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IMMUNOTHERAPY FOR PORCINE CYSTICERCOSIS: IMPLICATIONS FOR PREVENTION OF HUMAN DISEASE

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THE CYSTICERCOSIS WORKING GROUP IN PERU*

Abstract

Taenia solium cysticercosis is an important cause of human disease in many developing countries. Porcine cysticercosis is a vital link in the transmission of this disease and impairs meat production. A treatment for porcine cysticercosis may be an effective way of preventing human disease that would also benefit pig farmers, facilitating control programs in disease-endemic regions. Previous research suggests that reinfection with cysticercosis or immunotherapy with cysticercal antigens may cause degeneration of cysticerci, potentially curing porcine cysticercosis. Therefore, a blinded, randomized, controlled study to assess the efficacy and safety of immunotherapy in 28 naturally parasitized pigs was performed. Four groups of pigs with similar weights were inoculated twice with membrane-enriched cysticercal antigens (MA), saline, aqueous-soluble crude cysticercal antigens (AA) in adjuvant (Freund's complete then incomplete), or adjuvant alone. Immunotherapy was well tolerated but had no consistent effect on the macroscopic appearance of cysticerci or eosinophil count. Histopathologic findings were variable, with both

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severe and minimal inflammatory reactions seen in adjacent cysticerci in all pigs. Nine (64%) of 14 pigs given immunotherapy developed new antibody bands on electroimmunotransfer blot compared with one (7%) of 14 control pigs ($P < 0.01$). Treatment with AA in adjuvant caused a significant increase in the proportion of cysticerci that failed to evaginate and were, therefore, not viable for infecting humans (34% for pigs given AA in adjuvant compared with 10% for adjuvant alone; $P < 0.04$). Although immunotherapy caused a statistically significant decrease in the viability of cysticerci, this immunologic reaction was not great enough to prevent human disease.

Cysticercosis is a parasitic disease that results from ingestion of *Taenia solium* tapeworm eggs. When humans ingest these microscopic eggs they may develop cysticercosis, an important cause of disability and mortality in many developing countries, causing 20% of adult-onset epilepsy and filling 12% of neurologic hospital beds in Peru.[1-3] Porcine cysticercosis occurs when pigs ingest *T. solium* eggs and the resultant cysticercotic or measly pork is of greatly reduced value.[4] In the Andean region of Peru, up to 60% of the pigs have cysticercosis, contributing to economic hardship and human malnutrition.[5] When humans cat infected pork, they may develop *T. solium* tapeworms that release more eggs, contaminating the environment and completing the parasitic life cycle.

Improvements in public health and animal husbandry have led to the virtual eradication of human and porcine cysticercosis in developed countries, but such measures are too expensive for immediate implementation in less developed areas.[6] Experimental vaccination of healthy pigs with cysticercal antigens caused partial protection against porcine cysticercosis[7,8] but a vaccine is not commercially available and would be difficult to implement in disease-endemic regions where domestic pig-rearing predominates. In contrast to preventative measures, an inexpensive drug treatment for porcine cysticercosis might be sought and used by owners of infected pigs if it improved animal health, meat yield, and especially if it caused the degeneration of cysticerci in infected meat, thus increasing its value. Our group is evaluating cost-effective treatments for porcine cysticercosis with drug therapy[9, 10] and immunotherapy, which may be a way to break the life cycle of the parasite, thus preventing human as well as animal disease.

Cysticerci survive within host tissues by evading and modulating host immunity.[1, 11] The rationale for immunotherapy is the observation that immunologic intervention may alter this host-parasite interaction, causing destruction of cysticerci: pigs infected with two successive doses of *T. solium* eggs paradoxically developed significantly fewer cysticerci than pigs that had been infected with a single dose, implying that reinfection accelerated cysticercus degeneration and absorption.[12] Similarly, reinfection of cows infected with *T. saginata* and of sheep infected with *T. hydatigena* caused degeneration of established cysticerci[13-15] Furthermore, laboratory and field studies have reported that immunotherapy with cysticercal antigens caused the partial resolution of porcine cysticercosis.[16, 17] These encouraging results led us to further investigate the effect of immunotherapy on porcine cysticercosis in a randomized, controlled, and blinded study.

