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Method for Avoiding False-Positive Results Occurring in Immunoglobulin M Enzyme-Linked Immunosorbent Assays Due to Presence of Both Rheumatoid Factor and Antinuclear Antibodies

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In a double-sandwich immunoglobulin M (IgM) enzyme-linked immunosorbent assay recently developed for the detection of IgM antibodies to *Toxoplasma*, the presence of either rheumatoid factor or antinuclear antibodies did not cause false-positive results. We recently noted, however, that in certain sera containing both rheumatoid factor and antinuclear antibodies, false-positive results do occur. In experiments to define the nature of the cross-reaction, these false-positive results were not found to be a consequence of interactions of the sera with *Toxoplasma* antigens, but rather were due to interactions of rheumatoid factor-antinuclear antibodies with the Fc portion of IgG antibodies used for the enzyme conjugate. This was avoided when the $F(ab')_2$ fragment of IgG was used for the conjugate. The use of such $F(ab')_2$ conjugates did not affect the sensitivity and, thereby, the usefulness of the double-sandwich IgM enzyme-linked immunosorbent assay for the diagnosis of acute acquired or congenital *Toxoplasma* infections. We concluded that $F(ab')_2$ fragments of IgG antibodies can be used as enzyme conjugates to avoid false-positive results in sera positive for both rheumatoid factor and antinuclear antibodies in either the conventional test or in our double-sandwich IgM enzyme-linked immunosorbent assay.

The diagnostic value of the detection of immunoglobulin M (IgM) antibodies in acute acquired and congenital *Toxoplasma* infections is well recognized (1, 23, 26). Although the IgM-indirect fluorescent-antibody (IgM-IFA) test has been used successfully for this purpose, false-positive results do occur in some sera that contain rheumatoid factor (RF) or antinuclear antibodies (ANA) (3, 8, 9, 17). In addition, false-negative results occur and have recently been determined to be due to the presence in the sera of patients of IgG antibodies which compete for IgM antibody binding sites on the organisms (12, 22). Because of these factors, we have recently developed and described a double-sandwich (DS) enzyme-linked immunosorbent assay (ELISA) for the specific detection of IgM antibodies to *Toxoplasma gondii* (19, 20). Results in sera from acutely infected adults and from congenitally infected infants demonstrated that the DS-IgM-ELISA is significantly more sensitive and specific than the IgM-IFA test for diagnosing recently acquired and congenital toxoplasmosis (19, 20). The greater specificity of the DS-IgM-ELISA was evidenced by the fact that sera containing RF or ANA but not IgM antibodies to *T. gondii* were all negative in the DS-IgM-ELISA, whereas false-positive results were observed with these sera in the IgM-IFA test (19, 20). Interactions between IgM-RF and IgG antibodies to *T. gondii* or between IgG-ANA and *T. gondii* nuclear antigens are eliminated in the DS-IgM-ELISA, but not in the IgM-IFA test or in the conventional ELISA procedures described by others (6, 7, 24, 25). In view of these results, we replaced the IgM-IFA test with the DS-IgM-ELISA in routine testing in our laboratory.

Recently, we noted the occurrence of false-positive results in the DS-IgM-ELISA in sera that contain both RF and ANA. Because of the wide applicability of the DS-IgM-ELISA in the diagnosis of infectious diseases, the study reported here was carried out to examine the possible reasons for these false-positive results and to define those conditions necessary to avoid false-positive results.

MATERIALS AND METHODS

Antigens. Antigens were prepared from tachyzoites of the RH strain of *T. gondii* as previously described (20).

Serologic tests. The Sabin-Feldman dye test (DT) (13) and IgM-IFA (26) test were performed as previously described. Titers of RF were determined by latex agglutination (17), and titers of ANA were determined by immunofluorescence (5).

