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Hematologic and Clinical Response to Anaplasmosis Vaccines and the Comparative Efficacy of These Vaccines, As Measured by Field and Experimental Challenge*

K. L. Kuttler, H. Zaraza, E. D. Roberts

The first agent, and certainly the one most widely used to reduce losses from anaplasmosis, has been *Anaplasma centrale*. Theiler (11) as early as 1911, observed that premunition with *A. centrale* would reduce the severity of subsequent *A. marginale* infections. A recent report by Kuttler (6,7) confirms Theiler's original observations, and reviews the literature concerning the use of *A. centrale* by others.

In 1961, Kuttler (5) described a killed adjuvant vaccine against anaplasmosis, and showed a significant reduction in the severity of subsequent *A. marginale* challenge when compared to non-vaccinated controls. Brock *et al.* (2,3) in 1965 described a similar vaccine, using a lyophilized antigenic base combined with a commercial adjuvant and

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given in 2 injections 4-6 weeks apart. This vaccine was reported to give considerably greater protection, but was still limited to reducing the severity of clinical anaplasmosis.

The use of virulent *A. marginale* to premunize cattle was described by Schmidt (9) in 1937. Even though specific therapeutic agents were not available, his report indicates general success in this effort. Kuttler (7) reported the use of a mild *A. marginale* isolate to premunize splenectomized calves which resulted in a hematologic response comparable to that produced by *A. centrale*. Subsequent challenge of these calves with virulent *A. marginale* failed to result in any detectable reaction, suggesting that in areas where premunition appears to be the method of choice, such strains would be preferred over *A. centrale*.

One purpose of this study was to measure the hematologic response to premunition with an attenuated *A. marginale*, a virulent *A. marginale* controlled by drug therapy, and inoculation with a killed adjuvant vaccine. In addition, the relative protection afforded by these procedures was measured by natural and experimental challenge with virulent *A. marginale*.

MATERIALS AND METHODS

Experiment # 1:

Twenty, 3 month old Holstein Friesian female calves were divided into 4 equal groups. Group 1 was vaccinated on day 1 with 5 ml. of an attenuated *A. marginale* vaccine.* Group 2 was vaccinated on day 1 with 2 ml. of an adjuvant vaccine**, and again on day 42. Calves of group 3 were given 10 ml. whole blood intravenously, on day 1, from an intact calf showing evidence of acute anaplasmosis. Two calves of group 3 were treated with oxytetracycline on day 17 before any detectable drop in packed cell volume (PCV), using 2.7 mg/kg body weight intravenously (I.V.). The remaining 3 calves were treated with Compound 356-C61*** using 9.0 mg/kg. of body weight I.V., on day 24, after a marked drop in PCV had occurred. All animals of this group apparently had recovered by day 81. Calves of group 4 remained as untreated, non-vaccinated, fully susceptible controls.

On day 81 all calves were flown from their native environment, at about 8500 ft. above sea level, to an anaplasmosis endemic area on the north coast of Colombia at an elevation of less than 600 ft. During the 170 day observation period in this zone, no treatment specific for anaplasmosis was administered.

Throughout the experiment weekly blood samples were taken.

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* Obtained from Diamond Laboratories, Des Moines, Iowa. This product was shipped frozen and stored at −40°C for several weeks before use.

** Obtained from Ft. Dodge Laboratories, Ft. Dodge, Iowa. Available commercially under the trade name "Anaplaz."

*** Obtained from Burroughs Wellcome & Co., Tuckahoe, New York.

Packed cell volume determinations were made on all samples. In addition, serum samples were tested for the detection of specific *A. marginale* antibodies. The capillary tube-agglutination (CA) test was used (8) for the first 84 days. The complement-fixation test (CF) was used thereafter, serum titrations being conducted on all samples showing 2+ to 4+ reactions at the 1/5 dilaiton. The CF test was conducted using United States Department of Agriculture antigen in basically the manner described by the USDA (1), but employing a micro-technique similar to that described by Hidalgo (4).

For convenience in comparing the relative effects of natural *A. marginale* challenge between groups (Table 1), a day 0 was designated, based on the low PCV, associated with a marked increase in CF serum titers. Following arrival in the endemic zone, the day 0 average for group 1 was 95 ± 20 days, group 2 was 67 ± 18 days, and group 4 was 95 ± 25 days. Group 3 failed to show evidence of reaction to *A. marginale*. Day 0 was selected for this group on the basis of an average of the 3 other groups, showing anaplasmosis, and was 86 days following arrival in the anaplasmosis endemic zone.

