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Enzyme-Linked Immunosorbent Assays for Detection of Immunoglobulin M to Nontreponemal and Treponemal Antigens for the Diagnosis of Congenital Syphilis

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Sera from newborn infants born of mothers with a high risk of syphilis were examined for immunoglobulin M (IgM) antibodies in two different enzyme-linked immunosorbent assays (ELISAs), using either purified flagella from *Treponema phagedenis* biotype Reiter or the Venereal Disease Research Laboratory (VDRL) antigen as the antigen. All sera were also examined by the fluorescent treponemal antibody absorption (FTA-ABS) test for IgM. Three different groups of patients were studied. Group 1 consisted of 84 women and their newborn infants from a high-risk population for syphilis. Congenital syphilis was diagnosed in one child who had an IgM-positive cord blood specimen in both the ELISA and the FTA-ABS test. Group 2 consisted of 10 mothers and their newborn children. All mothers had positive syphilis-screening tests, and all children had signs of congenital syphilis. All but one child had positive IgM tests. Group 3 consisted of 15 mothers and their newborn children. These mothers had been treated for syphilis late in pregnancy, and all had a positive screening test at delivery. Two of the children had positive IgM tests, probably caused by reactivity after late intrauterine treatment of congenital syphilis. The specificities of the IgM tests were high when evaluated with sera from newborn children without signs of congenital syphilis. Even though IgM rheumatoid factor was found in all of the children tested with definite congenital syphilis, the rheumatoid factor did not cause false-positive results in either the VDRL ELISA or the flagellum ELISA. No significant IgG-IgM competition was noticed in the ELISAs. This study also confirmed that IgA antibodies do not cross the placenta; most newborn children with congenital syphilis were positive in the VDRL ELISA for IgA. Both the VDRL ELISA and the flagellum ELISA are very useful in the diagnosis of congenital syphilis and may be a substitute for the FTA-ABS test. The VDRL ELISA for IgM will be especially useful in developing countries with a high incidence of congenital syphilis.

The incidence of congenital syphilis is low in the developed countries, primarily because of the low incidence of acquired syphilis and the well-organized prenatal syphilis screening of pregnant women. In many developing countries, however, the incidence of congenital syphilis is very high, and it contributes significantly to the high rate of fetal wastage, neonatal mortality, and infant morbidity. In these countries, the incidence of acquired syphilis is also very high and the prenatal screening for syphilis is still not well organized. The prevalence of syphilis seroreactivity in pregnant women attending prenatal clinics in Africa may range from 5 to 15% (6, 15, 18), and it has been estimated that 6.5% of newborn children in Lusaka, Zambia, have a positive syphilis serological test and that 1% have signs of congenital syphilis (6, 15). Congenital syphilis may be responsible for 25 to 30% of the perinatal mortality rate of 50/1,000 births in Lusaka (5).

The diagnosis of congenital syphilis can be difficult, even in countries with advanced health systems, and often relies on serological testing. Newborn infants of mothers with reactive syphilis serological tests will themselves have reactive tests whether or not they have become infected, because of passive transplacental transfer of maternal immunoglobulin G (IgG) antibodies. If, however, specific IgM or IgA antibodies are present in the child's serum, it is a reflection of fetal antibody production and therefore infection, because IgM and IgA antibodies do not cross the intact placenta. The reference method for detection of specific IgM antibodies in congenital syphilis is the fluorescent treponemal antibody absorption (FTA-ABS) test, using a fluorescence-conjugated anti-human IgM antibody (1, 7, 19–21). The FTA-ABS IgM test is not widely used because it is laborious and because there have been doubts about both the specificity and the sensitivity of the test (4, 7, 9, 16). IgM rheumatoid factors in the child may give rise to false-positive results, and false-negative results may be the result of competitive inhibition of IgM by maternal IgG. Gel filtration and ultracentrifugation have been used to separate the immunoglobulin fractions in serum before testing, a time-consuming procedure (2, 9, 17).

