Vaccination of Calves with a Diaminopimelic Acid Mutant of *Salmonella typhimurium*

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ABSTRACT

The purpose of this study was to evaluate the safety and efficacy of a diaminopimelic acid mutant of *Salmonella typhimurium* as a vaccine for calves. Transposon techniques were used to create a stable nonreverting diaminopimelic acid mutant of a virulent *S. typhimurium* strain. Calves were vaccinated at weekly intervals with the diaminopimelic acid mutant given as an oral dose of 10^{10} organisms, followed by two subcutaneous doses of 10^9 organisms. The calves tolerated vaccination well and the vaccine strain was eliminated from the feces within four days. Of five vaccinated calves, three survived challenge with 5×10^9 organisms of the parent strain whereas all five unvaccinated calves that were challenged died. The surviving calves eliminated the challenge organism from the feces within three weeks.

Key words: *Salmonella typhimurium*, calves, diaminopimelic acid, salmonellosis, vaccine.

RÉSUMÉ

Cette expérience consistait à évaluer l'innocuité et l'efficacité d'un mutant de *Salmonella typhimurium* déficient en acide diaminopimélique, comme vaccin pour les veaux. Les auteurs utilisèrent un transposon pour créer un tel mutant, stable et non réversible, à partir d'un souche virulente de *S. typhimurium*. Ils vaccinèrent des veaux, à intervalles hebdomadaires, avec le mutant précité, en leur en administrant une dose buccale qui contenait 10^{10} organismes et, ultérieurement, une injection sous-cutanée qui en contenait 10^9. Les veaux tolérèrent bien cette vaccination et éliminèrent la souche vaccinale dans leur fumier, en l'espace de quatre jours. Trois des cinq veaux vaccinés survécurent à une infection de défi avec 5×10^9 organismes de la souche originale, tandis que les cinq témoins en moururent. Les deux survivants éliminèrent les organismes de l'infection de défi, dans leur fumier, en l'espace de trois semaines.

Mots clés: *Salmonella typhimurium*, veaux, acide diaminopimélique, salmonellose, vaccin.

INTRODUCTION

Research has shown that live *Salmonella* vaccines provide the best protection against challenge with virulent *Salmonella* organisms (1,2). This superior protection is especially evident when large challenge doses are used (3,4). Live cultures of rough mutants of *S. dublin* and *S. choleraesuis* have been used for many years to vaccinate cattle and pigs, respectively, in Britain and Europe (5,6). In recent years, an orally administered live culture of a *galE* mutant of *S. typhi* has given encouraging results in published reports of its experimental use in children (7). However, tests of *galE* mutants of *S. typhimurium* in calves have shown that these mutants tend to persist for a long time and to evoke serious adverse reactions (8,9,10). Experiments with *galE* mutants demonstrated problems with live vaccine organisms whose growth is not self-limited *in vivo* and which are not genetically stable (10). Aromatic mutants of *S. typhimurium*, created with transposon techniques, have been shown to be safe, effective and stable when used as vaccines in mice and calves (4,11,12). These mutants require nutrients not thought to be readily available in animal tissues, but they can multiply slowly in the initial stages of infection and persist for up to three weeks *in vivo* (4,12,13).

In order to reduce carcass and environmental contamination, the ideal live *Salmonella* vaccine should persist only long enough to stimulate protective immunity. A mutation was therefore selected that should result in a shorter period of persistence of the vaccine strain *in vivo* than that observed with *galE* or *aroA* mutants. Previous research by Curtiss and associates (14,15) demonstrated that diaminopimelic acid mutants (*dap*) of *Escherichia coli* K12 were unable to colonize and survive in the intestinal tracts of rats. Diaminopimelic acid (DAP) is needed to form the murein layer of the *E. coli* cell wall; therefore, strains which are defective in the ability to synthesize DAP form spheroplasts and lyse when they are replicating (14). Since DAP is not produced by eukaryotic cells, *dap* mutants are unable to persist in animal hosts. The purpose of this study was to evaluate the safety and immunogenicity of a *dap* mutant of *S. typhimurium* given to calves by the oral and subcutaneous routes.