An analysis of variance (10) was employed to determine the presence of significant differences in the PCV response of each group.

Experiment # 2:

A total of 23 adult Aberdeen Angus cows were divided into 4 groups. Group 1 received an attenuated *A. marginale*, group 2 an adjuvant vaccine, group 3 a virulent *A. marginale* followed by treatment, and group 4 a virulent *A. marginale* but no treatment. For the 4 months these cattle were under observation and test they were maintained on pasture at an altitude of approximately 8500 ft.

Group 1 consisted of 7 cows with an average age of 49 ± 25 months. These cattle, on day 1, were injected subcutaneously with 2 ml. of a 50% suspension, in physiological saline, of washed erythrocytes showing a 3% parasitemia. This inoculum was obtained from a splenectomized calf that had been infected 51 days previously with 20 ml. of the attenuated *A. marginale* vaccine described in experiment #1. All animals were challenged with virulent *A. marginale* 55 days after receiving the attenuated organism. This challenge consisted of 1 ml. injected subcutaneously, of a 0.75% suspension in physiological saline of washed erythrocytes showing a 4% parasitemia.

Group 2 consisted of 6 cows with an average age of 46 ± 22 months. Each animal received 2 ml. subcutaneously of the adjuvant vaccine, on day 1, and a second similar inoculum 28 days later. All animals were challenged with virulent *A. marginale* 27 days after receiving the last vaccine inoculum. The challenge dose was identical to that used in group 1.

Group 3 consited of 6 cows with an average age of 46 ± 28 months, which were not vaccinated, nor infected until day 55. From day 0 to

TABLE 1

Influence of Field Exposure to *Anaplasma marginale* on Vaccinated and Unvaccinated Calves.

DAYS

	No. of Animals		-42	-35	-28	-21	-14	-7	0	7	14	21	28	35	42
GROUP I		Avg. PCV	23.0	22.4	22.0	22.2	22.2	18.0	13.6	16.4	19.0	19.6	21.6	22.6	21.6
	5	Avg. CF	1/7	1/8	1/10	1/10	1/6	1/20	1/210	1/180	1/160	1/200	1/80	1/110	1/56
GROUP II		Avg. PCV	25.2	24.6	24.0	24.4	24.0	19.4	15.6	18.8	19.8	18.4	19.8	21.5	21.0
	5	Avg. CF	1/5	1/4	1/4	1/5	1/66	1/480	1/550	1/420	1/240	1/280	1/110	1/68	1/68
GROUP III		Avg. PCV	21.0	22.0	22.0	22.4	20.6	22.0	25.4	22.0	22.2	25.0	22.4	22.0	21.4
	5	Avg. CF	1/5	1/2	1/2	1/5	1/5	1/4	1/5	1/14	1/8	1/12	1/16	1/14	1/4
GROUP IV		Avg. PCV	21.0	20.0	25.0	21.0	22.0	18.2	12.4	20.6	19.4	17.2	19.6	22.2	19.6
	5	Avg. CF	-	-	-	-	1/5	1/190	1/450	1/450	1/80	1/40	1/100	1/64	1/100
Probability of error in PCV average differences			NS	NS	NS	NS	NS	<0.05	<0.01	<0.05	NS	<0.05	NS	NS	NS
Difference required for significance			-	-	-	-	-	4.0	3.5	4.9	-	4.7	-	-	-

Day 0: The time after arrival in the anaplasmosis endemic zone that the low PCV occurred, as a result of *A. marginale*.
Group I: Vaccinated with attenuated vaccine (Dimaond Laboratories) Day 0 = 95 days ± 20 after arrival in the endemic zone.
Group II: Vaccinated with adjuvant vaccine (Ft. Dodge Laboratories) Day 0 = 67 days ± 18 after arrival in endemic zone.
Group III: Premunized with virulent *Anaplasma*, and treated. Day 0 = 86 days after arrival in endemic zone.
Group IV: Unvaccinated controls. Day 0 = 95 days ± 25 after arrival in endemic zone.
PCV: Packed cell volume
CF: Complement-fixation Test
NS: No significant differences.

day 55 these animals served as untreated controls for the purpose of comparing the hematologic changes resulting from vaccination. On day 55 these animals were infected with virulent *A. marginale* using the same inoculum as described for group 1 challenge. Twenty five days after infection all animals were treated with the experimental Compound 356-C-61. Each animal was treated I.V. with 5 mg/kg. of body weight. Treatment in most instances was timed to coincide with early infection as evidenced by either parasitemia or CF response. Following infection and treatment this group (listed in Table 2 as group III-A), was then compared with other vaccination groups to evaluate the relative hematologic response.

