Cellular and Humoral Immune Response of Foals to Vaccination with Corynebacterium equi

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ABSTRACT

Transformation of peripheral blood lymphocytes from pony foals vaccinated and subsequently infected with Corynebacterium equi was studied. Three foals were vaccinated on two occasions using a formalinized C. equi vaccine with aluminum hydroxide as an adjuvant. Three nonvaccinated foals served as controls. Foals were challenged intratracheally with 9 x 10^9 C. equi six weeks after the initial vaccination. Foals survived this infection for one to two weeks. Significant lymphocyte transformation in response to C. equi antigens was detected in two vaccinated foals at the third week after initial vaccination and in all vaccinated animals at the fifth week. No statistically significant transformation was seen in nonvaccinated foals before infection. Vaccinated and nonvaccinated foals showed responsive lymphocytes following challenge. Vaccination offered no obvious protection against experimental challenge but this failure was probably due to an excessive infective dose of organisms. Low levels of humoral antibodies were detected in some challenged foals. The pathological changes in the lungs of infected animals were comparable with, but more fulminating than, changes observed in the natural disease.

RÉSUMÉ

Cette expérience visait à étudier les transformations des lymphocytes du sang périphérique de jeunes poneys, vaccinés et ensuite infectés avec Corynebacterium equi. On vaccina trois de ces poneys, à deux reprises, avec un vaccin formolé auquel on ajouta de l'hydroxyde d'aluminium, comme adjuvant. Trois poneys non vaccinés servirent de témoins. On soumit tous les poneys à une infection de défi intra-trachéale, en utilisant une dose de 9 x 10^9 C. equi, six semaines après la première vaccination. Ils survécurent à cette infection, pour une à deux semaines. On décela une transformation appréciable des lymphocytes, chez deux des poneys vaccinés, à partir de la troisième semaine après la vaccination, et chez tous les poneys vaccinés, à la cinquième semaine après la vaccination. On ne décela pas de transformation appréciable des lymphocytes, chez les poneys témoins, avant leur infection. Les poneys vaccinés, tout comme les témoins, manifestèrent une transformation de leurs lymphocytes, après l'infection de défi. La vaccination ne donna pas de protection appréciable contre une infection de défi, sans doute à cause de l'utilisation d'une dose excessive de C. equi. Certains des poneys soumis à une infection de défi développèrent un faible taux d'anticorps sériques. Les lésions pulmonaires des poneys infectés ressemblaient à celles que provoque la maladie naturelle, mais elles s'avérèrent plus marquées.

INTRODUCTION

Corynebacterium equi is a bacterium which causes a serious pneumonia in foals (2,5,11,16,18,24) and tuberculous-like changes in the cervical lymph nodes of pigs (10). It has been recovered infrequently from pneumonic lungs in sheep (1), cattle (12,20), pigs (23), immunosuppressed people (3) and from lymphadenitis in a cat (14).

Purulent pneumonia in horses caused by *C. equi* occurs throughout the world and is primarily a disease of one to three month old foals (2,8,11,16,18,24). The course of the disease is generally one to two weeks, is associated with a mortality rate of 65% and may be responsible for up to 1-3% of all foal deaths (9,22). Gross pathological changes include bilateral suppurative bronchopneumonia with extensive lymphadenitis and frequent involvement of the mucosa of the large bowel (7,11,18). The

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disease is mainly an individual farm or premises problem, is difficult to treat effectively and seems to be increasing in different parts of North America (22). The organism is thought to be an inhabitant of soil and to be widespread in the environment (11,24). It is distinctly different from typical members of the genus *Corynebacterium* and is related to the mycobacteria and nocardia, members of which include soil saprophytes (21).

Control of the disease presents several problems, in part because there is insufficient knowledge of the epidemiology and pathogenesis of the disease. Treatment is often unsatisfactory since severe pathological changes may be present before clinical disease is recognized. Vaccination offers one approach to control of the disease on affected premises. It has been known for years that horses sick with or recovered from *C. equi* pneumonia show no or only low agglutinating antibodies to *C. equi* (6,11,18). An intradermal hypersensitivity test was developed and about half the horses studied showed a marked reaction to the skin test, suggesting that immunity was in part cell-mediated and that infection was widespread in the population studied (24). This evidence, taken with the nature of the cellular response seen in infection (16), suggests that immunity to the disease is primarily cell-mediated rather than humoral and that, consequently, vaccination should be aimed at stimulating a cellular rather than a humoral response.