MATERIALS AND METHODS

BACTERIA

Salmonella typhimurium 3860C (10), a nalidixic acid resistant, virulent strain that was passaged in a calf was used as the challenge strain and also as

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the host for vaccine construction. A strain of *S. typhimurium* LT2 made Dap$^-$ by insertion of transposon Tn10 (strain π 1397) was received from Dr. J. Roth, University of Utah. The *dap*::Tn10 region of this strain was transduced into *S. typhimurium* 3860C using the high transducing phage P22 Ht105/*int* (16). Tetracycline resistant transductants were selected then replicated onto L agar plates with and without Dap (50 μg/mL) to detect Dap$^-$ mutants. The stability of these Dap$^-$ mutants was increased by selection for tetracycline sensitive Dap$^-$ mutants on Bochner media (16,17). Reversion rates were determined by spreading 0.2 mL of an overnight culture washed and concentrated tenfold in sterile phosphate buffered saline pH 7.4 (PBS) on each of ten large (150 mm) plates of NCE +glucose(1%) (16). After incubation at 37°C for 48 hours the number of revertants were determined. A tetracycline sensitive Dap$^-$ strain (RC256) with no detectable rate of reversion to Dap$^+$ ($<10^{-11}$) was selected for use as the vaccine organism in this study.

Vaccine was prepared by growing *S. typhimurium* strain RC256, overnight in L broth containing 50 μg/mL of DAP, at 37°C in a shaking water bath at 140 rpm. The culture was centrifuged at 8,000 × *g* for 20 minutes, then the pellet was resuspended in an equal volume of sterile PBS. Centrifugation and resuspension were repeated and the resuspended culture was used to inoculate the calves. Calves were vaccinated orally after an overnight fast by allowing them to suckle 5 mL of washed culture mixed with 30 mL of milk replacer. For subcutaneous vaccination a mixture of 1 mL of washed culture and 4 mL of sterile PBS was injected under the skin of the lateral chest wall.

The challenge dose was prepared by growing a culture of *S. typhimurium* 3860C overnight in L broth with nalidixic acid (50 μg/mL) in a shaking water bath at 37°C and 140 rpm. Calves were challenged orally after an overnight fast by allowing them to suckle 1 mL of the broth culture mixed with 30 mL of milk replacer.

EXPERIMENTAL DESIGN

Ten, one week old, colostrum-fed, Holstein-Friesian bull calves were used in this study. Each calf was housed in an elevated metal pen in an isolation facility, fed 2 L of milk replacer at each of three feedings daily and allowed free access to water and calf starter. Fecal consistency, rectal temperature, and clinical appearance were monitored daily and fecal samples were checked for the presence of *Salmonella* sp. (10). Calves of similar age and size were received in consignments of two, two and six, on three separate occasions. For each consignment, equal numbers of calves were assigned to each of two treatment groups. Five calves were vaccinated with *S. typhimurium* RC256. They received 2-3 × 10^{10} organisms orally at one week of age followed by 4-7 × 10^9 organisms subcutaneously at two and three weeks of age. The other five calves were used as unvaccinated controls. At 4.5 weeks of age, all vaccinated and unvaccinated calves were challenged orally with 5-9 × 10^9 nalidixic resistant *S. typhimurium* 3860C.

ENUMERATION OF *SALMONELLA*

Plate counts were made on brilliant green agar containing 50 μg/mL of DAP or 50 μg/mL of nalidixic acid. The numbers of live organisms in the vaccine and challenge inocula were determined by inoculating the appropriate plates with a spiral plater (Spiral Systems Inc., Bethesda, Maryland). The numbers of viable *S. typhimurium* in feces and tissues were determined by macerating 1 g of sample in 5 mL of PBS then spreading a 0.2 mL volume of the suspension over the surface of a plate and by dispensing dilutions of the suspension on plates by means of a spiral plater.

BLOOD CULTURE

Blood taken from the jugular vein at 24 and 48 hours after each subcutaneous vaccination was used to inoculate vented blood culture bottles which contained 40 mL of trypticase soy broth (Vacutainer, Becton Dickinson, Rutherford, New Jersey) to which DAP had been added at 50 μg/mL. Blood culture bottles were incubated at 37°C for one week. At two to three day intervals a 0.2 mL volume of broth was removed in a sterile manner and plated on brilliant green agar with DAP (50 μg/mL) to check for the presence of the vaccine strain.

