Evaluation of vaccination with *Neospora caninum* protein for prevention of fetal loss associated with experimentally induced neosporosis in sheep

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Objective—To evaluate the immunologic response of a killed tachyzoite vaccine against *Neospora caninum* and its effectiveness in preventing fetal loss associated with experimentally induced neosporosis in sheep.

Animals—30 Dorset ewes.

Procedure—Ewes were randomly allocated to receive vaccination on days 1 and 60 of the study with a killed *N caninum* tachyzoite preparation in a commercially available adjuvant or a saline-adjuvant mixture. A ram was placed on pasture with the ewes from days 15 to 60. Blood was collected from ewes before primary and booster vaccinations and prior to experimental challenge with *N caninum* tachyzoite performed on day 90; sera were assessed via *Neospora* agglutination (NA) and immunofluorescence antibody (IFA) assays. Blood was collected from lambs before they suckled, and sera were tested for antibodies against *N caninum*.

Results—Of the 14 vaccinated ewes that became pregnant, 12 gave birth to live-born lambs; in contrast, 5 of 11 pregnant control ewes gave birth to live-born lambs. Whereas vaccination improved fetal survival in pregnant ewes challenged with *N caninum* tachyzoites, it did not appear to have any appreciable effect on transmission of *N caninum* to offspring, as indicated by results of NA and IFA assays.

Conclusions and Clinical Relevance—The *N caninum* tachyzoite vaccine used in this study appeared to provide protection against fetal loss associated with experimentally induced neosporosis in a high proportion of pregnant ewes. (*Am J Vet Res* 2004;65:1404–1408)

Neospora caninum is an important cause of abortion, reproductive failure, and economic loss in cattle worldwide.[1] The only obvious clinical signs of neosporosis are abortion, stillbirth, or birth of calves with neurologic dysfunction. Prevention of neosporosis in dairy cattle may rely on vaccination with *N caninum* antigen to prevent in utero transfer of tachyzoites that can originate from a latent tissue cyst infection or from oocysts ingested during pregnancy. A commercial vaccine that is based on an extract of *N caninum* tachyzoites is being marketed as a means of preventing neosporosis in cattle, but the efficacy of this formulation against *Neospora*-associated abortion remains to be determined.[2,3] Vaccination with a mixture of either *N caninum* proteins or specific recombinant antigens, such as NcGRA7, NcSRS2, or NcMIC3, has protective efficacy in mice.[4-7] Results of a study[8] in ruminants suggest that exposure of cows to *N caninum* before pregnancy may protect against vertical transmission of the organism after an experimental challenge with the parasite at midgestation. Moreover, compared with naïve cows, the rate of fetal infection may be lower and the risk of pregnancy failure appears to be less in cows that are chronically infected with *N caninum*.[9] Although vaccination of cattle with *N caninum* tachyzoite extract elicits cellular and humoral immune responses,[2,10,11] there are no reports of complete protection against clinical disease or parasite transfer conferred via vaccination with tachyzoite protein. Several researchers have determined that the biologic features of *N caninum* and pathogenesis of infection in sheep are similar to that observed in cattle.[12-16] In a recent study,[17] vaccination of sheep with *N caninum* tachyzoite extract resulted in a modest reduction in the percentage of seropositive lambs; however, because the protozoal challenge was administered to ewes late in gestation, no effect on birth rate was observed. The purpose of the study reported here was to evaluate the immunologic response to a killed tachyzoite vaccine against *N caninum* and its effectiveness in preventing fetal loss associated with neosporosis induced experimentally early during gestation in sheep.

Materials and Methods

Vaccine preparation—*Neospora caninum* tachyzoites (NC-1) were cultured and harvested and total protein was extracted, as described.[17] The protein extract was frozen at –70°C in aliquots sufficient for each vaccination. Just prior to vaccination, the extract was thawed, diluted to an equivalent of 2×10^8 tachyzoites/mL, and mixed with adjuvant according to the manufacturer's instructions.[a] An adjuvant control preparation was prepared by substituting the tachyzoite extract with sterile PBS solution.

