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Study of Treatment of Congenital *Toxoplasma gondii* Infection in Rhesus Monkeys with Pyrimethamine and Sulfadiazine

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The efficacy of the combination of pyrimethamine and sulfadiazine for the treatment of congenital *Toxoplasma gondii* infection in rhesus monkeys was studied. The dosage regimen for pyrimethamine and sulfadiazine was established by pharmacokinetic studies in two monkeys. Those studies showed that the distributions of both drugs followed a one-compartment model. The serum elimination half-lives were found to be 5.2 h for sulfadiazine and 44.4 h for pyrimethamine. Sulfadiazine reached a maximum concentration in serum of 58.7 μg/ml, whereas a maximum concentration in serum of 0.22 μg/ml was found for pyrimethamine. Ten monkeys were infected intravenously with *T. gondii* at day 90 of pregnancy, which is comparable to the second trimester of organogenetic development in humans. Treatment was administered to six monkeys, in whose fetuses infection was diagnosed antenatally. From the moment that fetal infection was proven, the monkeys were treated throughout pregnancy with 1 mg of pyrimethamine per kg of body weight per day and 50 mg of sulfadiazine per kg of body weight per day orally. The therapy was supplemented with 3.5 mg of folinic acid once a week. No toxic side effects were found with this drug regimen. The parasite was no longer detectable in the next consecutive amniotic fluid sample, taken 10 to 13 days after treatment was started. Furthermore, *T. gondii* was also not found in the neonate at birth. The parasite was still present at birth in three of four untreated fetuses that served as controls. Both drugs crossed the placenta very well. Concentrations in fetal serum varied from 0.05 to 0.14 μg/ml for pyrimethamine and from 1.0 to 5.4 μg/ml for sulfadiazine. In addition, pyrimethamine was found to accumulate in the brain tissue, with concentrations being three to four times higher than the corresponding concentrations in serum. Thirty percent of the sulfadiazine was found to reach the brain tissue when compared with the corresponding serum drug concentration. When administered early after the onset of infection, the combination of pyrimethamine and sulfadiazine was clearly effective in reducing the number of parasites in the fetus to undetectable levels.

A congenitally acquired *Toxoplasma gondii* infection is generally treated with the combination of pyrimethamine and sulfadiazine (37). Both components are inhibitors of folate metabolism, acting synergistically to suppress the proliferation of the parasite by indirect impairment of DNA synthesis (16, 48).

Several studies in mice revealed that the combination of pyrimethamine and sulfadiazine reduces the parasitic load and prolongs the survival of mice after infection with a lethal dose of *T. gondii* (14, 31, 32). Studies on the effectiveness of pyrimethamine and sulfadiazine for the treatment of congenital *T. gondii* infections in humans, however, have been scarce. Those studies that have been reported in the literature are from France (10, 11, 20). Those studies described the effectiveness of treatment with pyrimethamine and sulfadiazine after fetal infection had been proven. The studies revealed that in utero treatment significantly reduced the number of infected offspring with severe toxoplasmosis. In addition, a relative decrease in benign to subclinical forms of infection was found. However, several of the offspring were still found to have intracranial calcifications or retinal scars which developed into chorioretinitis (10, 11, 20). It is not known whether these findings were due to the fact that the onset of treatment was started too late after fetal infection or whether the concentrations of pyrimethamine and sulfadiazine in the fetus were too low to be effective.

On the other hand, the effects described by the French investigators (10, 11, 20) cannot solely be attributed to pyrimethamine and sulfadiazine, since all patients were also treated with spiramycin throughout pregnancy, from the moment that maternal infection was diagnosed or suspected. An effect of the antibiotic cannot be ignored. A former study in the rhesus monkey showed that spiramycin reduces the parasitic load to undetectable levels in congenitally infected fetuses (40). Therefore, some of the effects in the French studies may have been caused by spiramycin.

In the present study, the efficacy of pyrimethamine-sulfadiazine was investigated in infected rhesus monkey fetuses while treatment was started early after the onset of fetal infection. Besides this, studies of the pharmacokinetics of these drugs were performed in this animal species for precise determination of the dosage regimen. Although extensive studies have been performed on the pharmacokinetics of both pyrimethamine and sulfadiazine in a variety of animals, little is known about the pharmacokinetics of the drugs in rhesus monkeys. The disposition of sulfadiazine has been investigated in young rhesus monkeys of 0.5 to 1.5 years of age (29, 30). However, results obtained in immature monkeys may be of limited relevance to adult monkeys, as has been found in pigs (15). The kinetics of sulfadiazine in adult monkeys have not been reported. The pharmacological studies of pyrimethamine in rhesus monkeys by Schmidt et al. (38) focused mainly on tissue localization.

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In the present study the kinetic parameters of pyrimethamine and sulfadiazine were determined in adult monkeys receiving a drug regimen that is applied to humans for the treatment of congenital *T. gondii* infections. Following this, transplacental transfer and the distributions of the drugs in tissue were investigated in pregnant monkeys. The effects of the treatment are related to the concentrations of pyrimethamine and sulfadiazine that are reached in the fetus.

MATERIALS AND METHODS

Monkeys. All monkeys were derived from the Laboratory Animals Breeding Experimental Farm of Shunde Guangdong, Beijing, People's Republic of China. Breeding of the monkeys and animal care have been described before (41).