DIRECT MICROAGGLUTINATION TEST FOR DETECTION OF *SALMONELLA* ANTIBODIES

The test was an adaptation of the method described for *Brucella* by Brown and associates (18). A suspension of a washed overnight broth culture of *S. typhimurium* 3860C was made in 1% formalized PBS and held at 4°C overnight. The cells were centrifuged and resuspended in 0.1% formalized PBS, then used as antigen preparation after adjustment to optical density 1.8 at 525 nm (Spectronic 20, Bausch and Lomb, Rochester, New York). Phosphate buffered saline containing 0.005% safranin O (25 μL) was added to each well of a sterile microtitration plate with 96 U-shaped wells (Flow Laboratories, McLean, Virginia). Serum (25 μL) was added to the first well in each row of 12, then doubling dilutions were made. Antigen (25 μL) was then added to each well. Each plate was incubated for two hours at 37°C then overnight at 4°C. The titers were expressed as the $\log_2$ of the reciprocal of the highest dilution which caused agglutination.

POSTMORTEM EXAMINATION

Postmortem examinations were conducted on all calves that died. Surviving animals were euthanized three weeks after challenge by intravenous injection of barbiturate (Euthanyl Forte, MTC Pharmaceuticals, Mississauga). Samples of the abomasum, jejunum, ileum, cecum and colon were removed and fixed in Bouin's fluid. Portions of the liver, spleen, kidney and lung were removed and fixed in formalin. The concentrations of challenge organisms in the liver, spleen, ileocecal lymph node and ileal mucosa was determined as described.

RESULTS

CLINICAL RESPONSE TO VACCINATION

A mild diarrheal illness affected three of the unvaccinated calves that were housed together on day zero (Fig.1). Rotavirus was detected by direct electron microscopic examination of feces. The infection was evident a few days later in three of the vaccinated calves in another isolation room (Fig.2). Direct electron microscopic examination of feces again confirmed rotavirus infection. In all calves, the

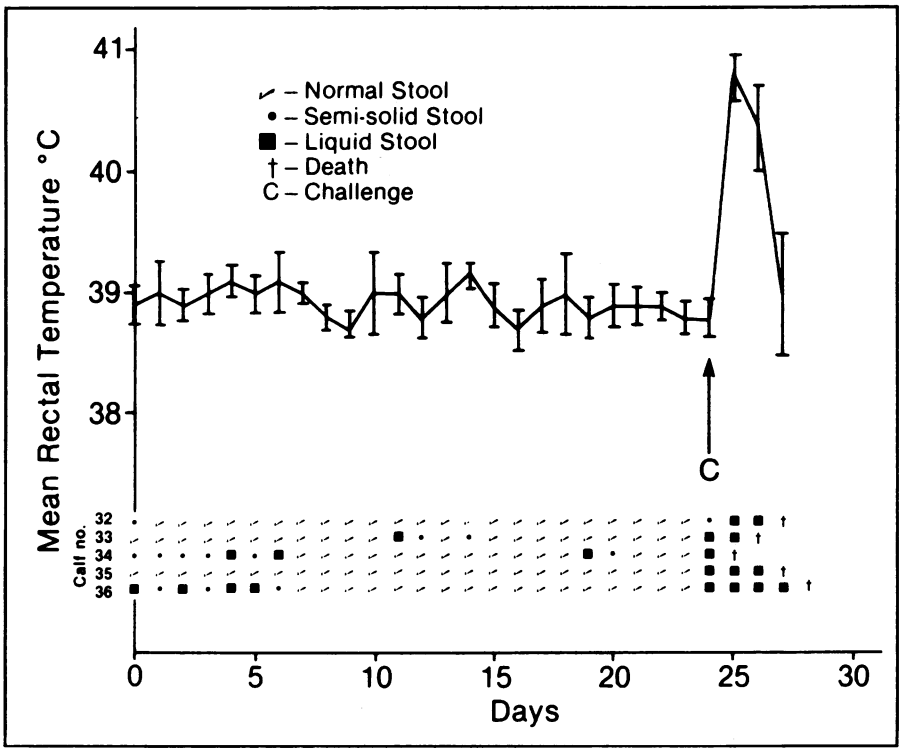

Fig. 1. Mean rectal temperatures and stool consistency of unvaccinated calves challenged with *S. typhimurium* 3860C. Rectal temperatures were taken daily. Stool consistency for each calf was classified daily as normal, semi-solid or liquid. Calves were challenged orally on day 24 with 5-9 × 10^9 *S. typhimurium* 3860C. Rotavirus was demonstrated in feces on day 3.