The purpose of the work described here was: 1) to develop suitable antigens of *C. equi* which could be used for *in vitro* lymphocyte studies of vaccinated or infected animals, 2) to use these antigens in a lymphocyte-stimulation test to study the cell-mediated response in such animals, 3) to determine the humoral antibody response to vaccination or infection and 4) to determine whether the vaccination procedures used would protect foals against experimental infection.

MATERIALS AND METHODS

Animals and Vaccination Procedures

Six purebred Welsh Mountain foals aged three weeks to two months were obtained from a herd with no history of pneumonic problems. They were divided randomly into two groups in adjacent pens. Three foals (numbered 1,2,3) were vaccinated at a time designated week 0. They were revaccinated four weeks later (week 3), and both groups of foals were challenged intrabronchially with *C. equi* after six weeks (week 5). Nonvaccinated foals were assigned numbers 4, 5, 6 for identification purposes.

The organism used to vaccinate the foals was obtained from the lung of a foal which died of *C. equi* pneumonia. It was designated *C. equi*, Isolate 1 and had been stored in liquid nitrogen since isolation. The organism was cultured on trypticase soy (TS) agar[1] in a Roux flask for 72 hours and washed off with 0.3% formal phosphate buffer saline (PBS). The organism was killed by suspension overnight at 37°C in formal PBS, washed twice in formal PBS and resuspended to give a concentration of approximately 3 x 10^{10} organisms per mL PBS (3.19 mg lyophilized bacterial/mL PBS). Aluminum hydroxide adjuvant (35% v/v) (Rehosorptar, 2% aluminum hydroxide absorptive gel)[2] was mixed with the bacterial suspension and 2.5 mL of the adjuvanted vaccine was injected deep into the muscle at one site in the neck of the three foals (nos. 1,2,3). The second vaccine, administered at week 3, was prepared and administered in the same way with the exception that a lower concentration of organisms was used (1 x 10^{10} organisms/mL, 2.16 mg lyophilized bacteria/mL PBS). Animals were observed following vaccination for signs of local reaction to the vaccine.

Experimental Infection of Foals

Foals were infected six weeks after the onset of the study by an intrabronchial inoculation of 9 x 10^9 *C. equi*, Isolate 1 (viable count), suspended in 40 mL PBS. Ponies were sedated with xylazine and placed in lateral recumbency and following local anaesthesia of the skin and underlying tissues of the neck a narrow polythene catheter was introduced into the trachea through a cannula and guided towards the bronchi. The suspension was

[1]Difco Laboratories, Detroit, Michigan.

[2]Reheis Chemical Corporation, Phoenix, Arizona.

administered once the animals coughed in response to the irritation of the tube. Animals were observed daily after infection. They were generally killed *in extremis* with an overdose of a barbiturate solution. Representative samples of all major organs were collected at postmortem and fixed in 10% buffered formalin. Fixed tissues were routinely processed and sections were stained with haematoxylin and eosin and Brown and Brenn stain for bacteria. Samples of lung, bronchial lymph nodes, liver, spleen, several levels of the intestinal tract and mesenteric lymph nodes were submitted for bacteriological examination.

Lymphocyte Stimulation Procedures

Preparation of antigens — C. equi, Isolate 1 was cultured in TSB broth for 72 hours at 37°C. The cultures were harvested by centrifuging at 10,000 x g for 30 minutes. The culture supernatant was filtered through filters of pore size 0.45 μm[3] and dialyzed against distilled water at 4°C for three days. The dialyzed material was lyophilized and used as antigen S. The sediment from the centrifugation was sonicated in a 100 watt ultrasonic disintegrator[4] until a homogenate was obtained. The homogenate was centrifuged twice at 15,000 x g for 15 minutes to remove cellular debris. The supernatant was dialyzed for three days at 4°C and lyophilized. This constituted antigen N. Antigens were stored at –20°C until used.