Antibodies to human IgM. The IgG fraction and the $F(ab')_2$ fragment of the IgG fraction of rabbit anti-human IgM (μ-chain specific) were obtained from Cappel Laboratories (Cochranville, Pa.). The specificity of these antibody preparations to human IgM was confirmed as previously described (20).

Enzyme-conjugated antibodies. Alkaline phosphatase conjugates were prepared with the IgG fraction or the $F(ab')_2$ fragment of the IgG fraction of rabbit anti-human IgM (μ-chain specific) as previously described (11). Alkaline phosphatase conjugates were also prepared with the IgG fraction or with the $F(ab')_2$ fragment obtained after pepsin digestion of rabbit anti-*Toxoplasma* immunoglobulins as previously described (4, 11).

IgM-ELISA. The DS-IgM-ELISA method used was that previously described for the detection of IgM antibodies to *Toxoplasma* in acute acquired and congenital toxoplasmosis (19, 20).

The conventional ELISA procedure was performed as described by others (6, 7, 24, 25), except that after the initial coating of wells with *T. gondii* antigens, the wells were postcoated with 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 for 1 h at 37°C.

Human sera. Eighteen sera obtained from patients diagnosed as having acute acquired or congenital toxoplasmosis (diagnosis established by previously described criteria [2, 10, 23]), systemic lupus erythematosus, or rheumatoid arthritis were studied. Sera obtained from seven healthy individuals were pooled to serve as a negative control; these sera were negative in the *Toxoplasma* DT test, the *Toxoplasma* IgM-IFA test, and the *Toxoplasma* DS-IgM-ELISA and were also negative for RF and ANA. A serum sample obtained from a patient 1 month after the clinical onset of acute toxoplasmosis and having titers of 1:16,384 in the DT test and 1:1,280 in the IgM-IFA test served as the positive control in all tests.

RESULTS

DS-IgM-ELISA in sera containing both RF and ANA. Six serum samples containing detectable concentrations of both RF and ANA were tested in the DS-IgM-ELISA under experimental conditions identical to those which yielded negative results in sera containing either RF or ANA. Five of the sera gave false-positive results in the DS-IgM-ELISA (Table 1). Thus, false-positive results occurred with certain sera that contained both RF and ANA.

Experiments performed to evaluate whether *T. gondii* antigens are involved in these false-positive results revealed that these results occurred even when *T. gondii* antigens were omitted from the test and phosphate-buffered saline was used instead of the antigen preparation. The false-positive results also occurred when an $F(ab')_2$ fragment of an IgG fraction of rabbit anti-human IgM antibodies was used in the test (Table 2). In view of these data, interactions among RF, ANA, and the Fc portion of the

TABLE 1. *DS-IgM-ELISA results in sera containing RF and ANA*

Patient no.	Serological test titers				
	RF	ANA	DT	IgM-IFA	IgM-ELISA
1[a]			16,384	1,280	16,384
2	2,560	64	<4	<16	1,024
3	5,120	256	<4	<16	4,096
4	10,240	64	<4	<16	4,096
5	20,480	64	<4	<16	256
6	80	256	<4	<16	<16
7	1,280	1,280	512	256[b]	64

[a] Serum sample obtained from a patient 1 month after clinical onset of acute toxoplasmosis.

[b] *Toxoplasma* IgM-IFA test was negative after absorption of this serum with latex particles coated with human IgG (9).

TABLE 2. *Effect of use of $F(ab')_2$ fragment on false-positive DS-IgM-ELISA results due to RF and ANA*

Patient no.	IgM-ELISA titers	
	Test A[a]	Test B[b]
1[c]	16,384	16,384
2	1,024	<16
3	4,096	<16
4	4,096	<16
5	1,024	<16
6	<16	<16
7	64	<16

[a] Performed with plates coated with $F(ab')_2$ fragment of rabbit IgG antibodies to human IgM and all other reagents as used in our IgM-ELISA.

[b] Performed with alkaline phosphatase-conjugated $F(ab')_2$ fragment of rabbit IgG antibodies to *T. gondii* and all other reagents as used in our IgM-ELISA.