illness was mild and resolved without complication. After oral vaccination all five calves were normal and there was no evidence of fever or diarrhea (Fig.2). The vaccine strain was shed in the feces for only two to four days, after which time it could not be recovered (Fig.3). Following subcutaneous vaccination a mild transient fever was noticed for one to three days (Fig.2), but the calves ate well and were active. Injection sites were inflamed for two to five days postvaccination but this did not seem to interfere with the activity of the calves. Blood cultures taken at one and two days after each subcutaneous vaccination were negative for *Salmonella*. Serum agglutination titers for *S. typhimurium* increased after the first and the second subcutaneous vaccination (Table I). The unvaccinated calves showed no increase in titer throughout a similar time period.

RESPONSE TO CHALLENGE

The unvaccinated calves developed high fevers (Fig.1) and profuse diarrhea within 12 hours after oral

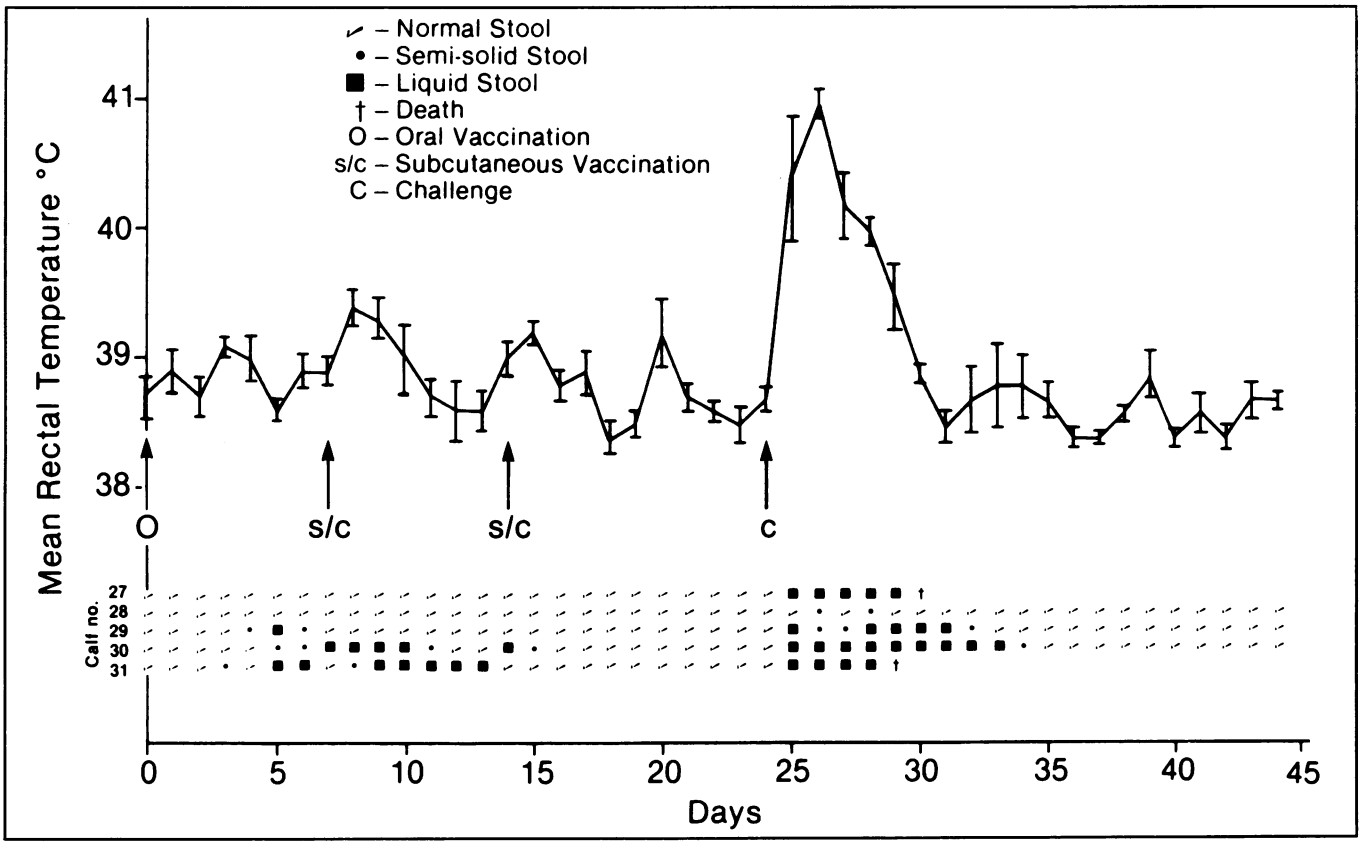

Fig. 2. Mean rectal temperature of calves vaccinated with *S. typhimurium* RC256 and challenged with *S. typhimurium* 3860C. On day 0 calves were vaccinatead orally with 2-3 × 10^{10} organisms orally, followed by 4-7 × 10^9 organisms subcutaneously on day 7, then 4-6 × 10^9 organisms subcutaneously on day 14. All calves were challenged with 5-9 × 10^9 *S. typhimurium* 3860C on day 24. Rectal temperatures were taken daily. Stool consistency for each calf was classified daily as normal, semi-solid or liquid. Rotavirus was demonstrated in feces on day 6.

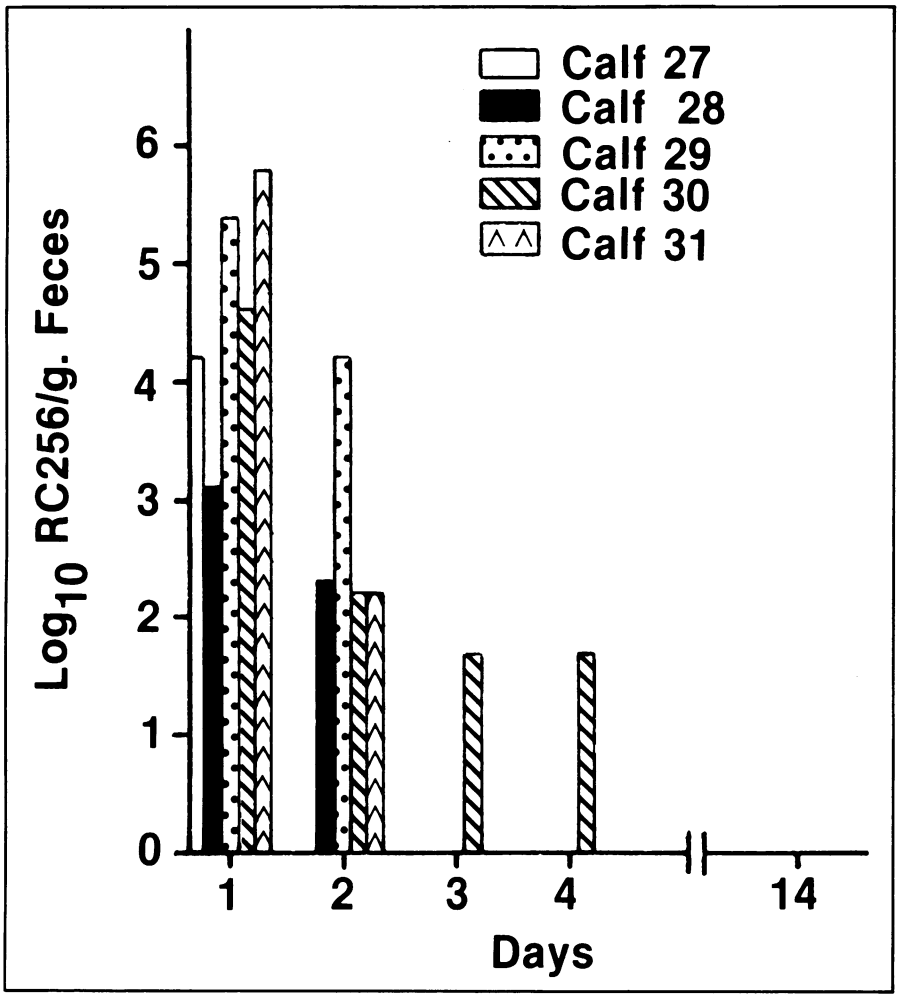

Fig. 3. Fecal excretion of *S. typhimurium* RC256 after oral vaccination of calves with 2-3 × 10^{10} live organisms. Fecal counts for each calf were recorded daily.