Previously, we have described an enzyme-linked immunosorbent assay (ELISA) for IgM antibodies against purified flagella from the nonpathogenic *Treponema phagedenis* biotype Reiter (13). No interaction of rheumatoid factors and no IgG-IgM competition was observed in this sensitive and specific ELISA, and it was suggested that this test could be useful for diagnosis of congenital syphilis in newborns.

The nontreponemal tests have so far not been very useful because they are not able to distinguish between IgG and IgM antibodies without fractionation of the serum. However, we recently described an easy ELISA that is able to detect IgG and IgM antibodies against the Venereal Disease Research Laboratory (VDRL) antigen (12).

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TABLE 1. Results of syphilis serological tests on sera from mothers and their newborn children

Patient group[a] (no. of serum samples)	No. (%) of positive serum samples by:						
	VDRL ELISA			Flagellum ELISA		FTA-ABS	
	IgG	IgA	IgM	IgG	IgM	IgG, IgA, and IgM	IgM
Group 1 (84)							
Mothers	14 (16.7)	8 (9.5)	15 (17.9)	29 (34.5)	Not done	30 (35.7)	Not done
Children	14 (16.7)	1 (1.2)	1 (1.2)	28 (33.3)	1 (1.2)	29 (34.5)	1 (1.2)
Group 2 (10)							
Mothers	10 (100)	9 (90)	10 (100)	10 (100)	7 (70.0)	10 (100)	1 (10)
Children	10 (100)	7 (70)	9 (90)	10 (100)	9 (90)	10 (100)	9 (90)
Group 3 (15)							
Mothers	13 (86.7)	8 (66.7)	10 (66.7)	15 (100)	5 (33.3)	15 (100)	0 (0)
Children	12 (80.0)	2 (13.3)	2 (13.3)	15 (100)	2 (13.3)	15 (100)	1 (6.7)

[a] Group 1, mothers admitted to labor ward and their newborn children; group 2, mothers with untreated syphilis and their newborn children with signs of congenital syphilis; group 3, mothers treated for syphilis late in pregnancy and their newborn children.

The purpose of the present study was to evaluate the VDRL ELISA and the flagellum ELISA for the diagnosis of congenital syphilis in newborns.

MATERIALS AND METHODS

Study population. Serum specimens for this study were obtained from different sources and stored at −20°C for various intervals until use. Three groups of patients were studied. Group 1 consisted of 84 consecutive pregnant women admitted to the labor ward of Maun Hospital, Botswana, in a 2-month period of 1986. Blood samples were obtained at delivery from the mothers, and cord blood samples were obtained from the children. Documentation of whether mothers had been screened for syphilis during pregnancy and had received antisyphilitic treatment was obtained from their prenatal card. Groups 2 and 3 consisted of mothers and children from Lusaka, Zambia. Over a period of 8 weeks in 1982, mothers and newborn babies admitted to the University Hospital of Lusaka, Lusaka, Zambia, were screened for possible syphilis. All mothers were asked about their past histories of genital ulcer or VDRL reactivity and about whether they had received antisyphilitic treatment. The mothers and the babies had a thorough clinical examination, and serum was examined by the rapid plasma reagin (RPR) test. Cases in which mothers or children had reactive serology were selected for detailed examination. If possible, babies were X rayed for defects of the long bones. Regardless of diagnosis, all children with a reactive RPR test were treated with penicillin. Group 2 consisted of 10 mothers and their children. The mothers all had a reactive RPR test, and none of them received antisyphilitic treatment during pregnancy. Group 3 consisted of 15 mothers and their children. All mothers had a reactive RPR test at delivery, and all received antisyphilitic treatment during pregnancy.