Collection of blood — Blood was collected weekly from the foals. Fifty mL of blood was collected in sterile tubes containing EDTA[5] and 10 mL collected in a sterile tube to obtain serum following clotting. The serum was stored at –20°C for subsequent serological studies.

Lymphocyte separation — Blood was processed for recovery of lymphocytes within two hours of collection. The lymphocytes were purified by a modification of the Ficoll-Hypaque (FH) method (4). Briefly, the blood was centrifuged at 400 x g for ten minutes and the top portion of plasma containing mainly platelets was discarded. The tubes were then centrifuged at 1500 x g for ten minutes, the buffy coat removed and suspended in 20 mL Seligman's balanced salt solution (SBSS). Eight mL FH was introduced under the buffy coat suspension and the tube centrifuged for five minutes at 1500 x g. The lymphocyte-rich fraction was removed from the surface of the FH and washed twice with SBSS and suspended in tissue culture medium.[6] The lymphocytes were counted in a haemocytometer and resuspended to give a concentration of 1 x 10^6 cells per mL RPMI-1640, containing 15% v/v fetal calf serum and penicillin (100 μg/mL) and streptomycin (40 μg/mL).

Lymphocyte culture — The cultured lymphocytes were dispensed in 2.5 mL quantities into sterile tubes.[7] Three tubes were not inoculated with mitogens or antigens and acted as control tubes. Phytohaemagglutinin (PHA-P)[8] was added to three tubes (5 μL/tube). Triplicate cultures were also inoculated with two antigen preparations designated S and N and prepared as described above. Crude antigen S was added to cultures to give a final concentration of 200, 100 and 50 μg of lyophilized antigen per mL of lymphocyte culture. Antigen N was used at a final concentration of 200, 100 and 50 μg of lyolyophilized preparation per mL of culture. After the third week (week 2) the concentration of N was changed to give 25, 12.5 and 6.25 μg/mL of culture medium.

Lymphocyte cultures were incubated at 37°C in tightly sealed tubes for 72 hours. Cultures were pulsed with 1 μCi/tube of methyl-tritiated-thymidine[9] 12 hours prior to the termination of the cultures. Cells were harvested for liquid scintillation counting as described (13) using a semi automatic cell harvester.[10]

Results for individual foals were expressed as the mean of the natural log (ln) of the counts per minute (cpm) of triplicate cultures either with additives

[3]Millipore Filter Corp., Bedford, Massachusetts.

[4]Virtis Model 16-850, Virtis Co., Gardiner, New York.

[5]Vacutainer, Becton, Dickinson & Co., Mississauga, Ontario.

[6]RPMI-1640, Grand Island Biological Company, Grand Island, New York.

[7]Falcon 12 x 75 mm, Fisher Scientific Co., Toronto, Ontario.

[8]Difco Laboratories, Detroit, Michigan.

[9]Amersham Corp., Oakville, Ontario.

[10]Titertek cell harvester, Flow Laboratories, Rockville, Maryland.

(mitogen or antigens) or without additives (control cultures). Stimulation ratios were calculated for comparison of group of vaccinated and nonvaccinated foals. A stimulation ratio was defined as: (mean ln cpm of stimulated cultures) — (mean ln cpm of control cultures).

Results were analysed statistically using a two tailed Student's t test.

Serological Studies

Tube agglutination test — Corynebacterium equi, Isolate 1, was cultured in Roux flasks on TS agar at 37°C for three days. The cultures were washed from the agar and washed three times in sterile PBS. They were suspended to an optical density of 1.0 at 525 nm. Sera from the foals taken weekly was serially diluted from dilutions of 1:5 to 1:5120 in PBS and an equal volume of the bacterial suspension added. Tubes were incubated at 50°C for one hour and agglutination recorded after overnight incubation at room temperature. Tubes were kept for indirect bacterial agglutination.