TABLE I. Titers[a] of Agglutinating Antibodies to *S. typhimurium* in the Sera of Calves Before and After Vaccination

Group	Treatment	Calf No.	Day of Experiment[b] 0	7	14	24	26	31	45
A	Vaccinated	27	1	2	6	7	5	NA	NA
		28	2	2	5	7	6	6	8
		29	3	3	4	8	8	9	7
		30	2	2	5	7	6	9	7
		31	1	4	6	8	7	NA	NA
B	Unvaccinated	32	4	3	2	1	1	NA	NA
		33	1	0	0	0	NA	NA	NA
		34	1	1	1	1	NA	NA	NA
		35	1	1	1	1	1	NA	NA
		36	1	1	1	1	1	NA	NA

[a]Titers are $\log_2$ of the reciprocal of the highest dilution of serum which caused agglutination of formalin killed *S. typhimurium*

[b]Calves were vaccinated with *S. typhimurium* RC256. At one week of age (day 0) they received 2-3 × 10^{10} organisms orally, followed by 4-7 × 10^9 organisms subcutaneously at two weeks of age (day 7), then 4-6 × 10^9 organisms subcutaneously at three weeks of age (day 14). All calves were challenged with 5-9 × 10^9 *S. typhimurium* 3860C on day 24

NA = Not applicable. Calf died

challenge with *S. typhimurium* 3860C. All calves underwent rapid deterioration in their clinical condition, became severely dehydrated, and died between 23 and 78 hours after challenge.

The vaccinated calves developed high fevers (Fig.2) which persisted for five days. All calves developed diarrhea, but the onset was not until 24 hours postchallenge and the volume of feces passed was noticeably less than in the unvaccinated calves. Two of the calves became anorectic and progressively weaker until they died; one at 113 hours and the other at 135 hours postchallenge. The three remaining calves ate well after challenge and were active. One calf had intermittent semi-solid stools for four days. The other two calves had diarrhea which lasted seven and ten days respectively. The numbers of challenge organisms shed in the feces of the vaccinated calves are shown in Figure 4. The surviving calves rapidly eliminated the challenge strain and by 13 days postchallenge it could only be detected following enrichment in tetrathionate broth. At 21 days postchallenge no challenge organisms could be detected in the feces from any of the three surviving calves.

POSTMORTEM EXAMINATIONS

Postmortem examination of the vaccinated and unvaccinated calves that succumbed to challenge revealed lesions characteristic of salmonellosis (10). The numbers of challenge organisms recovered from tissue samples taken from the calves at necropsy are shown in Table II. These counts demonstrate a pattern similar to that seen in other calves orally infected with *S. typhimurium* 3860C (10). Postmortem examination of the three surviving calves was conducted at three weeks postchallenge. *Salmonella* were not recovered from the tissues or feces at that time and the intestine and internal organs appeared normal. Histological examination revealed only a slightly increased infiltration of inflammatory cells in some areas of the distal small bowel and proximal colon.