The treatment group consisted of 10 female monkeys (monkeys D1 to D10) that were seronegative for antibodies to *T. gondii* before the experiments were started. A group of four untreated monkeys (monkeys A6 to A9), in whose fetuses infection had been proven, served as controls. This control group has been described in detail before (41). The purpose of the former study was to investigate whether the rhesus monkey was a suitable animal for use in the study of congenital *T. gondii* infections (41). In addition, we meant to use this group of monkeys as untreated controls. The outcome of fetal infection in the control group is summarized in this report. The fetuses and babies of the monkeys are indicated by D_f1 to D_f10 for the treatment group and A_f6 to A_f9 for the control group.

The pharmacokinetics of pyrimethamine and sulfadiazine were studied in two nonpregnant monkeys (monkeys E and F) after oral administration of a single dose of 1 mg of pyrimethamine per kg of body weight in combination with 50 mg of sulfadiazine per kg of body weight.

Pharmacokinetic studies with two nonpregnant monkeys. Tablets of pyrimethamine (Daraprim) were obtained from Wellcome Pharmaceuticals, Utrecht, The Netherlands, and capsules containing sulfadiazine were provided by the Department of Clinical Pharmacy of the University Hospital Nijmegen. Sulfadiazine was obtained from the Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands).

The pharmacokinetics of pyrimethamine and sulfadiazine were studied in nonpregnant monkeys E and F after oral administration of 1 mg of pyrimethamine per kg in combination with 50 mg of sulfadiazine per kg. These dosages were chosen because they are normally used to treat congenital *T. gondii* infections in humans (27, 34, 37).

Pulverized pyrimethamine and sulfadiazine were hidden in a small hole within a LIGA biscuit (General Biscuits Nederland BV, Rozendaal, The Netherlands) covered with marmelade or in a small piece of banana, and the monkeys were given the food.

Blood samples of 5 ml were collected from the vena mediana just before and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48, 72, and 96 h after drug administration. The blood samples were centrifuged at 800 × *g* for 10 min. The sera were collected and stored at −20°C until analysis.

Experimental design and collection of clinical samples. A time schedule of sample collection is shown in Fig. 1A. A *T. gondii* infection was induced in the monkeys at day 90 of pregnancy. The time point of infection is comparable to the second trimester of organogenetic development in humans. The infection was induced by intravenous inoculation of 5×10^6 tachyzoites of *T. gondii* (RH strain). Maternal parasitemia was tested for 8 days after inoculation of the monkeys, at amniotic punctures, and at the time of delivery by cesarean section as described before. In brief, all clinical samples (blood, amniotic fluid, placenta, and fetal tissues) were tested for the presence of *T. gondii* by inoculation of mice (44) and by nested PCR (39, 41).

Fetal infection in the control group was tested 10 days after inoculation of the mother by examination of fetal blood and amniotic fluid. The transmission of infection was examined again at birth by testing the amniotic fluid and the blood and tissues of the neonates (Fig. 1B) (41).

To monitor the effect of treatment during pregnancy, two additional samples obtained by amniotic puncture were included in the protocol for the treatment group. Amniotic fluid samples were thus obtained at 10, 25, and 40 days after inoculation of the mother (Fig. 1C). A cesarean section was performed at day 160 of pregnancy, which was about 10 days before the expected date of delivery. Amniotic fluid, placental tissue, and neonatal blood from the umbilical cord were collected and tested for the presence of *T. gondii*. The mothers and neonates were sacrificed, and autopsies were performed. The heart, brain, spleen, liver, and lungs were examined for the presence of *T. gondii*.

Processing of clinical samples. The protocols for the processing of the clinical samples have been described in detail previously (40, 41, 44). In brief, 6 ml of amniotic fluid was collected at each amniotic puncture. Two mice were each inoculated intraperitoneally with 1 ml of amniotic fluid to test for the presence of *T. gondii*. The remaining 4 ml was divided into portions of 1 ml each, and each portion was centrifuged at 800 × *g* for 10 min. The sediments of each portion were resuspended in 100 μl of physiological salt solution. DNA was isolated from two portions with guanidinium thiocyanate and silica particles exactly as described by Boom et al. (6). The remaining two portions were stored at −80°C.