Animals and experimental design—Adult Dorset ewes (n = 30) that had not been bred prior to inclusion in this study were randomly allocated to 1 of 2 treatment groups (designated as the vaccine and control groups) and raised on a single pasture. On day 1 of the study, ewes in the vac-

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cine group received an SC injection (0.5 mL) of *N caninum* tachyzoite protein extract (equivalent to 5×10^7 tachyzoites) emulsified in the commercial adjuvant[a] by use of a 20-gauge needle. On day 1 of the study, ewes in the control group received an SC injection (0.5 mL) of sterile PBS solution mixed with the same adjuvant. A Dorset ram was introduced into the pasture with all ewes on day 15 of the study and removed 45 days later (day 60). Ewes in both groups received a booster vaccination on day 60 of the study with the same preparation used in the primary vaccination. For the parasite challenge, tachyzoites of the *N caninum* Illinois strain were cultured and harvested, as described,[17] and suspended in sterile PBS solution to a final concentration of 2.5×10^6 tachyzoites/mL. On day 90, within 2 hours following parasite harvest, ewes in both groups received an SC injection of 2 mL of the live tachyzoite suspension (5×10^6 tachyzoites) administered by use of a 20-gauge needle. Ewes were challenged with *N caninum* tachyzoites during midgestation in an attempt to achieve high levels of fetal resorption and abortion.[13,18] On day 150, the ewes were removed from the pasture and confined in corrals equipped with shelters until lambing was completed. Ewes were tested for pregnancy by a commercial service company[b]; sera obtained on the day of parasite challenge (day 90) were evaluated via a radioimmunoassay for the presence of ovine pregnancy-specific protein B (a protein that is produced by the placenta).[19]

Prior to initial and booster vaccinations and parasite challenge, blood was collected from each ewe by jugular venipuncture into serum separator tubes and processed to obtain serum, as described.[17] During the lambing period, ewes were monitored on a regular basis for signs of labor. After witnessing parturition, blood samples were collected from lambs before they were allowed to suckle and processed, as described.[17]

Serologic analyses—Sera from the ewes and lambs in the vaccine and control groups were assessed for antibodies against *N caninum* by use of 2 different assays: a ***Neospora* agglutination (NA)** test and an **immunofluorescence antibody (IFA)** test. Anti-*Neospora* antibody titers were estimated by NA tests, as described.[20] An IFA test was performed on all sera at an initial dilution of 1:50 as recommended by the manufacturer.[c] Sera that were IFA-positive at a 1:50 dilution were further tested at a 1:200 dilution. On the basis of findings of a previous study,[21] sera from ewes and lambs were positive for *N caninum* antibodies when the NA titer was > 40 and a positive IFA reaction was noted at a 1:50 dilution. If

Table 1—Assessment of anti-*Neospora caninum* immunologic response in 15 ewes before vaccination (day 1) with a killed tachyzoite vaccine against *N caninum*, prior to receiving a booster vaccination (day 60), and prior to challenge with *N caninum* tachyzoites (day 90) and in their lambs (conceived after primary but before booster vaccination) via an NA test and an IFA test.

Vaccine group						
			Serologic status of ewe			
Ewe	Pregnancy	Assay*	Day 1	Day 60	Day 90	Serologic status of lambs†
V1	Yes	NA	20	< 20	≥ 160	≥ 160 and ≥ 160
		IFA	Neg	Neg	++	++ and ++
V2	Yes	NA	< 20	< 20	≥ 160	No lambs
		IFA	Neg	Neg	++	
V3	No	NA	≥ 160	≥ 160	≥ 160	No lambs
		IFA	++	++	++	
V4	Yes	NA	< 20	80	≥ 160	160 and ≥ 160
		IFA	Neg	++	++	++ and ++
V5	Yes	NA	< 20	20	≥ 160	< 20 and < 20
		IFA	Neg	+	++	Neg and neg
V6	Yes	NA	< 20	40	≥ 160	< 20 and < 20
		IFA	Neg	+	++	Neg and neg
V7	Yes	NA	< 20	80	≥ 160	≥ 160
		IFA	Neg	++	++	++
V8	Yes	NA	≥ 160	≥ 160	≥ 160	≥ 160, 40, and 80
		IFA	++	++	++	++, ++, and ++
V9	Yes	NA	< 20	40	≥ 160	< 20
		IFA	Neg	+	++	++
V10	Yes	NA	≥ 160	≥ 160 ≥	160	≥ 160 and ≥ 160
		IFA	++	++	++	++ and ++
V11	Yes	NA	< 20	80	≥ 160	No lambs
		IFA	Neg	++	++	
V12	Yes	NA	20	40	≥ 160	≥ 160
		IFA	Neg	+	++	++
V13	Yes	NA	≥ 160	≥ 160	≥ 160	< 20, < 20, and ≥ 160
		IFA	++	++	++	Neg, neg, and ++
V14	Yes	NA	≥ 160	20	≥ 160	No lambs
		IFA	++	++	++	
V15	Yes	NA	< 20	< 20	40	< 20 and ≥ 160
		IFA	Neg	Neg	++	++ and ++