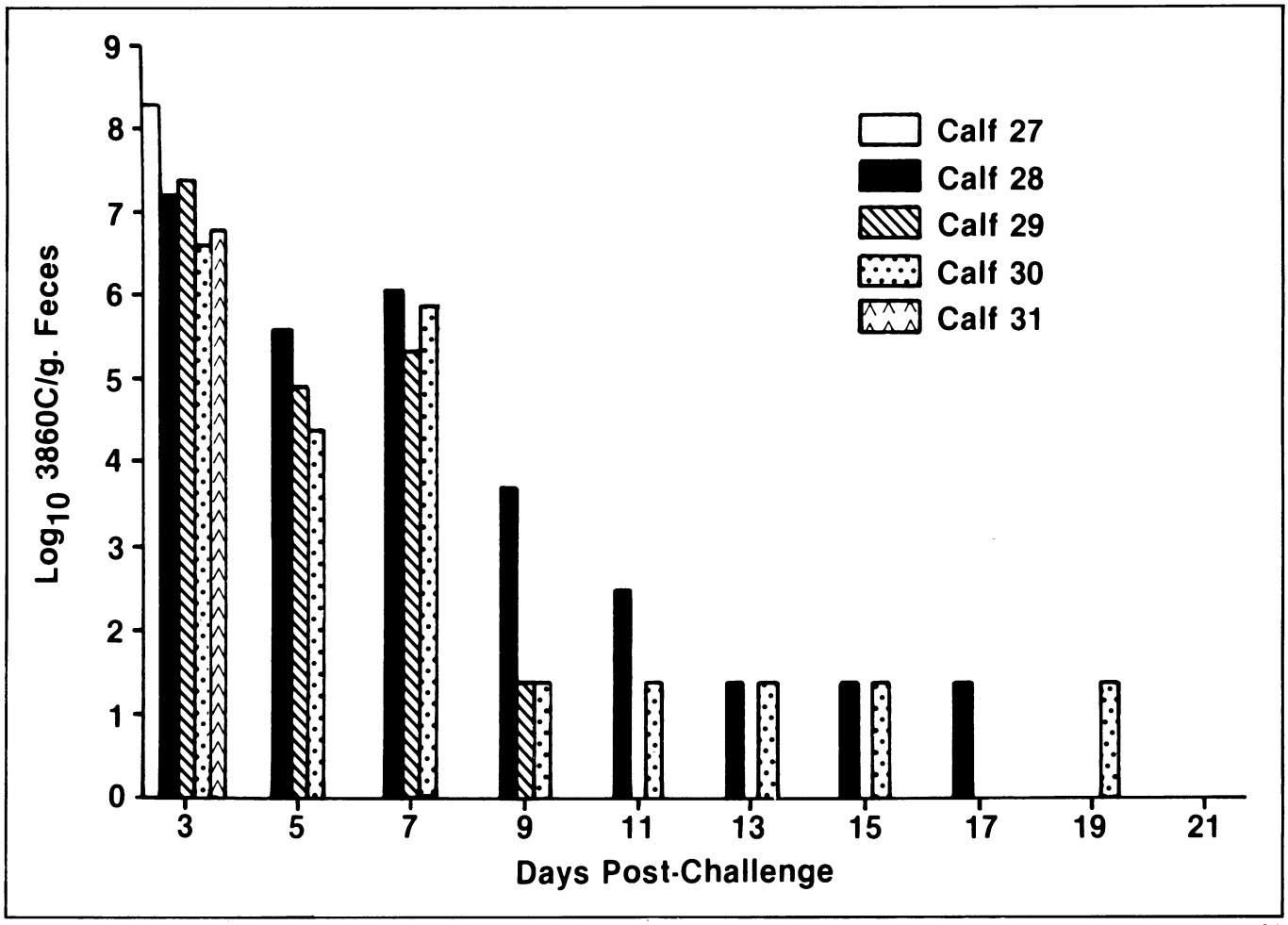

Fig. 4. Fecal excretion of challenge strain *S. typhimurium* 3860C by vaccinated calves. Calves were challenged with 5-9 × 10^9 organisms orally on day 24.

TABLE II. Numbers of Challenge Organism Recovered at Necropsy from Selected Tissues of Vaccinated and Control Calves

Group[a]	Calf	Time of Death (Hours)	Log_{10} *S. typhimurium* 3860C/Gram of Tissue			
			Ileal Mucosa	Ileocecal Lymph Node	Liver	Spleen
A	27	135	8.6	7.5	3.7	4.8
	28	NA	0	0	0	0
	29	NA	0	0	0	0
	30	NA	0	0	0	0
	31	113	8.5	7.5	5.3	5.0
B	32	53	8.0	6.0	3.0	2.7
	33	32	7.6	5.5	3.0	2.5
	34	23	8.0	5.5	2.0	[b]
	35	72	8.0	6.6	4.3	[b]
	36	78	7.8	6.5	5.3	5.3