Antiglobulin test — Antisera to horse globulins were prepared by injecting into rabbits an ammonium sulphate precipitation of horse serum suspended in Freund's complete adjuvant.[11] Antisera obtained was diluted 1:40 (v/v) in PBS prior to use in the antiglobulin test. Once titres had been recorded in the bacterial agglutination test the tubes were centrifuged and pellets washed once with PBS, centrifuged and resuspended in 0.5 mL rabbit antihorse globulin suspension. Tubes were left overnight at room temperature and agglutination titres reread.

Passive haemagglutination — The method used was that described previously (6) with the exception that the soluble capsular antigen, extracted from the bacteria by heating in PBS at 56°C for 30 minutes, was lyophilized after dialysis in distilled water for two days at 4°C. Packed human group O red blood cells (RBC) were incubated for two hours at 37°C in PBS with a quantity of lyophilized material added (10 mg/100 mL PBS). Cells were then washed three times in ordinary PBS. Foal sera were absorbed with human Group O RBC and tests were carried out as described.

All serological tests were done in duplicate.

[11]Difco Laboratories, Detroit, Michigan.

RESULTS

Vaccination Procedures

No local reaction was observed at the site of the first vaccine injection. After the second vaccination one foal (number 2) developed a large painful swelling at the site of inoculation. This was hand-sized three days after inoculation but was resolved by the seventh day. Foal number 1 developed a small swelling (five centimetres at three days) at the inoculation site which was slightly painful and also was resolved by the seventh day. The third pony (number 3) developed a pea-sized painless nodule at the site by the third day after inoculation which had disappeared by the seventh day.

Experimental Infection of the Foals

Little clinical difference was observed between the two groups of experimentally infected animals. The rectal temperatures of the vaccinated foals rose less quickly than those of nonvaccinated animals in the first six days after infection (102.5 ± 0.5°F on day 6 compared to 104.4 ± 0.4°F). Thereafter no difference was observed between the two groups in terms of the severity of the clinical disease and time of death or euthanasia. The clinical response was apparently a function of size since the smaller foals were more severely affected than the larger foals and died or were euthanized earliest. Foal number 4 (unvaccinated) was killed eight days after infection, foals number 2 and 3 (vaccinated) nine days after infection and number 5 (unvaccinated) died on day 9 after infection. Animals number 1 (vaccinated) and 6 (unvaccinated), the largest of the foals, were killed 17 days after infection. All foals ate well and remained bright and alert until shortly prior to death or euthanasia.

TABLE I. Individual DNA Synthesis Responses of Peripheral Blood Lymphocytes to PHA, *C. equi* Antigens S and N in Vaccinated and Nonvaccinated Foals