*Results of NA assay are antibody titers. Results of the IFA assay are as follows: Neg = No signal at 1:50 serum dilution, + = Positive signal at 1:50 serum dilution but negative at 1:200 dilution, and ++ = Positive signal at 1:50 and 1:200 serum dilutions. †Results of NA and IFA tests performed on samples of serum from each lamb born to that ewe.

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results of the 2 serologic assays were discordant, then an immunoblotting assay was performed as described.[22]

Statistical analyses—Pregnancy rates and lambing rates in the vaccine and control groups were compared by use of a 2-sided Fisher exact test. This statistical test was also used to compare the percentage of lambs that yielded positive results for *N caninum* via NA and IFA tests between the 2 groups. To determine whether there was any effect of prior exposure to the parasite, a Fisher exact test was also performed to compare ewes that were seropositive or seronegative for *N caninum* via NA and IFA tests before vaccination. An unpaired *t* test was used to compare the mean number of lambs produced per pregnant ewe between vaccinated and control groups. Mean values of 2 groups were considered significantly different for values of $P < 0.10$. All statistical analyses were performed by use of a statistical software package.[d]

Results

Overall, the mean age of the ewes was 4.5 ± 2.8 years; the mean age of the ewes in each treatment group was not significantly ($P = 0.27$) different. At the time of *N caninum* tachyzoite challenge, an anti-*N caninum* immunoglobulin response was detected via both NA and IFA testing in 14 of 15 ewes that were vaccinated with whole tachyzoite extract (**Tables 1 and 2**). Serum from 1 ewe in the vaccine group had a fairly low NA test response but yielded positive results via the IFA test. All ewes in the control group, except those with a high prevaccination anti-*N caninum* antibody titer, did not develop an appreciable NA titer or IFA test response.

Of the 15 ewes in the vaccine group, 14 became pregnant and 12 gave birth to live lambs; there were 21 lambs born in this group (mean number of lambs per ewe, 1.8). Results of serologic testing indicated that 10 of the 15 ewes in this group were seronegative for *N caninum* prior to the start of the study. Of these 10 seronegative ewes, 9 became pregnant and 8 gave birth to live lambs. There were 13 lambs born to these seronegative ewes (mean number of lambs per ewe, 1.6). Of the 15 ewes in the control group, 11 became pregnant and 5 gave birth to live lambs; there were 7 lambs born in this group (mean number of lambs per ewe, 1.4). Results of serologic testing indicated that 12 of the 15 ewes in this group were seronegative for *N caninum* prior to the start of the study. Of these 12 seronegative ewes, 9 became pregnant and 4 gave birth to live lambs. There were

Table 2—Assessment of anti-*N caninum* immunologic response in 15 control ewes before sham vaccination (day 1), prior to receiving a sham booster vaccination (day 60), and prior to challenge with *N caninum* tachyzoites (day 90) and in their lambs (conceived after primary but before booster vaccination), via a *Neospora* agglutination (NA) test and an immunofluorescent antibody (IFA) test.

Control group						
			Serologic status of ewes			
Ewe	Pregnancy	Assay*	Day 1	Day 60	Day 90	Serologic status of lambs†
C1	Yes	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	+	
C2	Yes	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	Neg	
C3	Yes	NA	< 20	< 20	< 20	< 20 and ≥ 160
		IFA	Neg	Neg	Neg	+ and ++
C4	Yes	NA	< 20	< 20	20	< 20
		IFA	Neg	Neg	+	++
C5	No	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	+	
C6	Yes	NA	≥ 160	≥ 160	≥ 160	No lambs
		IFA	++	++	++	
C7	No	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	Neg	
C8	Yes	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	Neg	
C9	Yes	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	Neg	
C10	No	NA	≥ 40	≥ 40	< 20	No lambs
		IFA	+	++	++	
C11	No	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	+	
C12	Yes	NA	≥ 160	≥ 160	≥ 160	≥ 160
		IFA	++	++	++	++
C13	Yes	NA	< 20	< 20	< 20	< 20
		IFA	++	+	++	++
C14	Yes	NA	< 20	≥ 160	≥ 160	< 20 and < 20
		IFA	Neg	++	++	Neg and neg
C15	Yes	NA	< 20	< 20	< 20	No lambs
		IFA	Neg	Neg	Neg	

See Table 1 for key.