Group 4 consisted of 5 cows with an average age of 79±50 months, which served as unvaccinated controls. Each animal was infected on day 55 with virulent *A. marginale* using the same level of exposure as described for the challenge of group 1. With 1 exception these animals remained untreated, and were allowed to undergo the full effects of anaplasmosis as produced by the challenge inoculum. The one exception was a cow that could not be sacrificed, which was treated I.V. with 5 mg/kg. of body weight of Compound 356-C-61 at a time when the PCV had reached 15%.

Blood samples were taken twice weekly at 3 and 4 day intervals. Packed cell volumes, parasitemia as determined on giemsa stained blood smears, and serum CF response were recorded on all samples. A micro CF technique was used for all tests as described in Experiment 1. All serum samples showing 2+ to 4+ positives at the 1/5 serum dilution were titrated. All CF titer averages and statistical comparisons were made on the basis of a logarithmic transformation of the dilution factor.

The relative response to vaccination is recorded in Table 2, and the efficacy of vaccination when animals were given a known challenge is recorded in Table 3. An analysis of variance as described by Snedecor (10) was conducted to establish the presence of significant differences.

RESULTS

Experiment # 1:

Calves of group 1 developed a positive CA response in every instance. The first positive occurred on day 25, and by day 47 all were positive. Individual PCV's, as well as group averages, remained normal until after day 84.

Calves of group 2 also developed positive CA reactions. This response was transient occurring in 3 of 5 calves following the first vaccination, but positive CA reactions were apparent in all calves 12 days following the second vaccination. These CA reactions persisted in serums collected up to and beyond day 84. No significant deviation from the normal PCV occurred until after day 84.

TABLE 2

Hematologic Response to Virulent, Attenuated and Killed Anaplasma Vaccines.

	GROUP I	GROUP II	GROUP III	GROUP IIIA	PROBABILITY OF ERROR
Number of animals	7	5	6	6	
Average age in months	47 ± 25	40 ± 22	46 ± 28	48 ± 28	
Average Pre-vaccination PCV	38.4	39.6	39.0	36.8	NS
Average time in days for 1st. CF Pos. reaction	11	11	0	22.0	<0.01
Average time in days for the 1st. measurable Parasitemia	19.7	0	0	28.5	<0.05
Average high CF titer	1/480	1/28	0	1/450	<0.01
Average high Parasitemia	0.69% ± 0.3	0	0	8.5%	<0.01
Duration, in days, of Parasitemia	17 ± 5	–	–	NT(1)	
Low PCV	31.7	34.4	35.2	21.8	<0.01 (4.21)*

(1)NT = Not tested, but parasitemias were sporadic and persistent even after treatment.
Group I: Vaccinated with Diamond Strain of Attenuated Anaplasma.
Group II: Vaccinated with Fort Dodge adjuvant vaccine.
Group III: Unvaccinated control.
Group IIIA: Vaccinated with Virulent Anaplasma followed by treatment 25 days later.
*4.21: The difference in tabulated averages required to indicate significance.

TABLE 3

Hematologic Response in Vaccinated and Unvaccinated Cattle to Virulent *A. marginale* Challenge

	Group 1	Group 2	Group 4*	Probability of error
Number of Animals	7	5	5	
Avg Age in months	49 ± 25	42 ± 22	79 ± 50	
Avg. Pre-challenge PCV	33.1	36.4	36.0	NS
Avg. time for CF response due to challenge inoculum	No Response	24 da.	24.8 da.	NS
Avg. time for Parasitemia due to challenge inoculum	No Response	36 da.	29.8 day	NS
High CF titer	1/66	1/1400	1/970	<0.01 (1)
Avg. high Parasitemia	3.3%	4.3%	20.8%	<0.01 (2)
Time to reach low PCV after CF response	No Response	29 da.	19 da.	NS
Low PCV (after challenge)	31.4	23.0	12.2	<0.01 (2)
Deaths	0	0	1	

Group 1: Vaccinated with an attenuated *Anaplasma*.
Group 2: Vaccinated with a killed adjuvant vaccine.
Group 4*: Unvaccinated, controls.
NS: No statistically significant differences occur.
(1): No significant difference between groups 2 and 4.
(2): All groups are significantly different.