Week Number	Mitogen or Antigen	Mean ln Counts per Minute ± Standard Deviation					
		Vaccinated Foal			Nonvaccinated Foal		
		1	2	3	4	5	6
0	PHA	10.5685 ± 0.1367[d]	10.5494 ± 0.1024[d]	9.5658 ± 0.5483[d]	10.6902 ± 0.0379[d]	11.0278 ± 0.0342[d]	10.3120 ± 0.1249[d]
	Control	6.6760 ± 0.1848	6.7215 ± 0.4624	5.5285 ± 0.2278	5.0353 ± 0.7931	6.5080 ± 0.2445	7.0376 ± 0.1124
	S200	6.7973 ± 0.1493	6.4821 ± 0.1767	5.5469 ± 0.4488	6.5766 ± 0.4707	7.0514 ± 0.3805	7.3267 ± 0.1410
	N12.5	6.1033 ± 0.2770[a]	5.0531 ± 0.1664[d]	5.3647 ± 0.3909	6.8393 ± 0.2202	6.9960 ± 0.3757	7.3938 ± 0.2017
1	PHA	9.5710 ± 0.0455[d]	9.8726 ± 0.0341[d]	9.8346 ± 0.2488[d]		9.2428 ± 0.2801[d]	10.3049 ± 0.1645[a]
	Control	5.1817 ± 0.1857	4.5656 ± 0.2887	5.3262 ± 0.3784		5.3894 ± 0.4584	8.6614 ± 0.5685
	S200	5.4709 ± 0.0618	4.9143 ± 0.0977	5.6160 ± 0.1207	Not done	5.5329 ± 0.6717	8.5700 ± 0.1498
	N12.5	4.9875 ± 0.5379	4.8139 ± 0.4784	5.2623 ± 0.5367		5.8859 ± 0.1766	8.7576 ± 0.2217
2	PHA	10.5182 ± 0.0042[d]	11.1467 ± 0.0364[d]	11.1355 ± 0.0314[d]	10.4522 ± 0.0743[d]	10.8835 ± 0.0230[d]	10.8297 ± 0.0884[d]
	Control	6.3834 ± 0.2513	7.4966 ± 0.2849	4.5536 ± 0.2077	8.0565 ± 0.2788	7.2673 ± 0.3329	7.6572 ± 0.4934
	S200	6.6139 ± 0.0315	7.5814 ± 0.0697	4.7897 ± 0.0881	7.8883 ± 0.2843	7.3905 ± 0.3199	7.6123 ± 0.5842
	N12.5	6.3472 ± 0.1503	6.6626 ± 0.1416[a]	5.3624 ± 0.1192[a]	6.7735 ± 0.2120[c]	6.5018 ± 0.2921[a]	6.5578 ± 0.2876[a]
3	PHA	10.8849 ± 0.1899[d]	10.8862 ± 0.0623[d]	7.5913 ± 0.9059[d]	11.0427 ± 0.0627[d]	4.7098 ± 0.1845[c]	6.0891 ± 0.0642[d]
	Control	8.5129 ± 0.2801	9.0761 ± 0.0276	4.1239 ± 0.1143	8.5799 ± 0.2748	4.0619 ± 0.1109	4.1972 ± 0.2538
	S200	8.7796 ± 0.2660	9.2312 ± 0.1636	4.4277 ± 0.5438	8.5226 ± 0.4479	4.0243 ± 0.0536	4.0348 ± 0.2612
	N6.25	8.7398 ± 0.2640	8.9010 ± 0.1355	4.5151 ± 0.0696	7.9739 ± 0.1682[a]	4.1106 ± 0.2522	4.0757 ± 0.0725
Booster Vaccination							
4	PHA	8.2743 ± 0.1780[d]	8.8712 ± 0.1920[d]	9.0772 ± 0.0869[d]	10.2021 ± 0.0869[d]	9.0458 ± 0.3366[d]	8.8925 ± 0.2879[d]
	Control	5.1332 ± 0.2067	7.0375 ± 0.3444	6.1601 ± 0.3330	8.0665 ± 0.0534	0.0166 ± 0.4034	5.5995 ± 0.1280
	S200	6.8221 ± 0.2424[d]	7.2440 ± 0.1912	6.6412 ± 0.2273	7.0825 ± 0.1880[d]	5.7317 ± 0.5116	5.6464 ± 0.1531
	N6.25	6.6312 ± 0.1708[d]	7.5436 ± 0.0117[a]	7.1741 ± 0.2002[c]	7.6604 ± 0.3077	5.2479 ± 0.2808[a]	5.4789 ± 0.2458
5	PHA	8.0723 ± 0.3189[d]	10.4597 ± 0.0438[d]	10.8035 ± 0.5617[b]	6.7112 ± 0.1083[d]	6.6979 ± 0.0950[d]	7.2762 ± 0.1037[d]
	Control	4.5891 ± 0.2244	7.9299 ± 0.1278	8.7594 ± 0.5750	4.2062 ± 0.2308	5.4063 ± 0.0059	4.6375 ± 0.3817
	S200	4.5093 ± 0.0778	Not done	9.0984 ± 0.3781	4.4112 ± 0.0600	5.3721 ± 0.3095	4.7108 ± 0.4764
	N6.25	5.1868 ± 0.0859[b]	8.1439 ± 0.0628	9.2459 ± 0.2285	4.3199 ± 0.1772	5.4789 ± 0.1532	4.7542 ± 0.3900
Challenge Infection							
6	PHA	9.2688 ± 0.1317[d]	10.3250 ± 0.1835[d]	9.4855 ± 0.1474[d]	7.9254 ± 0.3706[d]	8.7458 ± 0.0934[d]	7.6769 ± 0.2251[d]
	Control	5.3178 ± 0.3956	4.3917 ± 0.2453	5.1301 ± 0.2496	4.3001 ± 0.3060	4.2610 ± 0.0883	4.4863 ± 0.3370
	S200	6.0387 ± 0.5449	4.2241 ± 0.1240	5.2674 ± 0.1071	4.1473 ± 0.0553	4.9200 ± 0.6372	4.6332 ± 0.1811
	N6.25	7.3260 ± 0.3422[c]	4.6966 ± 0.1071	6.5845 ± 0.1371[d]	5.3572 ± 0.4530[a]	6.3671 ± 0.6389[c]	7.1496 ± 0.2434[d]
7	PHA	12.0698 ± 0.0341[d]		10.5048 ± 0.0189[d]			9.4458 ± 0.0003[d]
	Control	8.1225 ± 0.0355		8.2548 ± 0.0139			7.0678 ± 0.0922
	S200	9.1592 ± 0.0127[d]	Died	8.4445 ± 0.0167[d]	Died	Died	7.9834 ± 0.3340[c]
	N6.25	9.2066 ± 0.0385[d]		8.7776 ± 0.0069[d]			8.6170 ± 0.0707[d]