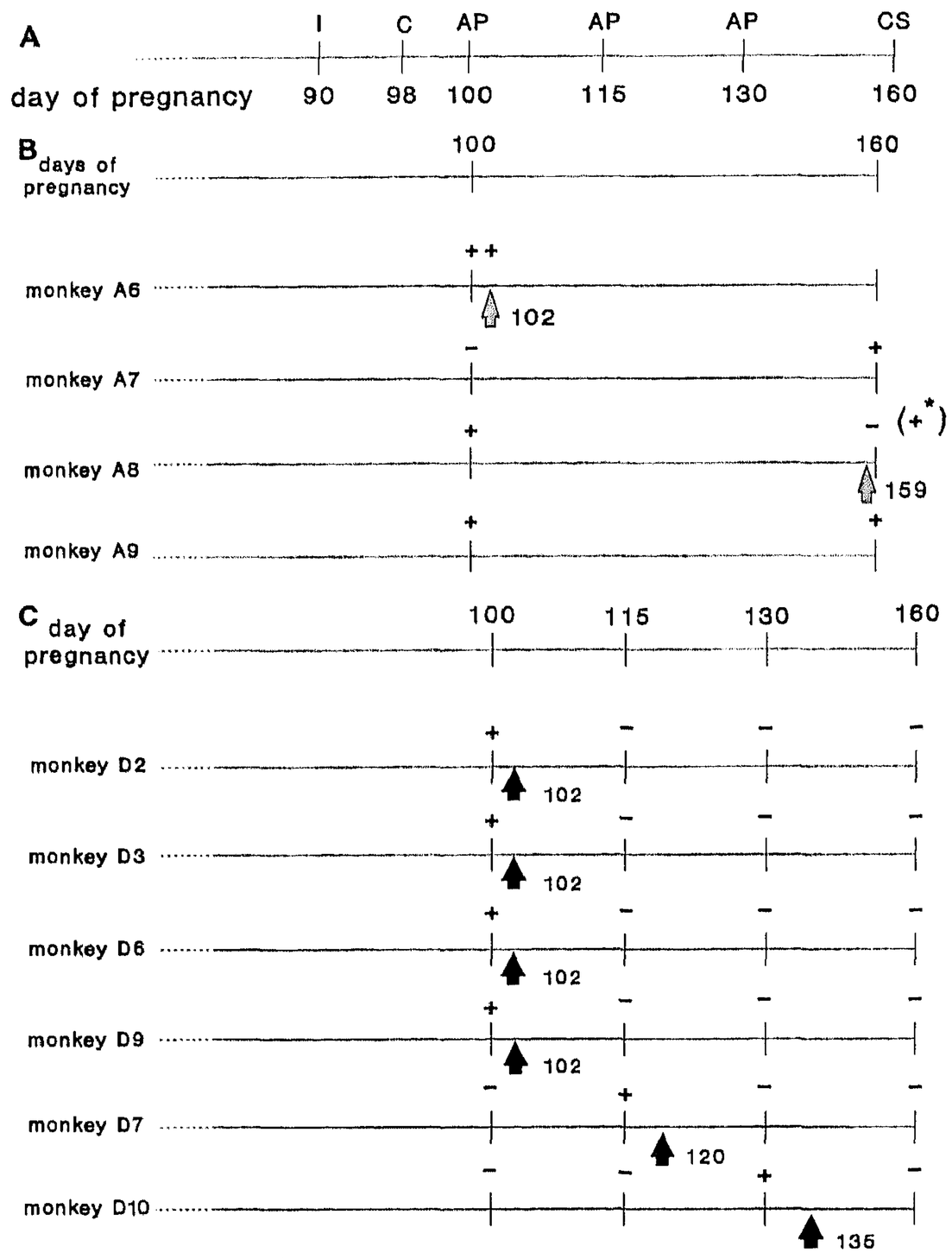

FIG. 1. (A) Time schedule of sample collection during pregnancy. I, infection, 90 days after conception; C, collection of maternal blood for control of parasitemia; AP, amniotic puncture; FBS, fetal blood sampling; CS, delivery by cesarean section. During fetal blood sampling both amniotic fluid and fetal blood were collected. During delivery by cesarean section amniotic fluid, neonatal blood, and the placenta were collected. Delivery by cesarean section was followed by autopsy of the neonate, and the heart, spleen, brain, and liver were obtained. All samples were tested for the presence of *T. gondii*. (B) Results for the four monkeys in the control group that received no treatment. The detection of *T. gondii* in the amniotic fluid and/or neonatal tissues is indicated with a plus sign. A premature birth is indicated by an arrow and the duration of pregnancy. +*, fetal infection proven by the demonstration of antibodies in the neonate at serological follow-up. (C) Results for the six monkeys in the study group that were treated with pyrimethamine-sulfadiazine. The detection of *T. gondii* in the amniotic fluid is indicated with a plus sign. The days at which treatment with pyrimethamine-sulfadiazine was started are marked with arrows.

The placenta and neonatal organs were homogenized in physiological salt solution as described before (40). Each sample was tested in duplicate for the presence of parasites by inoculation into mice. DNA was isolated from the tissues by incubation in 6% *p*-aminosalicylic acid–1% NaCl–10 mM EDTA with 0.5 μg of proteinase K per ml at 37°C for 3 h. After phenol extraction, the DNA was precipitated overnight at −20°C. One microgram of the tissue DNA was tested for the presence of *T. gondii* by a nested PCR on the ribosomal DNA gene as described before (39, 41).

Treatment with pyrimethamine and sulfadiazine. Treatment with pyrimethamine and sulfadiazine was started as soon as congenital infection was proven by detection of the parasite in the amniotic fluid and was continued until the mother gave birth. Transmission of infection occurred in 6 of the 10 monkeys of the treatment group (monkeys D2, D3, D6, D7, D9, and D10). The efficacy of pyrimethamine-sulfadiazine was studied in these six monkeys. The monkeys were treated orally with 1 mg of pyrimethamine per kg/day and 50 mg of sulfadiazine per kg/day. The drugs were administered as described above in the section on pharmacokinetic studies. In addition, folinic acid (3.5 mg) was administered orally once a week in order to counteract the bone marrow suppression caused by pyrimethamine (37). The cookies and bananas containing the drugs were always eaten eagerly, and the complete dose was consumed. Two monkeys, D3 and D9, refused the dose once.

Toxic side effects as a result of the treatment were monitored by making serial blood counts of leukocytes and differentiation of lymphocytes.

Determination of drug concentrations. The concentrations of pyrimethamine and sulfadiazine were determined in the sera and tissues of the mothers and fetuses and in amniotic fluids by high-performance liquid chromatography (HPLC). Tissue samples were homogenized in physiological salt solution (4 ml/g [wet weight] of tissue) with an Ultraturax apparatus (Ystral GmbH, Dottingen, Germany). The crude homogenate was pelleted by centrifugation at 1,000 × *g* for 20 min. The supernatant was used for determination of drug concentrations by HPLC. For determination of the pyrimethamine concentration, 200 μl of the samples (serum, supernatant of the tissues, and amniotic fluid) was deproteinized with 200 μl of acetonitrile. The samples were vortexed and centrifuged at 4,000 × *g* for 10 min. Deproteinization of the samples (200 μl) for determination of the sulfadiazine concentration was done with 300 μl of 0.33 M perchloric acid. Aliquots of 50 μl of the prepared samples were injected onto the analytical column.