Serological tests. All sera were tested by the RPR test according to the instructions of the manufacturers (Hinson, Westcott & Dunning). At Statens Seruminstitut in Copenhagen, sera were analyzed by FTA-ABS, flagellum ELISA, and VDRL ELISA. The conjugate used in FTA-ABS was a rabbit anti-human IgG, IgA, and IgM antiserum (code F 1009; DAKO Immunoglobulins, Copenhagen, Denmark) diluted 1:200 or a rabbit anti-human IgM antiserum (code F203, DAKO Immunoglobulins) diluted 1:80. The ELISA for IgG antibodies against purified flagella from *T. phagedenis* biotype Reiter was performed as described elsewhere (14). The ELISA for IgM antibodies against purified flagella was performed as described previously (13). Briefly, IgM in the diluted test sample was bound to anti-IgM-coated microdilution plates, and flagellum-specific IgM antibody was subsequently detected by incubation with a purified flagellum preparation and horseradish peroxidase-conjugated monospecific antiflagellum antibodies. The VDRL ELISA has been described elsewhere (13). Briefly, microdilution plates were coated with an ethanol solution of the VDRL antigen. Diluted serum was added to the wells, and bound IgG and IgM were detected by incubation with a horseradish peroxidase-conjugated anti-human IgG, anti-human IgA, or anti-human IgM conjugate.

Test results in the flagellum ELISA and the VDRL ELISA were based on twofold titration. The cutoff was defined as the absorbance value that represented 20% of the absorbance of a positive control serum. In the VDRL ELISA for IgG, the cutoff value was the 99.8 negative percentile, and for IgM the cutoff value was the 99.4 negative percentile, defined by testing sera from 500 blood donors. The cutoff value of the flagellum ELISA for IgG was the 99.0 percentile, and for IgM the cutoff value was the 99.5 percentile, defined by testing sera from 200 blood donors.

All sera from children in group 2 and group 3 and the serum from an IgM-positive child in group 1 were tested for rheumatoid factor by ELISA (23). Sera positive for IgM rheumatoid factor were absorbed by addition of anti-human IgG antibody (RF-absorbens, lot 407026 A; Behring-Werke, Federal Republic of Germany) according to the instructions of the manufacturer. The effectiveness of the procedure in eliminating the interaction of rheumatoid factor and IgG-IgM competition has previously been described (3, 10, 24). After absorption, the sera were tested in the VDRL ELISA for IgM antibodies and the obtained titers were compared with those of nonabsorbed sera.

RESULTS

The results of the serological testing of 84 mothers admitted for delivery and of their newborn children (group 1) are shown in Table 1. Fourteen of the mothers had not been screened for syphilis during pregnancy, and two of these had positive syphilis serological tests at delivery. One mother had a weak reactivity in the VDRL ELISA for IgG and IgM

but was strongly positive in the flagellum ELISA and the FTA-ABS test. Her child did not have specific IgM antibodies and was apparently healthy at birth. The other mother was strongly reactive in the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test. Her child had specific IgM and IgA antibodies detected by the VDRL ELISA and specific IgM antibodies detected by the flagellum ELISA and the FTA-ABS test, and the child died within hours after birth from congenital syphilis.

Twelve of the mothers in group 1 had received antisyphilitic treatment during pregnancy because of a reactive RPR test. At delivery nine of these mothers were still reactive in the VDRL ELISA, and all but one were reactive in the flagellum ELISA and the FTA-ABS test. None of their children had specific IgM or IgA antibodies. Three mothers in group 1 were found to be reactive by the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test at delivery, although they had been screened by RPR in pregnancy and were found to be negative. One of these mothers had a very strong reactivity in all syphilis serological tests, indicating untreated, early syphilis. None of the children of these three mothers had specific IgA or IgM antibodies. The specificities of the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test for IgM antibodies in the cord sera was high because no false-positive results were found.