[a] $P < 0.05$
[b] $P < 0.025$
[c] $P < 0.01$
[d] $P < 0.005$

Pathology

Gross pulmonary lesions, which were unilateral, comprised massive consolidation involving almost all the affected lung in three ponies and at least 2/3 of the organ in the other animals. Areas of necrosis were prominent in the lungs of two ponies (#5 and 6) and in one of these (#5) liquefaction of necrotic areas was advanced. The microscopic changes were those of a suppurative bronchopneumonia with varying degrees of necrosis. Gram-positive coccoid bacteria were present within the pulmonary macrophages which occurred in large numbers in the exudate and also in multinucleated giant cells, present at lower frequency. The bronchial lymph nodes of all ponies were markedly enlarged and in two cases (#1 and 5) contained foci of necrosis. Histologically, there was extensive pyogranulomatous lymphadenitis with variable degrees of necrosis. Generalized lesions consistent with septicaemia were seen in the only pony to die naturally (#5). In one pony only (#5), microscopic pathology in the colon indicated *C. equi* infection. Detailed description of the pathology of these experimental infections with *C. equi* will be presented elsewhere.

C. equi was isolated in large numbers from the affected lungs and bronchial lymph nodes of all ponies and also, in lesser numbers, from the lesion-free lungs. Bacteraemia, indicated by positive cultures from liver or spleen, occurred in all six animals. *C. equi* was isolated in high numbers from the large intestines of three ponies (#1, 2 and 6).

Lymphocyte Stimulation

Results of lymphocyte stimulation studies are shown in Table I. A trial concentration of 200 μg per mL of culture gave the greatest response for preparation S. Antigen N was toxic to lymphocytes at higher doses (50, 20 μg/mL) and on occasion a concentration of 12.5 μg per mL culture medium was toxic. A concentration of 6.25 μg per mL was therefore used in the later weeks.

Significant stimulation with antigen N was observed in two vaccinated foals (numbers 2 and 3) at week 2 and in all vaccinated foals at week 4. No significant stimulation was detected in nonvaccinated foals prior to challenge. All experimentally infected foals, except foal number 2, showed significant lymphocyte stimulation with antigen N from the first week following challenge. Significant lymphocyte stimulation in infected or vaccinated foals was seen less commonly with antigen S.

Table II shows the comparison of the group mean stimulation ratios between vaccinated and nonvaccinated foals. No statistically significant difference could be detected between the two groups at each week.

Serological Studies

Table III shows the results of the bacterial agglutination and passive haemagglutination tests in vaccinated and nonvaccinated foals.

Results of the indirect bacterial agglutination (antiglobulin) test are not shown. No obvious difference was detected in the antiglobulin test between the two groups of horses. Both groups showed indirect agglutination titres of at least 1:160 to 1:320, which on occasion rose to 1:1280 in foals of either group. Results were variable and difficult to reproduce. The highest indirect agglutination titres were obtained with freshly grown bacteria.