[a]Calves in Group A were vaccinated as described in footnote b, Table I. Calves in group B were not vaccinated

[b]Organisms were not recovered on direct plating but were recovered following tetrathionate enrichment

NA = Surviving calves were euthanized three weeks after challenge

DISCUSSION

Vaccination of calves with the *dap* mutant of *S. typhimurium* RC256 either orally or subcutaneously appears to be safe and the side effects appear to be less serious than those reported for *galE* mutants (8-10). Elimination of the *dap* mutant strain from the feces of orally vaccinated calves was more rapid than that reported for *galE* (8,10) or *aroA* (4) mutants of *S. typhimurium* administered at similar doses. This short survival of the *dap* mutant in the host is the likely reason for its greatly reduced virulence. Stability of the mutation and selection for revertants *in vivo* are important considerations in the development of live vaccines based on mutations. *Salmonella typhimurium* RC256 appears to be a stable *dap* mutant since no DAP^+ revertants were detected in *in vitro* reversion tests or in the feces of vaccinated animals.

Three of the five calves vaccinated with the *dap* mutant *S. typhimurium* RC256 were protected against death following challenge with *S. typhimurium* 3860C whereas all of the unvaccinated calves died. The small number of calves used in this trial and others reported in the literature (4,8,9,12) make it very difficult to compare the degree of protection elicited by different live *Salmonella* vaccines. It is probable, however, that the inability of the *dap* mutant to multiply *in vivo* may account for its failure to protect all the vaccinated calves. Ushiba (19) found that live streptomycin dependent mutants of *S. enteritidis* could not protect mice against challenge unless streptomycin was given to allow vaccine replication *in vivo*. The immunogenic qualities of the host strain which carries the mutation may also play a critical role and make comparison of specific types of mutations difficult. For example, Smith and colleagues (12) reported that only one of three *aroA* mutant strains that were tested proved effective, even though all three strains had complete loss of function of gene *aroA*. Preliminary trials with the aromatic mutant *S. typhimurium* SL1479 have shown it to be safe and to provide significant protection against death when given orally (4) or intramuscularly (12). Vaccination of calves with large doses of *galE* mutants can also protect against death but significant side effects have been documented (8-10).

All of the vaccinated calves in the present study developed fever and diarrhea of varying severity following challenge. Despite the excellent protection against death achieved by some live *Salmonella* vaccines in calves, none have been completely effective in preventing the development of mild clinical signs such as fever or diarrhea after oral challenge with large doses of virulent *Salmonella* strains. This deficiency has been demonstrated with *S. dublin* strain 51 (6), *galE* mutants of *S. typhimurium* (8-10) and to a lesser extent with *aroA* mutants (4,12). In all of the above reports vaccinated calves developed fever after challenge indicating that invasion had taken place. Even calves vaccinated orally on three occasions with large doses of the aromatic mutant *S. typhimurium* SL1479 developed fever for up to five days after challenge (4).

Fecal cultures of the surviving vaccinated calves in the present study showed that the challenge organism *S. typhimurium* 3860C was rapidly eliminated from the feces. These results are similar to those reported by Robertsson and associates (4) who demonstrated that calves vaccinated with the live aromatic mutant *S. typhimurium* SL1479 completely eliminated the challenge organism *S. typhimurium* SVA44 within three weeks after challenge. In contrast, calves vaccinated with a heat-killed bacterin and challenged with a lower dose (10^6) of the same challenge strain were still heavily infected when postmortem examinations were conducted, three weeks after challenge (4).

There was an obvious increase in antibody titer in the vaccinated calves (Table I). The levels of antibody produced were of a similar magnitude to those produced by the surviving calves three weeks after challenge, indicating that the vaccine was a potent stimulator of humoral antibody.

The results of this study indicate that the *dap* mutant *S. typhimurium* RC256 is safe when given orally or subcutaneously and elicits a degree of protection against challenge by large oral doses of virulent *S. typhimurium*. The challenge dose of *S. typhimurium* 3860C which was used was a severe challenge and caused death within 78 hours for all unvaccinated calves to which it was administered. In order to more accurately determine the degree of protection elicited by this vaccine, controlled field trials would be appropriate.

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