A total of 25 mothers in groups 2 and 3 from Lusaka, Zambia, had a positive RPR test at delivery (Table 1). Ten of these (group 2) had not been screened for syphilis and had not received antisyphilitic treatment during pregnancy, while 15 (group 3) had received antisyphilitic treatment during pregnancy because of a positive RPR test. All of the untreated mothers (group 2) except two had signs suggestive of syphilis, 10 had generalized lymphadenopathy, and 2 of these also had condylomata and one had a skin rash. All children of these mothers had clinical signs of congenital syphilis (that is, hepatomegaly [n = 10], jaundice [n = 5], skin rash [n = 6], and abnormal X ray of the long bones [n = 7]), and all children were premature (birth weight, <2,500 g). The serological data of the mothers and children are shown in Table 1, and the titers of the VDRL ELISA and flagellum ELISA for IgM on paired serum samples from mothers and children are shown in Fig. 1. All but one of the children in group 2 had serological findings indicative of congenital syphilis, since they had IgM antibodies according to the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test. Typical titration curves from the VDRL ELISA for IgG, IgA, and IgM of serum specimens from a child with congenital syphilis and from its mother are shown in Fig. 2. Five of the children with IgM reactivity had significantly higher titers in the VDRL ELISA for IgG than did their mothers, and none had a significantly lower titer (Fig. 1). In the flagellum ELISA for IgG, none of the children with IgM reactivity had significantly higher titers than did their mothers, whereas six had significantly lower titers (Fig. 1). Two children had specific IgM antibodies in the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test but did not have IgA antibodies in the VDRL ELISA (Table 1). The one child (Fig. 1, number 10) without specific IgM antibodies had hepatomegaly and abnormal X ray of the long bones and was regarded as having congenital syphilis.

In the group of mothers that had been treated in pregnancy (group 3), three mothers had received a treatment that was regarded as inadequate. Three had been treated in the second trimester, while 12 had been treated in the third trimester; 11 of the mothers had lymphadenopathy, while four had no signs indicative of syphilis. Three of the children were premature, while the rest were full term. Four children had hepatomegaly, including one premature child. None of the children had abnormal X rays of the long bones. The serological results are shown in Table 1, and the titers for paired samples from mothers and children in the VDRL ELISA and the flagellum ELISA for IgG and IgM antibodies are shown in Fig. 1. Only two of the children had serological findings indicative of congenital syphilis (Fig. 1, numbers 24 and 25). One child was born to a mother who was treated in the 28th week of pregnancy; this child was full term but had hepatomegaly. The other child was born to a mother who had received therapy during the 30th week of pregnancy. The child was premature but had no other signs of congenital syphilis.

Eight of the ten children in group 2 were tested for IgM rheumatoid factor, and six were found to be positive. Two sera were not tested due to lack of serum. None of the 15 tested children in group 3 had sera with IgM rheumatoid factor. The child in group 1 with congenital syphilis also had an IgM rheumatoid factor. The median concentration of rheumatoid factor in the seven positive children was 26 IU/ml (range, 14 to 87 IU/ml). After absorption with anti-human IgG, none of the seven sera contained rheumatoid factor. The titer in the VDRL-ELISA for IgM antibodies was not significantly different before and after absorption, since five serum samples had the same titer and two serum samples had a twofold-increased titer.

DISCUSSION

Congenital syphilis in the newborn may be clinically indistinguishable from other congenital infections, notably rubella, cytomegalovirus infections, and toxoplasmosis. Hepatomegaly, petechial rashes, thrombocytopenia, and anemia are common in all these congenital infections.

The results of the present study indicate that both the VDRL ELISA and the flagellum ELISA for IgM antibodies may be useful in the diagnosis of congenital syphilis, especially the VDRL ELISA, which is inexpensive and easy to perform and is therefore useful in developing countries with a high incidence of congenital syphilis. The flagellum ELISA for IgM antibodies is somewhat more laborious because it uses purified flagella from *T. phagedenis* biotype Reiter and a monospecific horseradish peroxidase-conjugated antibody against flagella, but it is a useful test in a reference laboratory.