Determination of the pyrimethamine concentration was performed on a column packed with Spherisorb 5 ODS (particle size, 5 μm; 4.6 by 250 mm [inner diameter]; Chrompack, Middelburg, The Netherlands). The mobile phase consisted of 2 g of H_3PO_4 (85%; wt/wt) and 0.6 g of tetramethylammoniumchloride in 1 liter of water mixed with 1 liter of acetonitrile (vol/vol) and was run at a flow rate of 1.5 ml/min. UV detection was achieved at 220 nm. The quantitation limit of pyrimethamine was 0.05 μg/ml at a signal-to-noise ratio of 3. The pure pyrimethamine that was used as a reference in HPLC was kindly provided by The Wellcome Foundation (Kent, England).

Sulfadiazine was measured by gradient HPLC analysis. The HPLC system consisted of a Dynamax 6.0-nm C8 column (particle size, 8 μm; 4.6 by 250 mm [inner diameter]; Rainin Instruments Co. Inc., Woburn, Mass.). At time zero, the eluent consisted of 10% acetonitrile and 90% of a 1% acetic acid solution in water. In 18 min the eluent changed linearly to 28% acetonitrile and 72% acetic acid (1%). The flow rate for the solvent was 1.5 ml/min, and detection was achieved at 272 nm. The detection limit of sulfadiazine was 0.2 μg/ml at a signal-to-noise ratio of 3.

The intraday and interday coefficients of variation were calculated for serum samples. The coefficients were found to be 6.4 and 7.7%, respectively, for pyrimethamine and 3.2 and 1.9%, respectively, for sulfadiazine.

Curve fitting ($r^2 > 0.97$) and estimation of pharmacokinetic parameters were done with the aid of the computer programm MW/PHARM (33).

Statistical evaluation. Fisher's exact test for the comparison of proportions was used to compare the probabilities of infection in the treatment and control groups. Furthermore, 80 and 95% confidence intervals were calculated for an infection-free outcome of therapy. The confidence intervals were based on a binomial distribution (5).

RESULTS

Pharmacokinetic studies. The pharmacokinetics of pyrimethamine and sulfadiazine were determined after oral administration of 1 mg of pyrimethamine per kg with 50 mg of sulfadiazine per kg to nonpregnant monkeys E and F. The concentrations of pyrimethamine and sulfadiazine in serum are presented in the Fig. 2 and 3, respectively.

The serum concentration-time curves of both pyrimethamine and sulfadiazine fit best to a one-compartment model. The pharmacokinetic parameters that were calculated from these data are shown in Table 1. The serum elimination half-lives were found to be 44.4 h for pyrimethamine and 5.2 h for sulfadiazine. A maximum concentration in serum of 58.7 μg of sulfadiazine per ml was reached at 3.7 h after administration. At 2.7 h after administration of 1 mg of pyrimethamine per kg a peak concentration of 0.22 μg/ml in serum was found.

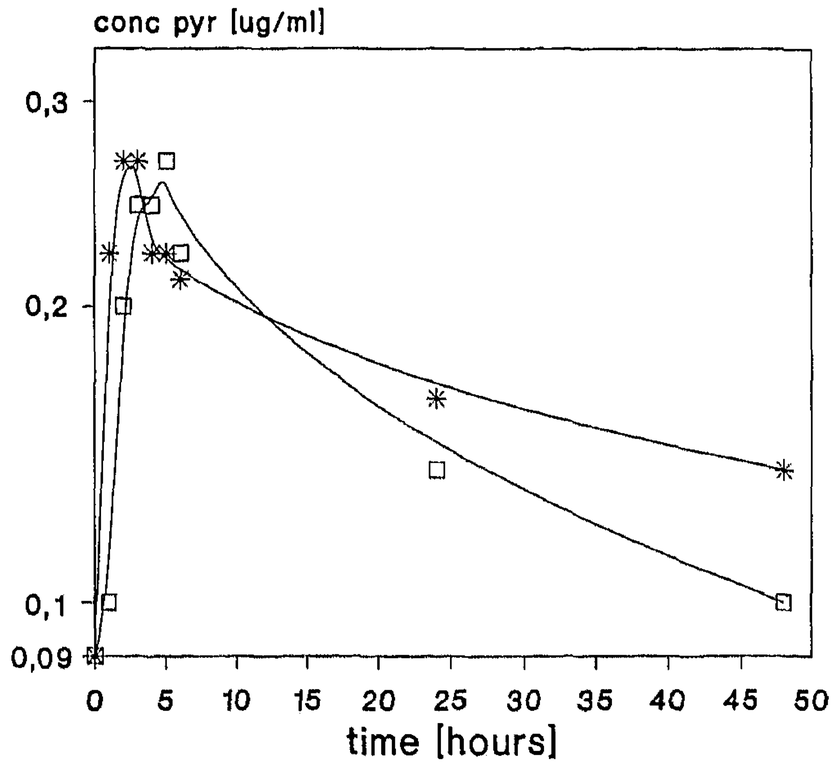

FIG. 2. Concentrations of pyrimethamine (pyr) in the sera of monkeys E (*) and F (□) after oral administration of 1 mg/kg when given in combination with 50 mg of sulfadiazine per kg.

Treatment of congenital *T. gondii* infections. The 4 monkeys in the control group and the 10 monkeys in the treatment group all developed a parasitemia that lasted for about 10 days. Parasitemia was demonstrated by the isolation of parasites from maternal blood by both mouse inoculation and detection by PCR.