[c] Serum sample obtained from a patient 1 month after clinical onset of acute toxoplasmosis.

enzyme conjugate were then considered.

Effect of using an enzyme-conjugated F(ab')$_2$ fragment of IgG antibodies to *Toxoplasma*. When an alkaline phosphatase-conjugated F(ab')$_2$ fragment of IgG antibodies to *Toxoplasma* was used instead of the IgG enzyme conjugate, false-positive results in sera positive for both RF and ANA did not occur (Table 2). Moreover, the use of the enzyme-conjugated F(ab')$_2$ fragment did not affect the ability to demonstrate high titers of IgM antibodies in the positive control serum as well as in other sera obtained from individuals with acute acquired and congenital toxoplasmosis (Table 3).

Effect of the enzyme-conjugated F(ab')$_2$ fragment on false-positive results observed in a conventional IgM-ELISA. We next examined whether the same effect of the enzyme-conjugated F(ab')$_2$ fragment would correct for the false-positive results obtained in other ELISA procedures. For this purpose, we employed the widely used ELISA in which wells are first coated with antigen and thereafter the human sera to be tested are added (6, 7, 24, 25). After the plates are incubated and washed, enzyme-conjugated antibodies to human IgM are added to determine the presence of reaction between IgM antibodies and the specified antigen (Table 4). It is noteworthy that the use of the enzyme-conjugated F(ab')$_2$ fraction abolished false-positive results due to ANA (e.g., patient 18) and the combination of ANA and RF (e.g., patients 2 through 5) only if the sera did not contain antibodies to *Toxoplasma* as measured in the DT (mainly IgG antibodies).

DISCUSSION

In an effort to find a highly sensitive and specific test for the detection of IgM antibodies to *T. gondii*, we developed a *Toxoplasma* DS-IgM-ELISA (19, 20). This test avoids the false-positive results that occur in the IgM-IFA test, in the conventional IgM-ELISA, and in the IgG-ELISA with sera that contain either RF or ANA (3, 6–9, 17, 24, 25). We were surprised, therefore, when we observed that some sera containing both RF and ANA gave false-positive results both in our DS-IgM-ELISA and in the conventional test.

In attempting to define the interactions that occurred between the sera containing a combination of RF and ANA and the various components used in our DS-IgM-ELISA, several possibilities were considered. First, we examined the possibility of interactions between the sera containing both RF and ANA and the *Toxoplasma* antigen preparations. When phosphate-buffered saline was substituted for the *T. gondii* antigens and the false-positive results still occurred, it became obvious that the false-positive results were not due to a reaction of these sera with *T. gondii* antigens.

We next considered the possibility of interactions between the sera containing both RF and ANA and the IgG antibodies to human IgM used for coating the plates. When plates were coated with the F(ab')$_2$ fragment of IgG antibodies to human IgM rather than with intact IgG antibodies, this substitution did not eliminate the false-positive results. Thus, the false-positive results could not be attributed merely to a reaction between the IgM-RF or IgG-RF

TABLE 3. *Effect of enzyme-conjugated F(ab')$_2$ fragment on detection of IgM antibodies in DS-IgM-ELISA*

Patient no.	Mo from onset of clinical disease to serum collection	Serological test titers			
		DT	IgM-IFA	Test A[a]	Test B[b]
1	1	16,384	1,280	16,384	16,384
8	0.5	8,000	<16	256	256
9	0.75	4,096	16	512	1,024
10	4	4,096	256	1,024	4,096
11	6	4,096	<16	256	256
12	6	16,384	32	64	64
13	7	4,096	<16	64	64
14	8	4,096	<16	64	64
15[c]	5 days after birth	256	128	4,096	4,096
16[d]	3 mo after birth	5	10	4,096	4,096

[a] Performed with alkaline phosphatase-conjugated IgG fraction of rabbit antibodies to *T. gondii* and all other reagents as used in our regular IgM-ELISA.