MATERIALS AND METHODS

Twenty-eight privately reared, naturally parasitized pigs that were being sold for slaughter were purchased from Huancayo, a city in the Peruvian Sierra. All pigs tested seropositive for cysticercosis and had palpable tongue nodules, implying heavy infection.[18] Swine cholera vaccine was given immediately after purchase, and three weeks acclimatization was allowed, during which the pigs were fed freely and no other medications were given. The animals were housed together, so the investigators were blind to the treatment group. The study was approved by the San Marcos Veterinary College ethical committee.

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Immunotherapy

The pigs were randomly divided into four treatment groups: 1) membrane-enriched cysticercal antigens (MA), five pigs; 2) saline control, seven pigs; 3) aqueous-soluble crude cysticercal antigens (AA) in adjuvant, nine pigs; and 4) adjuvant alone, seven pigs. The MA was prepared by the method of Molinari and others.[16] Briefly, 2,000 *T. solium* cysticerci were homogenized for 3 sec, centrifuged at 1,500 × *g*, and the pellet was resuspended in 0.02 M phosphate buffer (pH 7.4) containing 0.4% sodium deoxycholate (Merck, Rahway, NJ) and deoxyribonuclease (Sigma, St. Louis, MO) at a concentration of 20 μg/ml and ground in a mortar. The material was centrifuged at 30,000 × *g* and the supernatant was removed and dialyzed against several changes of 0.02 M phosphate buffer (pH 7.0) for six days. After the material was centrifuged at 30,000 × *g* for 20 min, the supernatant was lyophilized. The same amount of antigen was administered as in previous studies:[16], [17] a subcutaneous injection of 0.25 mg of protein (in 0. 1 ml of saline) was given behind the ear on days 1 and 7 (group 1). Protein was estimated by the method of Lowry and others.[19] Saline was administered in the same way as for group 1, as a control (group 2).

To maximize the expected immune response, we also evaluated larger doses of a different antigen, AA, given in adjuvant with a longer interval between doses. The AA in adjuvant was prepared by the method described by Estrada and Kuhn.[20] Briefly, the supernatant was taken from 2,000 homogenized, sonicated, centrifuged cysticerci, and the concentration was adjusted so that 2.4 mg of protein (measured as above) was given in 1 ml of Freund's adjuvant (Sigma) (group 3). This preparation was divided into five equal volumes that were injected into different subcutaneous sites on day 1 and again on day 14. Freund's complete adjuvant was used on day 1, and Freund's incomplete adjuvant was used on day 14. The cysticerci used for both MA and AA preparations were dissected from 17 pigs naturally infected in the same region as the pigs we treated with immunotherapy. Freund's complete adjuvant alone on day 1, followed by incomplete adjuvant alone on day 14, were administered in the same way as in group 3 (group 4).

Hematology and serology

Blood was taken from each pig immediately before the first immunization, at week 5, and immediately before being killed for measurement of differential white blood cell counts and electroimmunotransfer blot (EITB).[21] Briefly, EITB detects *T. solium*-specific antibodies by using seven lentil-lectin-purified glycoprotein (LLGP) bands (molecular weights = 50, 42–39, 24, 21, 18, 14, and 13 kD) commonly recognized by serum of humans and pigs with cysticercosis.[22], [23] A similar immunoblot method as the LLGP-EITB was also performed using the same AA that was administered as immunotherapy.[24]

Necropsy

Pigs were anesthetized and humanely killed 10–12 weeks after the first immunization. Sections through the right psoas muscle, heart, and right half of the brain were fixed in 10% buffered formalin (0. 15 M, pH 7.2), dehydrated, set in paraffin, and stained with hematoxylin and eosin. At least three cysticerci from each of these three tissues were examined for every pig. Predetermined criteria were used by a histopathologist who was blind to the treatment group to assess the host inflammatory response (cell density, predominant cell type, granulomatous reaction, and degree of fibrosis) and the degree of inflammation of the cyst membrane, spiral canal, and scolex (none, edema, mild-to-severe inflammation, presence of host cells, and degeneration or necrosis). Host cell type was identified by morphology.