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6 lambs born to these seronegative ewes (mean number of lambs per ewe, 1.5).

No significant difference in the pregnancy rate between vaccinated and control groups was detected, regardless of whether all ewes ($P = 0.33$) or only ewes that were seronegative for *N caninum* prior to vaccination ($P = 0.59$) were analyzed. Among the ewes that were seronegative for *N caninum* prior to vaccination, the delivery rate in ewes vaccinated with *N caninum* tachyzoite antigen was nearly twice that of ewes in the control group (8 lambs/9 ewes and 4 lambs/9 ewes, respectively). Also, evaluation of ewes that were seropositive for *N caninum* before vaccination revealed that prior exposure to *N caninum* did not appear to have an appreciable effect on the delivery rate in ewes that were vaccinated, compared with the rate in ewes that were not vaccinated. Analysis of data for all ewes regardless of prevaccination status revealed that the delivery rate of liveborn lambs in the vaccinated ewes (12 lambs/14 ewes) was twice that observed in ewes administered adjuvant alone (5 lambs/11 ewes). The delivery rate in vaccinated ewes was significantly higher than that of the control group, regardless of whether the ewes were seropositive ($P = 0.07$) or seronegative ($P = 0.08$) for *N caninum* prior to vaccination. The mean ± SD number of lambs born per ewe in the vaccinated group was slightly greater than that of the control group (1.9 ± 0.7 and 1.4 ± 0.6, respectively), but this difference was not significant ($P = 0.18$).

In contrast to the delivery rate, no significant ($P = 0.33$) difference was detected in the percentage of *N caninum*-seropositive lambs born in the vaccinated or control groups (Table 1). In the vaccinated group, 15 of the 21 lambs yielded positive results via the NA or IFA tests (or both). In the control group, a similar proportion of lambs (5/7) yielded positive results via NA or IFA tests (or both). Although there was excellent agreement between seropositivity results from NA and IFA tests of ewe sera, 4 lambs yielded positive results via the IFA test and negative results via the NA test (Table 1). Immunoblotting analysis of lamb sera, which confirmed the IFA test results, indicated that this NA test result might be a false-negative reaction (data not shown).

Discussion

These results indicated that in sheep, vaccination with *N caninum* tachyzoite protein extract protected against fetal loss, but did not prevent in utero transmission of *N caninum* tachyzoites from ewes to lambs. This conclusion was based on the fact that placental transfer of immunoglobulin from dam to fetus does not occur in ruminants. Therefore, lambs that yielded positive results via NA or IFA tests would have acquired *N caninum* tachyzoites in utero and produced antibodies against the parasite before birth. Our results are consistent with those of another study in sheep[17] in which marginal protection against transfer of the parasite to fetuses in utero resulted after vaccination of ewes during late gestation (at 120 days) with *N caninum* antigen, prior to tachyzoite challenge. Also, vaccination of preparturient cattle with a commercial *Neospora* vaccine that is similar to the antigen preparation used in the study of this report (except for the adjuvant used) elicited a serologic response but did not prevent infection of the fetuses in utero after *N caninum* tachyzoite challenge.[10] Inoculation of cattle and sheep with *N caninum* tachyzoites prior to pregnancy can provide some protection against abortion and vertical transmission of parasites associated with a subsequent challenge.[8,23] In contrast, natural exposure of cattle to *N caninum* in utero or by ingestion of oocysts does not appear to protect against abortion and, in fact, may lead to further transmission of the parasite.[24] Although the number of sheep used in the study of this report was low, there did not appear to be any appreciable effect of prior exposure to *Neospora* organisms on the delivery rate in vaccinated or control ewes. These findings suggested that, regardless of serologic status, vaccination of ewes with *N caninum* tachyzoite protein before breeding may be required to prevent neosporosis-associated abortion. Whether the vaccine-adjuvant combination used in the present study will be useful for protecting cattle against *N caninum*-associated abortion remains to be determined.

[a]Immumax S-R, Zonagen Inc, The Woodlands, Tex.
[b]BioTracking LLC, Moscow, Idaho.
[c]VMRD Inc, Pullman, Wash.
[d]Instat, version 3.05, GraphPad Software, San Diego, Calif.

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