Calves of group 3 injected with virulent *A. marginale* developed evidence of early acute anaplasmosis. All calves were positive to the CA test 21 days after exposure. Even following treatment these calves showed an average low PCV of 19.2±6.6, as compared to an average of 38.0±2.2 for all other calves on the same date. In addition to the drop in PCV, a significant reduction in weight gain for the 84 day period was observed in this group. By day 144 the weight gains for each of the 4 groups were very nearly the same with no significant differences apparent. On day 84 the PCV average had returned to 30%, but even so was significantly lower than the corresponding PCV average of group 2. Although lower than the averages of groups 1 and 4 the differences were not significant.

Calves of group 4 failed to show any evidence of anaplasmosis or other blood parasites, remaining negative to the CA test, with no significant alteration from the accepted normal PCV, until after day 84.

Following arrival in the anaplasmosis endemic zone, a marked decrease in PCV occurred in all groups that could not be attributed any specific blood parasitic infection. The average PCV for all animals on day 84 was 34%, whereas 2 weeks after arrival in the endemic

zone, on day 98, the average for all animals was 28% and on day 112 was 24%.

As the result of field exposure, active cases of anaplasmosis were observed in all animals of groups 1, 2 and 4, as evidenced by marked increases in CF serum titers, reductions of PCV, and the appearance of *Anaplasma* bodies in giemsa stained blood smears.

The response of each group based on day 0, or the low PCV, is tabulated in Table 1 for a period of 42 days before and 42 days after reaching the low PCV resulting from anaplasmosis. In addition, the average CF titers for each group at each examination, are recorded.

Significant differences in PCV occurred at days −7, 0, +7, and +21. Otherwise, no significance could be detected. In every instance involving significant differences, group 3 showed higher PCV values.

Marked increases in serum CF titers accompanied the anaplasmosis resulting from field challenge. This CF response was greatest in groups 2 and 4. Group 3, while showing some slight increase in CF titer, did not at any time show the sudden 3-6 fold increase so characteristic of acute anaplasmosis in the other groups. A gradual decline in serum CF titers occurred following the peaks associated with the low PCV in all reacting groups.

Premunition with a virulent *A. marginale*, followed by treatment, resulted in complete protection to field exposure to anaplasmosis. No detectable protection to a field challenge was produced by vaccinating 3 month old calves with either an adjuvant vaccine or with an attenuated *A. marginale*.

Experiment # 2:

Cattle of group 1, vaccinated with attenuated *A. marginale*, developed evidence of infection on an average of 11 days after inoculation as determined by the CF test. A measurable parasitemia occured in each animal, even though the average high parasitemia was only 0.69%. The average low PCV was 31.7% ± 3.3. The CF response was comparable to that produced by virulent *A. marginale* in group III-A, and significantly higher than the CF response to the adjuvant vaccine. No obvious evidence of illness was observed in any of the animals of this group (Table 2). The challenge results of group 1 are recorded in Table 3. No evidence was seen of any challenge response in animals of this group for the 64 days they remained under observation.

Cattle of group 2 gave a prompt but low level response to the serum CF test following vaccination. In every instance a detectable CF response occurred after the first vaccination, which increased following the second. As expected no parasitemia or deviation in PCV occurred with this group, (Table 2). Challenge with virulent *A. marginale* was followed by evidence of anaplasmosis as determined by parasitemia, increase in CF serum titers, and a reduction in PCV. This response was significantly less severe than observed in the unvaccinated con-

trols. The average low PCV was 23% ± 5.6. A very marked CF response was observed with the average high titer reaching 1/1400. The average high parasitemia was 4.5% ± 3.5.

Group 3 remained negative and free of any apparent natural infection until day 55. These same animals, when exposed to virulent *A. marginale* (Group III-A) and then treated, developed evidence of infection. Of the 3 methods used to establish an anaplasmosis resistant animal, this procedure was the most severe. A low PCV of 21.3% ± 3.2 and an average parasitemia of 8.5% ± 4.7 developed which was significantly different from the milder response occurring with the attenuated organism (Table 2). The response of this group to infection was, however, significantly milder when compared to the untreated controls (Tables 2 and 3).

The low PCV of 21.8%, occurring in animals given virulent *A. marginale* then treated, was not significantly different from the low PCV of 23.0% occurring in group 2, which were first vaccinated and then exposed to virulent infection.

Cattle of group 4 developed acute anaplasmosis (Table 3) following exposure to the challenge inoculum. One death occurred as the result of anaplasmosis and a second would likely have occurred had treatment not been administered. The average incubation time of 24.8 days for the first CF response and 29.8 days for the first parasitemia, are suggestive of a mild challenge.