Parasite viability

The left psoas and left anconeal muscles, tongue, and left half of the brain were weighed and dissected. Cysticerci were removed, graded according to macroscopic appearance, and evagination was attempted using a standard protocol. Briefly, cysticerci were washed insterile phosphate-buffered saline (PBS) (0.15 M, pH 7.2) supplemented with antibiotics and a fungistatic agent, and then incubated in 1% pepsin (pH 2.0, 37°C)for 30 min to digest the cyst membrane. The cysts were then washed with PBS and incubated at 37°C in the evagination medium (1/1,000 pH 8.9 trypsin solution with bile salts [Sigma]) for 1 hr. Cysticerci that failed to evaginate were considered not to be viable, and therefore would not be infective for humans.[25]

Data analysis

Data were analyzed with the Statistical Package for the Social Science (SPSS) software (SPSS Inc., Chicago, IL). Associations between categorical variables were analyzed by the Fisher exact test and associations among continuous variables by the Mann-Whitney and Kruskall-Wallis one-way analysis of variance nonparametnic tests.

RESULTS

There were no serious adverse reactions to immunotherapy. Two pigs developed a transient (< 1 day) limp following vaccination in the forelimb but no malaise, fever, or seizures were noted.

Hematology and serology

Differential peripheral blood white cell counts (including eosinophil counts) did not change significantly during the study or differ among treatment groups. The EITB with LLGP and AA confirmed that all pigs were antibody positive at the start of the study and repeat LLGP-EITB testing at the end of the study did not reveal any changes. Nine (64%) of 14 pigs given immunotherapy (MA plus AA group combined) developed new antibody bands during the study (12–13, 19, and 24 kD), as detected by AA-EITB, in contrast to only one (7%) of 14 control pigs ($P < 0.01$). The presence or development of one or more of these bands was not significantly related to white cell count, eosinophilia, or cysticercal viability.

Necropsy

Necropsy confirmed that all pigs were heavily parasitized with *T. solium* cysticercosis. The mean (SD) number of cysticerci per kilogram dissected from the tissues of all pigs were muscle, 356 (418); tongue, 70 (65); and brain, 14 (18). Overall, the mean number of cysticerci/kg varied considerably among pigs and was highest for the saline control group, but there were no significant differences among treatment groups (Table 1). Some inflammation was present around all of the cysticerci examined from all treatment groups, but this was so variable, even between adjacent cysticerci, that it was not possible to correlate inflammatory changes with treatment group or cysticercal viability. In approximately 50% of the sections from all treatment groups, 100–1,000 inflammatory cells were seen surrounding the cyst membrane, > 1,000 in 25%, and < 100 cells in the remaining > 25% of all sections. Macrophages were the most common cell type, especially when > 1,000 inflammatory cells were seen. Large numbers of eosinophils were also seen but predominated in less than one quarter of the cases, most commonly in the presence of < 100 inflammatory cells. Lymphocytes and plasma cells were seen in much smaller numbers.

Parasite viability

The macroscopic appearance of cysticerci revealed only six early degenerated cysticerci, all of which were found in two pigs that had been given AA in adjuvant. The percentage of cysticerci that failed to evaginate and were presumably not infective was calculated for each tissue from each pig (Table 1). Significantly fewer of the cysts from pigs given AA in adjuvant were viable compared with those from the adjuvant ($P < 0.04$) or saline ($P < 0.05$) control groups. Adjuvant alone had no significant effect upon viability compared with saline. There was a trend towards an effect of MA to decrease parasite viability in muscle, where the parasites were most numerous, but this was not significant when compared with adjuvant, saline, or combined control groups. Immunotherapy (AA and MA groups combined) was associated with a 50% increase in the percentage of nonviable cysticcrci when compared with the combined control groups ($P < 0.04$). Immunotherapy (MA and AA combined) was associated with a three-fold increase in the proportion of nonviable cysticerci in tongue tissue ($P < 0.03$) and muscle ($P < 0.06$), but there was no effect of immunotherapy detected in the brain ($P < 0.9$) (Table 1). Relatively few cysticerci in brain tissue were found, and these were of low viability for all treatment groups (Table 1).

The large variation in the number of cysticerci found in the pigs in each treatment group (from 34 to 2,413 cysts/kg) led us to base the statistical analysis on the percentage of viability of cysts for each tissue from each pig. This avoided potential bias introduced by the small number of heavily infected pigs and by the amount of each tissue dissected. Moreover, we repeated the above analyses using the total number of viable and nonviable cysticerci dissected from pigs in each treatment group and the pattern of significance was unchanged: overall, 35% of the cysts dissected from pigs given immunotherapy were nonviable compared with 10% of those from all of the control animals ($P < 0.05$). In spite of these effects, at least 25% of the cysticerci were viable in every pig and more than 50% were viable in 11 of the 14 pigs given immunotherapy (MA or AA).