DISCUSSION

Immunity to *C. equi* infection is thought to be primarily cell-mediated rather than humoral (6,16) and for this reason it was decided to use an adjuvant with the bacterial cell vaccine. Cell-mediated immunity is greatly stimulated by Freund's adjuvant but in horses the use of this material results in severe local and systemic effects (17). Aluminum hydroxide was therefore chosen as an adjuvant since it is not toxic and in some cases appears to stimulate a local humoral and cell-mediated, as well as a systemic humoral, immune response (19). Local, but temporary reactions were seen to the second vaccination injection but whether this represented an immunolo-

TABLE II. Comparison of DNA Synthesis Responses of Peripheral Blood Lymphocytes to PHA and *C. equi* Antigens S and N Between Groups of Vaccinated and Unvaccinated Foals

Week Number	Group mean ln Stimulation Ratio[a]					
	Vaccinated Foals (3)			Nonvaccinated Foals (3)		
	PHA	Antigen S	Antigen N	PHA	Antigen S	Antigen N
0	3.9242 ± 0.1068	$\bar{0}$.0347 ± 0.1840	$\bar{0}$.8017 ± 0.8779	4.1893 ± 0.7845	0.4893 ± 0.1793[b]	$\bar{0}$.2389 ± 0.7308
1	4.7349 ± 0.4490	0.3152 ± 0.0289	$\bar{0}$.0997 ± 0.3671	2.7485 ± 1.5626	0.0259 ± 0.1658	0.2963 ± 0.2830
2	4.7889 ± 1.5715	0.1839 ± 0.0854	0.0035 ± 0.8580	3.0614 ± 0.6178	$\bar{0}$.0299 ± 0.1460	$\bar{1}$.0474 ± 0.2619
3	2.5498 ± 0.8428	0.2418 ± 0.0774	0.1476 ± 0.2913	1.6825 ± 0.9033	0.0607 ± 0.0100	$\bar{0}$.1572 ± 0.4631
Booster Vaccination						
4	2.6306 ± 0.6992	0.5782 ± 0.9887	1.0060 ± 0.4957	2.8192 ± 0.6066	$\bar{0}$.4073 ± 0.5263	0.4318 ± 0.3248
5	2.5253 ± 0.6783	0.1296 ± 0.2961	0.4327 ± 0.1974	2.1451 ± 0.7421	0.0813 ± 0.1198	0.1010 ± 0.0246
Challenge Infection						
6	4.7465 ± 1.0474	0.2302 ± 0.4518	1.2558 ± 0.8688	3.7702 ± 0.6575	0.3304 ± 0.2850	1.9283 ± 0.8114
7	3.0986 ± 1.2002	0.6132 ± 0.5989	0.8035 ± 0.3969	2.3772	0.9156	1.5492

[a]Stimulation ratio for each horse is the (mean ln cpm of stimulated culture) — (mean ln cpm of control cultures)
[b]$P < 0.05$

gically-mediated reaction remains to be determined.

It is clear from the results shown in Table I that animals experimentally infected for one week with *C. equi* (week 6) showed significant lymphocyte stimulation with antigen N. In most cases the stimulation response was more marked than that generally seen in vaccinated but uninfected animals. It is clear that the vaccination procedure used also gave rise to sensitized lymphocytes even though not all foals showed this response until the fifth week after initial vaccination.

Sensitized lymphocytes from vaccinated or infected animals generally responded more markedly to antigen N than to antigen S, in which the concentration of antigen present was probably low. Antigen N was thus a suitable antigen for use in these lymphocyte studies although it was sometimes toxic to lymphocytes at concentrations higher than 6.25 μg per mL culture. This was most marked, for example, in week 2 where the antigen concentration of 12.5 μg was more toxic for lymphocytes from nonvaccinated when compared to vaccinated foals. The reason for this difference is not known.

Sensitized lymphocytes were detected in two vaccinated foals as early as the third week after vaccination, were not detected in the fourth week but were detected again in the fifth week following a booster vaccination. The lymphocyte stimulation response following infection with *C. equi* was in general more marked than that observed following vaccination. This increased response is apparent in Table II where the group mean stimulation ratio for infected foals (weeks 6 and 7) was compared to group mean stimulation ratios of the previous weeks. Foal number 2 responded poorly to antigen N one week following infection. The reason for this apparent poor response is not clear.