DISCUSSION

Experiment # 1:

It is not fully understood why calves vaccinated at 3 months of age failed to show the same degree of protection to field challenge as adult cattle vaccinated with essentially the same products and later given an artificial challenge.

The obvious difference in age, the stress of a tropical climate, the interval between vaccination and challenge are all factors which may be involved. In the case of attenuated vaccine the delay in serological response in the calves (34 days as compared to 11 days in adult) suggests a possible low potency in the material used. It is also possible that *Babesia* infections occurred in some calves. If, for example, both piroplasmosis and anaplasmosis occurred simultaneously, an increase in CF titer would lead us to the conclusion that anaplasmosis was involved, whereas the drop in PCV may have been due to the *Babesia* infection. Care was taken to prevent such incorrect assumptions by observations for other signs of piroplasmosis such, as hemoglobinuria and the examination of stained blood smears, but still, under the conditions prevalent with field challenge, the possibility of error does exist.

On the basis of the results obtained premunition with virulent *A.*

marginale and treatment produced the only satisfactory results. No significant protection was produced by either of the 2 vaccination programs when compared to the unvaccinated controls.

Experiment # 2:

Adult cattle maintained under close supervision for 4 months, during which time they were vaccinated and then challenged, yielded more positive results as to the relative value of killed vs. live attenuated vaccines.

The adjuvant vaccines appeared to reduce the severity of but failed to prevent, infection by the challenge organism. The duration of the partial protection afforded by this product assumes some importance in tropical areas where transmission may occur throughout the year.

Premunization with the attenuated vaccine gave complete protection in adult cattle under the conditions of these trials. In view of experiment 1 results, a question occurs as to how long infections with the attenuated organism can be maintained, particularly in young calves.

It would appear that premunition, followed by drug therapy, can be safely used to protect susceptible adult cattle about to be introduced into an endemic zone. It is probable, however, that such a method would only be practical where cattle are maintained under close supervision, in order to treat at the proper time.

It is apparent, however, that methods do exist for the protection of susceptible cattle from acute anaplasmosis. The area, distribution and type of vectors, age of cattle, and the circumstances under which the work can be done are all factors to be considered in the selection of the proper immunization procedure.

SUMMARY

Twenty, 3-month-old calves were divided in 4 equal groups. Group 1 was inoculated with an attenuated *Anaplasma marginale*, group 2 received an *A. marginale* adjuvant vaccine, group 3 was infected with virulent *A. marginale* followed by treatment, and group 4 remained as unvaccinated controls. All animals were moved into an *Anaplasma* endemic zone 3 months later and allowed to undergo natural field challenge. Evidence of acute anaplasmosis was observed in all calves, except those premunized by virulent *A. marginale*. No significant evidence of protection was produced by either the attenuated *A. marginale* or the adjuvant vaccine when compared to the unvaccinated controls. The group premunized with virulent *A. marginale* failed to respond to natural exposure.

Hematologic response to virulent, attenuated and killed *A. marginale* vaccines was measured in 18 mature cattle divided into 3 groups. The group receiving virulent *A. marginale* was treated 25 days after

infection (Burroughs Wellcome Compound 356-C-61). No death losses occurred in this group, but moderate infections were observed to result in a significant reduction of PCV. The attenuated *A. marginale* vaccine produced a low level parasitemia, a marked serological response, as measured by the complement-fixation test (CF), and a very slight drop in PCV, which was not significantly different from values observed in an unvaccinated, non-infected, control group. The group receiving adjuvant vaccine showed only a low level, transient, CF serological response.

An experimental challenge was administered 8 weeks after vaccination to cattle receiving the attenuated and adjuvant vaccines along with a group of 5 unvaccinated controls. All controls reacted to challenge with severe acute signs of anaplasmosis. One animal was allowed to die, a second would probably have died, had it not been treated. Cattle receiving the attenuated vaccine showed no signs of active infection resulting from challenge. Cattle receiving the adjuvant vaccine reacted to challenge, but less severely than did the controls.

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Questions, Answers and Comments

QUESTION: Dr. Smith, Stillwater, Oklahoma.

I wonder if Dr. Dimopoullos has any idea if the decrease in acetylcholinesterase activity has anything to do with an ultimate demise in

the red cell? It is stated in human medicine that when acetylcholinesterase is inhibited, it doesn't have any effect on red cells.

ANSWER: Dr. Dimopoullos, Baton Rouge, Louisiana.

Dr. Smith, the only evidence that we have of acetylcholinesterase inhibition is circumstantial. As far as we know, this is the first time this has been demonstrated in a hemotropic disease.