[b] Performed with alkaline phosphatase-conjugated F(ab')$_2$ fragment of rabbit IgG antibodies to *T. gondii* and all other reagents as used in our regular IgM-ELISA.

[c] Serum obtained from infant with severe congenital toxoplasmosis.

[d] Serum obtained from infant with subclinical congenital toxoplasmosis.

TABLE 4. *Effect of enzyme-conjugated F(ab')$_2$ fragment on false-positive results due to RF or ANA or both in a conventional IgM-ELISA*

Patient no.	Serological test titers				
	RF	ANA	DT	IgM-ELISA[a]	
				A	B
1[b]	−	−	16,384	1,024	1,024
2	+	+	<4	256	<16
3	+	+	<4	1,024	<16
4	+	+	<4	1,024	<16
5	+	+	<4	256	<16
17	+	−	128	256	64
18	−	+	<4	<16	<16

[a] Sera from the specified patients were added to plates coated with *T. gondii* antigens. The attachment of IgM antibodies to wells was tested by the addition of either (A) alkaline phosphatase-conjugated IgG fraction of rabbit antibodies to human IgM or (B) alkaline phosphatase-conjugated F(ab')$_2$ fragment of the IgG fraction of rabbit antibodies to human IgM.

[b] Serum obtained from patient 1 month after clinical onset of acute toxoplasmosis.

and the Fc portion of the IgG used for coating the plates. This conclusion agreed with our previous observations (19, 20) that, in sera containing only RF or ANA, false-positive results do not occur in our *Toxoplasma* DS-IgM-ELISA. These findings and the results of our subsequent experiments reported above suggested that the false-positive results were likely due to interactions between the sera containing both RF and ANA and the enzyme-conjugated antibodies. As demonstrated by the experiments reported here, these false-positive results were eliminated in both tests when the F(ab')$_2$ fragment of IgG antibodies to human IgM (used in the conventional assay) or the F(ab')$_2$ fragment of IgG antibodies to *Toxoplasma* (used in our assay) are employed instead of intact IgG for preparing the enzyme conjugates. Fortunately, the use of the enzyme-conjugated F(ab')$_2$ fragment did not affect the sensitivity of our DS-IgM-ELISA in detecting IgM antibodies to *Toxoplasma*. Thus, sera obtained from individuals with acute acquired or congenital toxoplasmosis exhibited the same IgM antibody titers when tested in the DS-IgM-ELISA with either the F(ab')$_2$ fragment or the IgG antibodies to *T. gondii*. Our ability to eliminate false-positive results by using an F(ab')$_2$ fragment instead of the intact IgG as the enzyme conjugate in both the conventional IgM-ELISA and our DS-IgM-ELISA led to the conclusion that the Fc portion of the IgG used as the enzyme conjugate is the crucial factor for the false-positive results. In addition, it was evident that antibodies to the Fc portion [or the Fc-plus-F(ab')$_2$ portion, but not the F(ab')$_2$ portion alone] of IgG are also essential in the test (patient) serum for these false-positive results to occur. However, as mentioned above, the presence of only IgM-RF or IgG-RF does not cause a false-positive result. Therefore, more complex reactions involving IgM exhibiting anti-IgG activity (but differing in some way from conventional IgM-RF) occurred in sera containing both RF and ANA. There are a number of possible explanations for this phenomenon. It has been shown by Mach et al. (18) that, in sera containing both IgM-RF and IgG-ANA, high-molecular-weight complexes are formed due to the interaction between IgM-RF and IgG-ANA. These authors suggested that in such complexes, IgM-RF acts as an agglutinator by reacting with determinants on the F(ab) portion of IgG complexed with antigens. Moreover, it has been reported (14–16) that, in sera obtained from rheumatoid arthritis patients, approximately 50% of ANA activity can be attributed to a cross-reacting antibody, usually of the IgM class but in all likelihood also of the IgG class, that reacts with the Fc portion of IgG as well as with nucleohistone. Thus, a cross-ligating or highly cross-reactive antibody that has antibody determinants on its F(ab')$_2$ portion and is capable of binding to antigenic determinants on dissimilar molecules can be demonstrated in sera from patients exhibiting both RF and ANA. Furthermore, naturally occurring anti-F(ab')$_2$ antibodies which are cross-reacting, auto-anti-idiotypic antibodies have been demonstrated recently (21) in sera from patients with systemic lupus erythematosus. Thus, in sera positive for RF and ANA, several phenomena may occur (Fig. 1). There may be complexes of IgM-RF, IgG-ANA (18), and anti-F(ab')$_2$ (21) antibodies which bind the solid-phase F(ab')$_2$ portion or the F(ab')$_2$-plus-Fc portion of the rabbit IgG antibody against human IgM to the Fc portion of the alkaline phosphatase-labeled rabbit IgG antibody to *Toxoplasma*. It is also possible that the cross-reactive ligands (data not shown) with RF-ANA activity (14–16) and anti-F(ab')$_2$ antibodies (21) may bind the solid-phase F(ab')$_2$ portion or the F(ab')$_2$-plus-Fc portion of the rabbit IgG antibody against human IgM to the alkaline phosphatase-labeled Fc portion of the rabbit IgG antibody to *Toxoplasma*. Either of these possibilities would produce false-positive results only if there were an Fc portion available on the