DISCUSSION

This blinded, randomized, controlled study confirmed that administration of cysticercal antigens (AA) to pigs naturally infected with *T. solium* cysticercosis caused a significant reduction in the viability of cysticerci that can cause human tapeworm infection. The percentage of nonviable cysticerci was increased three-fold in pigs given AA in adjuvant and most of these animals developed new antibody bands detected by EITB.

In a previous study of immunotherapy, two cysticercotic pigs inoculated with MA developed eosinophilia and necropsy four and eight weeks later revealed histologic findings suggestive of cyst degeneration.[16] However, the viability of cysticerci was not assessed in this study and the histopathologic findings may have been biased by the small number of pigs and cysticerci studied, particularly in view of the highly variable inflammation we observed after administering the same dose of MA.

In a field trial in Mexico,[17] 1,076 doses of MA that was almost identical to the preparation we used were administered repeatedly to 447 pigs, although it was not clear how many doses each pig received. The prevalence of cysticercosis as assessed by tongue palpation decreased significantly in the villages studied but there was no control group, limiting interpretation of these results. Furthermore, their reliance on tongue palpation alone to assess response to immunotherapy may have been misleading since we found the effect of immunotherapy to be greater in tongue than in other tissues. In this field trial, seven cysticercotic pigs given MA immunotherapy were studied in more detail and 73% of the cysts excised from treated pigs failed to evaginate compared with 5% in seven untreated cysticercotic pigs. The ineffectiveness of MA in our study contrasts with these results and

might be explained by the smaller number of vaccinations given, which may have induced a weaker immune response.

The partial effectiveness of AA immunotherapy and the variable inflammatory changes seen in adjacent cysticerci in our study might be explained if the cysticerci infecting the pigs differed antigenically from the cysticerci used to prepare the immunotherapy. Morphologic heterogeneity and antigenic diversity among cysticerci dissected from different naturally infected pigs have both been noted, and DNA probes have revealed genetic variation among different geographic isolates of porcine cysticerci.[26-28] However, our antigens were prepared from cysticerci obtained from pigs from the same area as those receiving immunotherapy, so antigenic heterogeneity is not as likely to be relevant.

At necropsy, control pigs that had been treated with saline generally had more cysticerci/kg tissue than pigs from the other groups. This is most likely due to random variations rather than treatment effects because the differences did not approach statistical significance and there were no scars or calcifications present in the tissues of pigs given immunotherapy. In contrast, 12 weeks after drug therapy for porcine cysticercosis, calcifications were clearly visible at the site of degenerated cysticerci in previously infected meat, so it is unlikely that immunotherapy caused the disappearance of cysticerci without a trace in the same period.

The statistically significant effect of immunotherapy (AA) on *T. solium* viability illustrates the dynamic nature of the host-parasite interaction and the potential for manipulating this relationship to control parasitic infection. Genetically engineered, recombinant vaccines cause greater immunity against other tapeworm species.[29, 30] and identification and synthesis of the appropriate antigens for porcine cysticercosis may allow more effective immunotherapy. However, despite the statistically significant effect of immunotherapy on cysticercal viability in our study, all of the pigs remained macroscopically heavily infected with predominantly viable cysticerci. This immunotherapy, alone, therefore, is unlikely to prevent human taeniasis.

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Table 1

Effect of immunotherapy*

Exdperimental groups	No. of pigs	Total cysts/kg	% of cysticerci that failed to evaginate			
			Muscle	Tongue	Brain	All cysts
Membrane-enriched antigens (MA)	5	245 (164)	15 (14)	13 (15)	48 (37)	17 (16)
Saline	7	524 (855)	7 (8)	13 (21)	41 (39)	18 (19)
Aqueous-soluble antigens (AA)	9	287 (274)	28 (29)	46 (36)	44 (41)	34 (28)†
Adjuvant	7	292 (261)	7 (8)	8 (11)	53 (40)	10 (9)†
Immunotherapy (MA, AA)	14	272 (235)	23 (25)	35 (34)	46 (38)	28 (24)‡
Controls (saline, adjuvant)	14	408 (558)	7 (8)	11 (16)	47 (38)	14 (15)‡

*The percentage of cysts that failed to evaginate and were presumably not infective was calculated for each pig. The mean (SD) of these percentages for each treatment group are shown with the total number of cysticerci identified per kilogram of tissue.

†These values are statistically different from each other, $P < 0.04$.

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