As described in detail in our previous report (41), *T. gondii* was detected in four of the nine fetuses of the control group (A_f6 to A_f9). The results are summarized in Figure 1B.

Fetal infection in the treatment group was monitored prenatally by sequential testing of amniotic fluid samples obtained by puncture at days 100, 115, and 130 of pregnancy. Transmission of infection was found in 6 of the 10 monkeys (D_f2, D_f3, D_f6, D_f7, D_f9, and D_f10). The parasite was detected by PCR in the amniotic fluid of all six monkeys, whereas mouse inoculation was positive only for monkeys D_f2, D_f9, and D_f10. In four of six monkeys, the transmission of infection occurred within 10 days after inoculation of the mother, in monkey D7 between days 10 and 25, and in monkey D10 between days 25 and 40 (Fig. 1C).

TABLE 1. Pharmacokinetic parameters of pyrimethamine and sulfadiazine in rhesus monkeys[a]

Drug and monkey	T_{max} (h)	C_{max} (μg/liter)	V (liters)	MRT (h)	AUC (h · μg/ml)	AUC, tr (h · μg/ml)	$t_{1/2}$ (h)	CL (liters/h)
Pyrimethamine								
E	1.0	0.22	4.5	87.0	19.3	8.2	60.2	0.05
F	4.3	0.22	4.1	42.7	8.2	8.0	28.6	0.10
Mean (n = 2)	2.7	0.22	4.3	64.9	14.6	8.1	44.4	0.08
Sulfadiazine								
E	4.3	53.2	0.54	10.3	711	680	5.3	0.07
F	3.0	64.1	0.54	8.9	676	675	5.0	0.07
Mean (n = 2)	3.7	58.7	0.54	9.6	694	678	5.2	0.07

[a] The values for the pharmacokinetic parameters were determined after oral administration of 1 mg of pyrimethamine per kg with 50 mg of sulfadiazine per kg. T_{max}, time to maximum concentration of drug in serum; C_{max}, maximum concentration of drug in serum; V, volume of distribution of the central compartment; MRT, mean residence time; AUC, area under the concentration-time curve; AUC, tr, area under the concentration time curve, trapezoidal rule; $t_{1/2}$, half-life; CL, total plasma clearance.

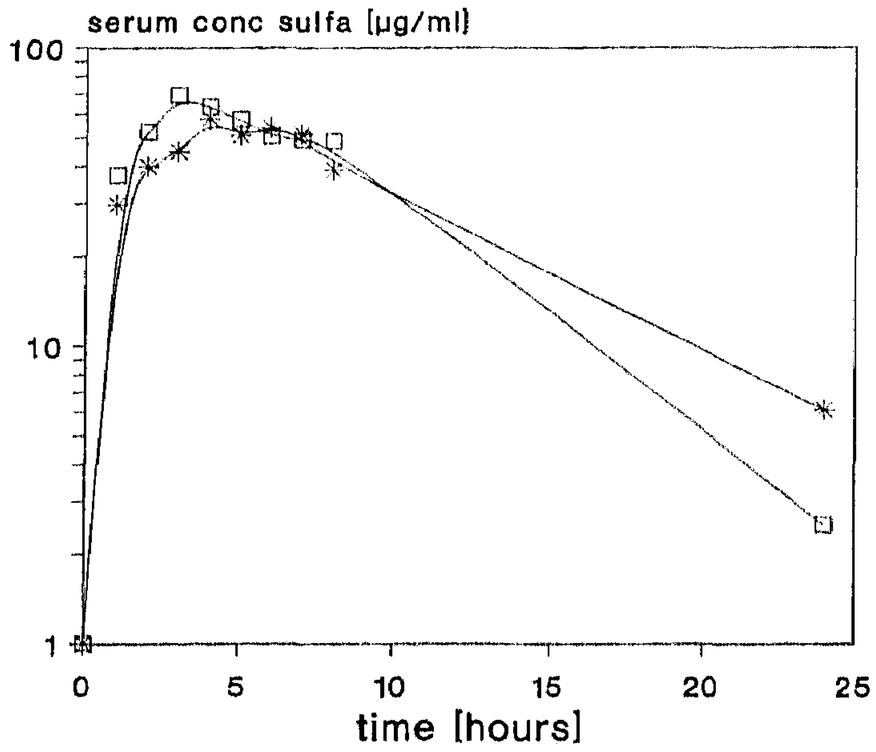

FIG. 3. Concentrations of sulfadiazine (sulfa) in the sera of monkeys E (*) and F (□) after oral administration of 50 mg/kg when given in combination with 1 mg of pyrimethamine per kg.

As shown in Fig. 1C, treatment was started in the six monkeys shortly after collection of the amniotic fluid samples and infection was proven. In four monkeys (monkeys D2, D3, D6, and D9) the time interval between collection of the amniotic fluid sample and the initiation of treatment was only 2 days. The parasite was no longer detectable in the next consecutive amniotic fluid sample from any monkey collected 10 to 13 days after the initiation of treatment (Figure 1C).

Postnatally, the parasite was not found in neonatal tissues or amniotic fluids. The placenta of monkey D10, which received treatment for 25 days, was the only sample found to be positive at birth. The placenta was found to be positive by PCR but not by mouse inoculation. No hematological side effects from the administered drug regimen were found.