The specificities of the tests were compared on paired serum samples from mothers and their newborn infants (Table 1, group 1). Neither the VDRL ELISA, the flagellum ELISA, nor the FTA-ABS test for IgM antibodies revealed false-positive results. One infant in this group was correctly diagnosed as having congenital syphilis, since IgM antibodies were detected by all three tests and IgA antibodies were detected by the VDRL ELISA. The sensitivities of the tests for IgM antibodies were evaluated with paired sera from mothers found to be RPR positive (Table 1, group 2) and their newborn premature children. These children had all clinical findings suggestive of congenital syphilis, and all but one had IgM antibodies detected by the VDRL ELISA, the flagellum ELISA, and the FTA-ABS test (Table 1 and Fig. 1). Seven of the nine tested children with definite congenital syphilis had IgM rheumatoid factors, which are known to be frequent in congenital infections in newborn children (11, 16, 22). Rheumatoid factors, however, did not cause false-positive results in the VDRL ELISA for IgM, and IgG-IgM competition did not cause lower titers, since the titer in the

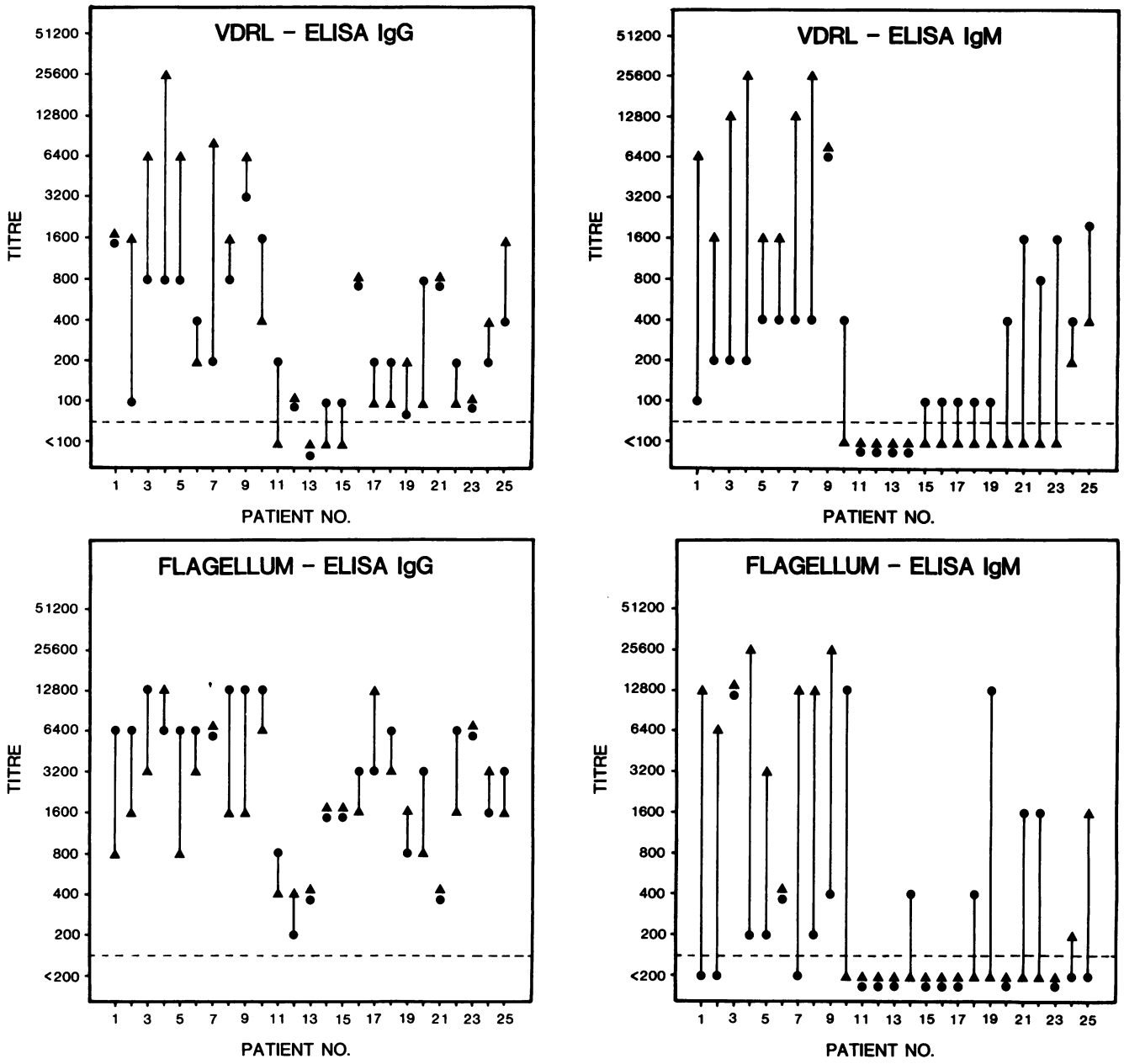
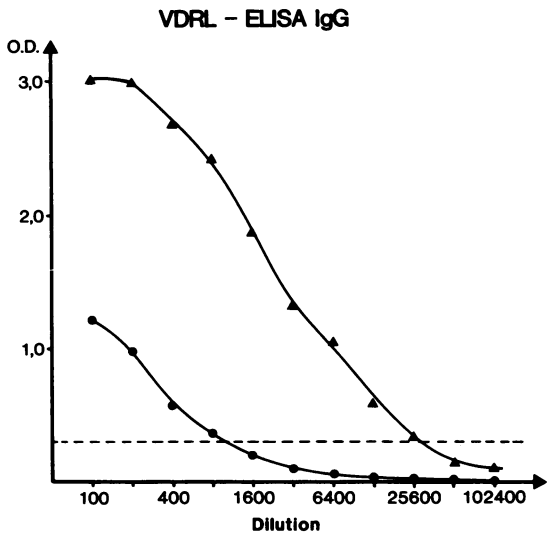
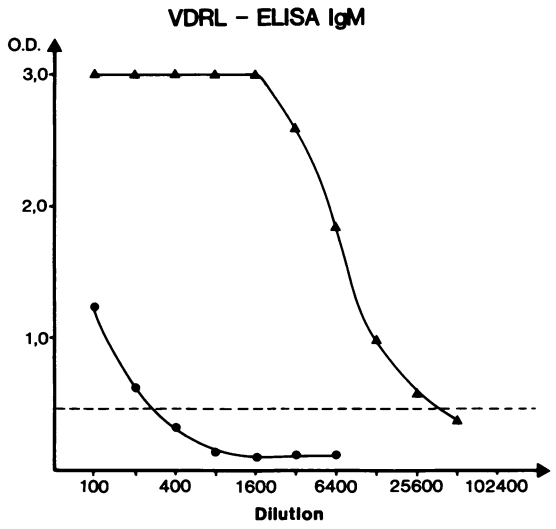


