG gave a false-positive IgM reaction in test A, but it was clearly negative in the IgM-capturing test system. One of the advantages of this assay, with respect to interfering factors, also seems to be its high sensitivity which thus allows sera to be tested at a dilution of 1:1,000. This high dilution seems to eliminate most of the possible interference by RF. As a reliable indicator for virus-specific IgM antibodies, the test should also be of great value for the type-specific serodiagnosis in cases in which different flaviviruses may have caused the disease, since it has been shown that IgG antibodies cross-react broadly with other members of the same serological subgroup, whereas IgM antibodies show much higher type specificity for the infecting virus (18–20).

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