Statistical evaluation of these results revealed that the infection rate in the treatment group was similar to the rate in the control group ($P = 0.66$). Furthermore, the outcome of fetal infection after treatment was significantly different from the outcome in the control group ($P = 0.033$). The confidence intervals were calculated for this small number of animals; the 80% confidence limits were 0 to 28%, whereas the 95% confidence limits were 0 to 40%. The drug concentrations at which these effects occurred are given in Tables 2 and 3. The time interval between the last dose and the collection of samples was 4 h for monkeys D3 and D10 (peak levels) and about 26 h for monkeys D2, D6, D7, and D9 (trough levels). The concentrations in fetal serum varied from 0.05 to 0.14 µg/ml for pyrimethamine and from 0.6 to 5.4 µg/ml for sulfadiazine. Pyrimethamine was found to accumulate in the soft tissues, especially in the spleen and the lungs. Sulfadiazine was also found in the tissues, but the concentrations were always less than the concentrations in serum. Both pyrimethamine and sulfadiazine crossed the placenta of the rhesus monkey very well, as shown in Table 4. Pyrimethamine penetrated into brain

TABLE 2. Concentration of pyrimethamine

Sample (day of collection)[a]	Pyrimethamine concn (µg/ml or µg/g)					
	Monkey D2	Monkey D6	Monkey D9	Monkey D7	Monkey D3[b]	Monkey D10[b]
Maternal blood (115)	<0.05	<0.05	<0.05	NT[c]	<0.05	NT
Maternal blood (130)	<0.05	0.09	<0.05	0.09	<0.05	NT
Maternal blood (160)	<0.05	0.11	<0.05	0.11	0.21	0.30
Maternal brain[d]	0.30	<0.05	0.25	0.30	<0.05	0.85
Maternal heart[d]	<0.05	1.3	0.35	<0.05	<0.05	0.30
Maternal spleen[d]	0.95	0.8	0.50	1.40	<0.05	2.40
Maternal liver[d]	0.70	1.0	0.90	1.60	2.0	2.50
Amniotic fluid (115)	<0.05	<0.05	<0.05	NT	<0.05	NT
Amniotic fluid (130)	<0.05	<0.05	<0.05	<0.05	<0.05	NT
Amniotic fluid (160)	<0.05	0.06	<0.05	0.07	<0.05	0.13
Fetal blood (130)	<0.05	<0.05	<0.05	<0.05	<0.05	NA[e]
Fetal blood (160)	<0.05	<0.05	<0.05	0.09	0.08	0.14
Placenta (160)[d]	<0.05	<0.05	<0.05	0.60	0.40	2.50
Fetal brain[d]	0.30	<0.05	<0.05	<0.05	0.30	0.65
Fetal heart[d]	<0.05	<0.05	<0.05	0.35	<0.05	0.95
Fetal spleen[d]	1.10	0.50	0.50	1.0	1.05	ND[f]
Fetal liver[d]	<0.05	<0.05	<0.05	<0.05	<0.05	0.50
Fetal lung[d]	1.15	1.05	1.6	1.95	0.45	6.0
Duration of treatment (days)	58	58	58	40	58	25

[a] The day of sample collection corresponds to pregnancy duration.
[b] The average time interval between the last dosing and sample collection was 26 h. Thus, the concentrations in serum presented here are mainly trough levels. For monkeys D3 and D10 at day 160 of pregnancy, the time interval was 4 h.
[c] NT, no treatment at that moment.
[d] The concentrations in tissue were determined by using tissue extracts which were diluted five times in physiological salt solution. If a concentration in tissue was found to be <0.05 µg/ml, this means that this was the concentration in the diluted sample.
[e] NA, sample not available.
[f] ND, not done.

TABLE 3. Concentration of sulfadiazine

Sample (day of collection)[a]	Sulfadiazine concn (μg/ml or μg/g)					
	Monkey D2	Monkey D6	Monkey D9	Monkey D7	Monkey D3[b]	Monkey D10[b]
Maternal blood (115)	4.1	7.4	17.6	NT[c]	3.9	NT
Maternal blood (130)	5.3	2.3	2.2	1.0	2.5	NT
Maternal blood (160)	4.6	1.9	3.3	3.4	5.7	6.0
Maternal brain[d]	1.75	<0.2	1.6	1.2	2.3	1.95
Maternal heart[d]	2.35	<0.2	1.2	1.4	2.1	1.25
Maternal spleen[d]	3.45	<0.2	2.0	2.3	4.55	3.2
Maternal liver[d]	2.85	<0.2	2.25	<0.2	2.15	3.6
Amniotic fluid (115)	7.5	4.0	0.75	NT	3.9	NT
Amniotic fluid (130)	5.3	2.6	1.7	1.2	2.9	NT
Amniotic fluid (160)	4.0	2.7	2.0	5.4	4.2	4.4
Fetal blood (130)	4.8	2.0	1.7	0.6	1.0	NA[e]
Fetal blood (160)	4.0	1.9	3.2	3.7	5.1	5.4
Placenta[d]	3.1	<0.2	1.55	2.4	2.45	2.4
Fetal brain[d]	1.3	0.55	1.0	1.45	1.2	2.35
Fetal heart[d]	2.7	<0.2	1.4	2.0	1.9	1.95
Fetal spleen[d]	3.95	<0.2	2.15	2.3	2.65	3.5
Fetal liver[d]	2.75	<0.2	2.0	2.0	2.85	3.75
Fetal lung[d]	3.0	1.0	1.95	2.2	2.25	2.75
Duration of treatment (days)	58	58	58	40	58	25

[a] The day of sample collection corresponds to pregnancy duration.
[b] The average time interval between the last dosing and sample collection was 26 h. Thus, the concentrations in serum presented here are mainly trough levels. For monkeys D3 and D10 at day 160 of pregnancy, the time interval was 4 h.
[c] NT, no treatment at that moment.
[d] The concentrations in tissue were determined by using tissue extracts which were diluted five times in physiological salt solution. If a concentration in tissue was found to be <0.2 μg/ml, this means that this was the concentration in the diluted sample.
[e] NA, sample not available.

tissue better than sulfadiazine; the concentrations of pyrimethamine in the brain were about three to four times greater than the corresponding concentrations in serum, whereas the concentrations of sulfadiazine were found to be about 30% of the corresponding concentrations in serum (Table 4).