FIG. 1. VDRL ELISA and flagellum ELISA results for paired serum specimens from mothers (●) and their newborn children (▲). Pairs 1 to 10 constitute group 2 (mothers with untreated syphilis and their newborn children with signs of congenital syphilis). Pairs 11 to 25 constitute group 3 (mothers treated for syphilis late in pregnancy and their newborn children). Sera with titer values above the dashed horizontal line were regarded as reactive. Each point is the mean value of two determinations.

VDRL ELISA for IgM did not change significantly after removal of rheumatoid factors and IgG by an anti-human IgG antibody. Neither IgG-IgM competition nor rheumatoid factor interaction was observed in the flagellum ELISA for IgM antibodies (13). However, in the FTA-ABS test for IgM antibodies the reactivities were weaker; one serum gave only a 1+ reaction, while the rest gave 2+ reactions. These relatively weak reactions were most likely caused by IgG-IgM competition, which is a well-known phenomenon in other indirect immunofluorescence tests (4). The infant (Fig. 1, number 10) who was IgM nonreactive in all tests had hepatosplenomegaly, jaundice, anemia, and abnormal X ray of the long bones. The infant was number one of twins; the other baby was stillborn and macerated. In other studies, FTA-ABS tests for IgM have been found to be positive whenever an infant had obvious syphilis (1, 8, 19, 20), and it is difficult to explain why all the IgM tests were negative. The child did not have rheumatoid factor, which was found in all tested children with definite congenital syphilis. We regard this child as having congenital syphilis, but it cannot be ruled out that the child could have another congenital infection.