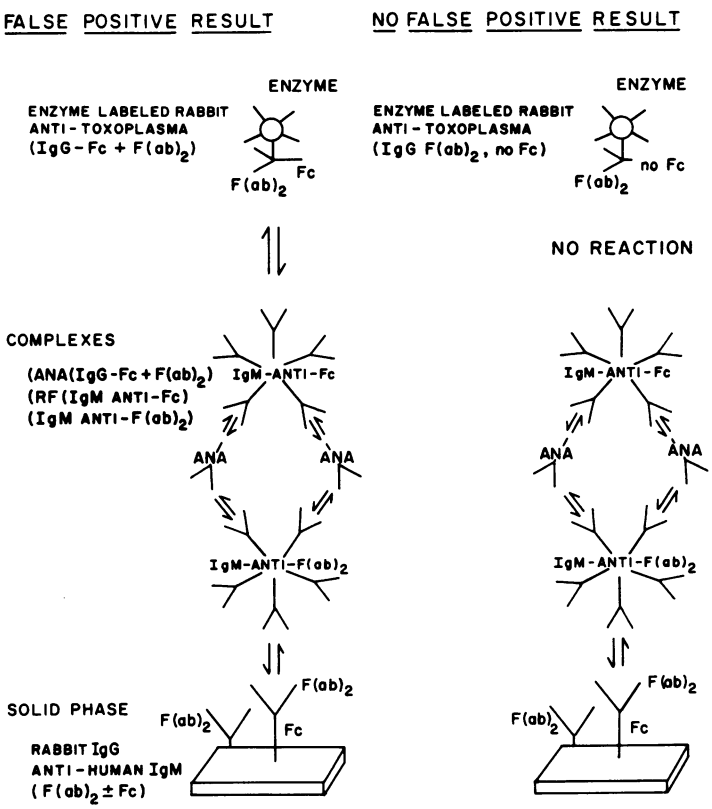

FIG. 1. *Hypothetical mechanism for false-positive result in DS-IgM-ELISA due to RF and ANA.*

alkaline phosphatase-labeled anti-*Toxoplasma* antibody (Fig. 1). Which of these phenomena are involved in the false-positive results we have observed is not clear and is under study in our laboratories. It is of importance that these false-positive results can be avoided by using the $F(ab')_2$ fraction instead of intact IgG for the enzyme conjugate, and this should be considered in any ELISA system.

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