DISCUSSION

The antitoxoplasma activities of pyrimethamine and sulfadiazine have been demonstrated both in vitro and in vivo. Pyrimethamine and sulfadiazine act synergistically on *T. gondii*, and it appeared that the combination of these drugs inhibits the growth of the parasite in vitro (12, 24, 42). The inhibition of growth was associated with a reduction in the number of parasitized cells and the number of intracellular parasites (12, 24). Pyrimethamine and sulfadiazine also cause morphological changes in the parasite, as demonstrated by electron microscopy (42).

Studies in mice have shown that treatment with pyrimethamine and sulfadiazine prolongs survival after infection with a lethal dose of *T. gondii* by reducing the parasite load (14, 32). Nguyen and Stadtsbaeder (31) found that mice treated with pyrimethamine and sulfadiazine acquired no resistence to a lethal challenge with *T. gondii*. Treatment with pyrimethamine

TABLE 4. Transplacental passage and penetration into the brain of pyrimethamine and sulfadiazine

Drug[a]	Drug ratio between the samples[b]	Day of collection[c]	Monkey D2	Monkey D6	Monkey D9	Monkey D7	Monkey D3	Monkey D10
P	Fetal blood/maternal blood	160	ND[d]	ND	ND	0.81	0.38	0.46
P	Fetal brain/fetal blood	160	ND	ND	ND	ND	3.75	4.6
P	Maternal brain/maternal blood	160	ND	ND	ND	2.7	ND	2.8
S	Fetal blood/maternal blood	130	0.90	0.87	0.77	0.60	0.40	ND
S	Fetal blood/maternal blood	160	0.87	1.0	0.97	1.0	0.89	0.90
S	Fetal brain/fetal blood	160	0.33	0.29	0.31	0.39	0.24	0.44
S	Maternal brain/maternal blood	160	0.38	ND	0.48	0.35	0.40	0.32

[a] P, pyrimethamine; S, sulfadiazine.
[b] Transplacental passage is expressed as the ratio between the concentration of the drug in fetal serum and the corresponding concentration in maternal serum. The ratio between the concentration in the brain and the corresponding concentration in serum gives an impression of the level of penetration of the drug into the brain.
[c] The day of sample collection corresponds to pregnancy duration.
[d] ND, not determined because the drug concentration was below the detection level or because there was no treatment given at that moment.

and sulfadiazine also prevented squirrel monkeys from dying of an acute toxoplasmosis (17).

Studies in humans revealed that treatment with pyrimethamine and sulfadiazine significantly reduced the number of offspring with severe congenital toxoplasmosis (9–11, 20). Several children, however, were born suffering from subclinical or even symptomatic toxoplasmosis, despite treatment with pyrimethamine and sulfadiazine (7, 10, 11, 20). The treatment of these children was started after fetal infection had been proven. The prenatal diagnosis of infection was time-consuming most of the time, and the studies contained no information about the duration of infection at the moment that therapy was started. The time interval between the onset of fetal infection and the initiation of therapy was therefore possibly too long. In the present study the moment of infection was adequately known. Because antenatal detection of *T. gondii* was performed by PCR, a result can be obtained within 2 days of receiving the sample, and treatment could be started early after the onset of fetal infection in our experimental setup. By PCR, transmission of infection was detected antenatally in six monkeys, whereas mouse inoculation was positive for only three monkeys. The high degrees of sensitivity and reproducibility of the PCR were also found in our former study (40). It has been stated before that PCR cannot discriminate between viable and dead parasites. However, since transmission of dead parasites is unlikely, the detection of *T. gondii* in amniotic fluid by PCR is considered to be of clinical importance.

Statistical analysis of the results revealed that the rate of transmission of infection to the fetuses in the control group was similar to the rate in the treatment group. In addition, treatment with pyrimethamine and sulfadiazine significantly reduced the number of infected offspring compared with the number of infected untreated controls.

The efficacy of treatment was related to the concentrations of the drugs that were reached in the fetus. Both pyrimethamine and sulfadiazine can cross the placenta freely and are therefore thought to be appropriate drugs for use in the treatment of congenital *T. gondii* infection (13). The concentrations of pyrimethamine in the sera of neonates varied from 38 to 81% of the corresponding concentrations in the sera of the mothers. The concentrations of sulfadiazine in the sera of the fetuses were similar to those in the sera of the mothers. The same results have also been found for transplacental passage of pyrimethamine and sulfadoxine in humans (13). This was expected because rhesus monkeys and humans both possess a placenta of the hemochorial type (35). Since brain damage is the most severe consequence of congenital toxoplasmosis, the concentrations in the brain are of special interest. It was found that pyrimethamine and sulfadiazine both can cross the blood-brain barrier in the rhesus monkey. The concentrations of sulfadiazine in the brain were found to be about 35% of the concomitant concentrations in serum for both the fetus and the mother. These percentages are in close agreement with the levels of sulfadiazine that have been found in the cerebrospinal fluid of dogs (45) and humans (46). Pyrimethamine was found to penetrate and accumulate in monkey brain tissue, and concentrations up to 4.5 times the concomitant levels in serum were found. This was already expected from experiments in humans (23), rhesus monkeys (38), and rats (8), in which similar ratios of levels in brain to levels in serum were found.