Five of the children in group 2 had a significantly higher titer in the VDRL ELISA for IgG than their mothers did (Fig. 1), indicating that these children produced IgG against the VDRL antigen in addition to the passively transferred





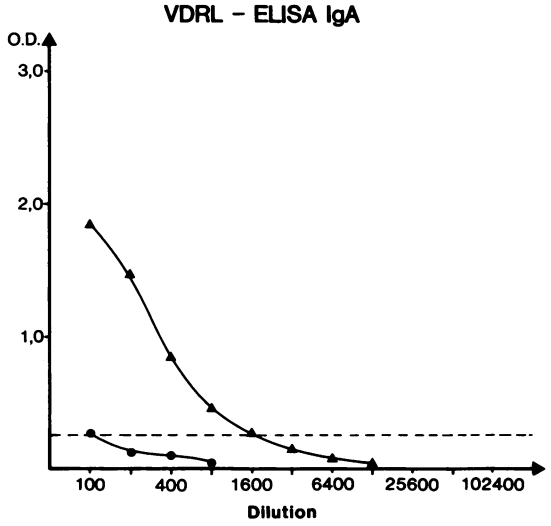


FIG. 2. Titration curves in the VDRL-ELISA for IgG, IgA, and IgM with paired sera from a child with congenital syphilis (▲) and its mother (●). The reactive titer is the highest dilution that gives an optical density value above the dashed horizontal line. O.D., Optical density at 492 nm.

IgG from their mother. In the flagellum ELISA for IgG, none of the children had a significantly higher titer than their mother. However, six mothers had significantly higher titers than their children did. This probably reflects a difference in the antibody response in these premature children against a lipid antigen and a protein antigen. In adults, the antibody response against the VDRL antigen may be T-cell independent, since IgM antibodies are found even in long-lasting untreated infections (12) and in reinfection (5) (unpublished results). However, IgM antibodies against the flagella are seldom found in reinfections and long-lasting infections (13). It is characteristic that both IgG and IgM antibodies against VDRL antigen decline very quickly after antisyphilitic treatment, whereas only IgM antibodies against the flagellum antigen decline quickly. IgG antibodies against flagella may persist for many years.

The present study also confirms that IgA antibodies do not cross the intact placenta and that detection of specific IgA in the newborn indicates congenital infection. The results of this study suggest that the VDRL ELISA for IgA may be a useful supplement to the VDRL test for IgM antibodies when the serological diagnosis of congenital syphilis is to be made.

In the group of mothers treated for syphilis during pregnancy (group 3) and their newborn infants, two of the children had IgM antibodies according to the VDRL ELISA and the flagellum ELISA. It was not possible to determine whether the IgM antibodies detected indicated reactivity after a sufficiently treated intrauterine infection or whether they indicated active congenital syphilis. None of these children had rheumatoid factors.

Diagnosis and management of congenital syphilis in newborns will continue to be a problem. Although the IgM-specific serological tests may be very useful, some infected babies may not show clinical signs at birth and may also be nonreactive in the tests. If the fetus first becomes infected late in pregnancy, the child may be born apparently healthy without specific IgM antibodies at birth. A negative IgM test thus does not exclude incubating syphilis and, in rare cases, may not even exclude symptomatic congenital syphilis. In countries with a high prevalence of infection and a poor follow-up of patients, all children born of mothers with a positive screening test for syphilis should receive antisyphilitic treatment as soon as possible after birth.

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