No difference could be shown statistically between the groups of foals in terms of response to antigens, mitogens or to challenge for any particular week. Failure to show a difference was in part owing to the small numbers of animals and the large standard deviation between the two groups. Since this work was carried out the test has been refined and far more uniform data have been

TABLE III. Direct Bacterial Agglutination and Passive Haemagglutination Titres in Sera from Vaccinated (1, 2, 3) and Nonvaccinated (4, 5, 6) Foals

Week	1		2		3		4		5		6	
Number[a]	A[b]	H[c]	A	H	A	H	A	H	A	H	A	H
5	—	20	—	—	—	—	—	—	—	—	10	—
6	10	160	5	—	10	320	—	—	—	—	10	160
7	10	160	Died		ND[d]	ND[d]	Died		Died		10	160

[a]Sera from week 0 to 4 were negative in both tests
[b]Bacterial agglutination titre
[c]Passive haemagglutination titre
[d]Not done

obtained than that seen in the present study. It is clear however from Table I that individual foals showed significant sensitization of lymphocytes following vaccination and challenge infection.

The lymphocyte response to the mitogen phytohaemagglutinin showed great variation between animals and from week to week. No effect of vaccination on this response could be detected (Table II). In animals following challenge with *C. equi* no change was apparent in the response to phytohaemagglutinin, suggesting that gross depression of T-lymphocyte reactivity was not involved in the pathogenesis of the infection.

The cellular immune response detected in vaccinated animals preceded the humoral response, which was observed to any degree only in some of the experimentally infected animals. Results of the serological response to vaccination and infection (Table III) are in accord with the findings of other authors that at best only low levels of agglutinating antibody develop (11, 18). The passive haemagglutination test is more sensitive than the agglutination test (6), but it only detected a minor response in one vaccinated and uninfected foal. Moderate levels of indirect haemagglutinating antibody were detected in only half the infected foals, although titres were as high or higher than the titres described in a previous study (6). In that study it was also observed that serum from a foal which died from *C. equi* pneumonia showed no detectable serological response. Results of the antiglobulin test were difficult to interpret but suggested that no incomplete antibodies developed in vaccinated or infected horses.

Vaccination offered no protection against experimental infection. The fact that no protective effect was evident was probably due to the large number of organisms used to challenge the foals. The nature of the pathological changes also indicated that these provided an overwhelming challenge.

The pathological changes observed were compatible with those described by others (7, 11, 15, 18) but changes in the lungs were more fulminating than usually observed in the natural disease. Challenging the animals in lateral recumbency resulted in severe changes being limited to one side of the lung. A large number of organisms was used to challenge foals in view of the difficulty some workers (8) experienced in infecting foals. A more realistic method of challenge was probably that adopted by Flatla (11) who observed the disease about three weeks following the feeding of *C. equi* cultures to young foals.

There is evidence that *C. equi* behaves as an intracellular parasite and that immunity is cellular rather than humoral (16, 24). The results presented here suggest that it is possible to produce a cell-mediated response to *C. equi* in foals using an adjuvant vaccine. The bacterium possesses both species specific capsular (6) and somatic antigens (5) and it is probable that one typical *C. equi* isolate could be used in a vaccine for general use in horses; on a premise where *C. equi* pneumonia is a problem then an isolate originating from this source might offer the additional advantage of appropriate type-specific antigens (5). It is not known however which antigens confer protection in horses against disease caused by *C. equi*

Further work is required on the optimal means of producing artificial immunity to *C. equi* pneumonia in foals. Information is needed as to the role of the various antigens of *C. equi* in the immune response, and the number of vaccinations and the appropriate type of

adjuvant required to produce protection also requires investigation. The vaccination of the dam with *C. equi* prior to parturition and the effect of passive transfer of immunity through colostrum may also be worth exploring. Antigen N described in this paper seems to be a preparation suitable for assessing the effect of vaccination on the sensitization of lymphocytes. We are currently assessing the use of antigen N in lymphocyte stimulation tests for the diagnosis of *C. equi* infections.

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