Pharmacokinetic studies revealed that pyrimethamine and sulfadiazine have serum half-lives of 44.4 and 5.2 h, respectively, in the rhesus monkey. Sulfadiazine has a shorter elimination half-life in rhesus monkeys than in humans (1, 25, 36). In addition, the elimination half-life found in adult monkeys in the present study is shorter than that reported for young monkeys (29, 30). A similar finding was reported by Friis et al. (15) in pigs, who found that the half-life of sulfadiazine decreased with an increase in the ages of the pigs.

The pyrimethamine half-life of 44.4 h found in rhesus monkeys is shorter than the 85 to 114 h reported in humans (8, 26). When compared with those in babies, however, the half-life in serum was only slightly shorter than the reported 64 ± 12 h (28). On the basis of the serum half-life of pyrimethamine observed in the rhesus monkey, a frequency of one dose every 1 or 2 days would be appropriate. Because the half-life of sulfadiazine is 5.2 h, however, a more frequent dosing would be required.

The pharmacokinetic studies were performed with nonpregnant monkeys. Given the effects of pregnancy on the volume of distribution and renal blood flow, drug clearance could be different. Further investigation of these parameters needs to be performed to establish the dosages of the drugs in the treatment of humans.

Weiss et al. (47) demonstrated that the concentration of pyrimethamine in the sera of mice should be at least 0.5 μg/ml to be effective in inhibiting *Toxoplasma* growth. When pyrimethamine was used in combination with sulfadiazine, the concentrations in serum should be at least 0.1 μg/ml for pyrimethamine and 25 μg/ml for sulfadiazine. The concentrations that were found to be effective in vivo were in close agreement with those that were found to be effective in vitro (24, 42). Lower concentrations of 0.02 μg of pyrimethamine per ml in combination with 0.1 μg of sulfadiazine per ml, however, also had an inhibitory effect (12). Such concentrations were easily reached in the rhesus monkeys by the investigated dose regimen. Concentrations of ≥25 μg of sulfadiazine per ml were present in the sera of adult monkeys for about 10 h, and concentrations of ≥0.1 μg of pyrimethamine per ml were present in sera for more than 24 h after single oral dosing. Given the high rate of transplacental passage of both drugs, it is assumed that such effective concentrations in serum are also present in the fetus. On the basis of these findings a single dose pyrimethamine given every day would be appropriate. The half-life of sulfadiazine of 5.2 h and the time during which effective concentrations are present in serum, however, would require two doses per day. For practical reasons pyrimethamine and sulfadiazine were administered together in a single dose.

Concentrations of 0.1 to 0.5 μg of pyrimethamine per g were also reached in the tissues of the rhesus monkey. Because in most cases trough levels were measured, concentrations in serum were often below the quantitation limit of 0.05 μg/ml. In two monkeys (monkeys D3 and D10), the levels in serum were measured approximately 4 h after administration of the last dose. In these cases concentrations in serum greater than 0.1 μg/ml were reached. No toxic side effects from the administered drug regimen were found in the rhesus monkeys. Pyrimethamine, however, should not be given in excess of dosages of 1.25 mg/kg/day since higher dosages cannot be tolerated by rhesus monkeys when they are administered repeatedly (38). It should be mentioned, however, that in the study of Schmidt et al. (38) the treatment was not supplemented with folinic acid, as was the case in the present study.

At high doses pyrimethamine has been found to have teratogenic effects in rats (19, 21, 43) and miniature pigs (18). Besides this, sulfadiazine should not be administered during the last month of pregnancy because it increases the risk of kernicterus (48). Kernicterus was not investigated in the monkeys used in the present study, which were killed immediately after birth.

The risk of teratogenic effects caused by pyrimethamine and

the risk of kernicterus caused by sulfadiazine create a reluctance to use this drug combination for the prevention of transmission of infection. Therefore, the safer antibiotic spiramycin is given as long as fetal infection has not been proven. A former study in rhesus monkeys showed that spiramycin is able to reduce the number of parasites in amniotic fluid to undetectable levels (40). However, spiramycin had to be administered for at least 3 weeks to be effective (40). Treatment with pyrimethamine and sulfadiazine, on the other hand, showed the same effect within 10 to 13 days.

Because of the risk of malformations, the use of pyrimethamine and sulfadiazine during pregnancy has been discussed. The published work, however, revealed no grounds for contraindication during pregnancy (2). On the basis of these findings, the drug combination instead of spiramycin should be used to prevent the transmission of infection because of the potent activity of the combination against *T. gondii* parasites. During the last month of pregnancy (i.e., after 36 weeks or earlier if an anamnesis exists for premature birth) sulfadiazine should be left out because of the risk of kernicterus.

It appears from the results of the present study that the combination of pyrimethamine and sulfadiazine is an effective drug regimen for the treatment of congenital *T. gondii* infection. Pyrimethamine and sulfadiazine act on the proliferative stage of the parasite but not on cysts in tissues. The eradication of cysts is very important because of the risk of developing chorioretinitis later in life (22). Studies should focus on new drugs that cross the blood-brain barrier and that act on the cysts as well. Initial studies with atovaquone showed promising results (3, 4). On long-term probation, however, atovaquone was not able to eradicate the cysts from infected mouse brains (2a).

In conclusion, when administered early after the onset of fetal infection the combination of pyrimethamine and sulfadiazine is an effective drug regimen for the treatment of congenital *T. gondii* infections.

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