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Refinement of Fluoroacetate Detoxification by Genetically Modified Rumen Bacteria

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Test of Fluoroacetate Toxicity in Sheep Inoculated with Detoxifying Rumen Bacteria

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SUMMARY

Four genetically-modified strains of the rumen bacterium *Butyrivibrio fibrisolvens* were inoculated into the rumens of two sheep (T1 & T2). All four bacterial strains had been shown to produce the detoxifying enzyme: fluoroacetate dehalogenase with activity between 10 - 20 nmol fluoroacetate/min/mg bacterial protein. For direct comparison of poison sensitivity, two uninoculated sheep were used as "control" animals (C1 & C2).

For the week preceding the toxicity tests, samples of rumen contents were taken daily from all four sheep and analysed to measure the number of bacteria containing the detoxification gene. Immediately prior to the test, inoculated sheep showed levels around 10^7 (T1) and 10^6 (T2) modified bacteria per millilitre of rumen fluid, whereas C1 and C2 contained no recombinant bacteria.

Sheep were weighed the day before the poisoning experiment and doses were adjusted for each animal, to ensure that each received a specific amount of poison, per kilogram of body weight. The four sheep were fed a series of small doses of fluoroacetate over a period of three days, to a total amount calculated as an average lethal dose. They were allowed to recover for 3 days, and then fed repeat doses over the following day. Fluoroacetate was fed in a series of small doses to allow observation of any significant difference between the test-sheep and the control-sheep

During day 2, the control-sheep both showed symptoms typical of acute fluoroacetate toxicity. Both were extremely nervous, reacting to even familiar noises with a panic response. For several hours on day 2, both control-sheep had to be restrained from breaking loose and possibly damaging themselves. Both test-sheep remained calm throughout this period, but showed some physiological signs of chronic toxicity (raised heart-rate and respiration rate) for 1 - 2 hours.

On day 3, the control-sheep remained very nervous, again requiring physical restraint on several occasions. However, the test-sheep showed no symptoms of fluoroacetate poisoning on day 3.

During the second major poisoning period (day 7) the control-sheep again showed extreme nervousness, and periods of panic. Test-sheep remained calm, with just a minor detectable increase in their wariness of sudden noises.

At around 5 a.m. on day 8, the control-sheep succumbed to acute poisoning, underwent a panic response, broke free of the metabolism crates in which they were housed and died.

At 8.53 a.m., test-sheep T2 died without muscular or nervous spasms. Autopsy showed that death was from pulmonary oedema (fluid on the lungs) typical of chronic fluoroacetate poisoning.

At 9:00 a.m. test-sheep T1 was visibly affected by the poison, with a slightly elevated heart-rate (130 beats/min) and respiration rate (30 resp/min). These were approximately 50% above normal resting levels. Within 4 hours, both were back to normal and T1 appeared unstressed.

The conclusion was that test-sheep displayed significantly greater resistance to fluoroacetate poisoning than the control-sheep. Between the two test-sheep, T1 showed higher levels of detoxifying bacteria than T2 at the beginning of the experiment. T1 also showed greater toxin resistance than T2.

These results are highly encouraging, but need to be tested in a repeat experiment before definite conclusions can be made about the protective effect of the bacteria.

Background

During 1996, several strains of *Butyrivibrio fibrisolvens* were transformed with a recombinant plasmid containing the fluoroacetate dehalogenase gene. Three strains expressed moderate levels of the enzyme (each defluorinating approximately 10 nmol fluoroacetate/ml/mg bacterial protein), while six others expressed low levels of activity (<1 nmol/min/mg). Modification of the existing gene promoter produced a significant improvement in one strain, which now expresses enzyme with an activity of approximately 20 nmol/min/mg. The total activity available in the best four strains, therefore, was approximately five-fold greater than the original single strain.

A ten-fold increase was thought preferable, before *in vivo* testing for toxin protection was performed. The original schedule planned to reach this stage by May 1997. However, reduced funding from July 1996 dictated that if work was to be extended to June 1997, it would be necessary to reduce staff numbers from 4 to 2. A staff of four researchers was seen as the minimum "critical mass" required to make significant progress. A staff of only two researchers would have been insufficient to maintain the necessary research momentum. It was decided, therefore, to retain four research staff and attempt the *in vivo* tests within the first six months, with funding terminating at December 1996.

A primary goal was to obtain gene expression in the second rumen bacterial species *Prevotella ruminicola*. During 1996, several altered forms of the dehalogenase gene were inserted into *P. ruminicola* strains AR20 and AR29. Unfortunately, gene regulatory sequences used in these experiments were non-functional in the rumen bacteria. At present, expression of the dehalogenase gene to produce active enzyme in *P. ruminicola* remains to be achieved.

A second important goal was to develop specific tracking systems for the useful bacterial strains. DNA from these strains resisted cloning as plasmid inserts, as much rumen bacterial DNA has in the past. Despite repeated attempts, within the restricted time-table, it was not possible to develop systems for tracking bacterial strains individually.

The decision was made to proceed with the experiment under these difficult conditions, provided the total number of plasmid-bearing bacteria in the rumen was 10^6 or higher. The presence of multiple engineered strains was expected to smooth out the population fluctuations seen with individual strains.

The only method available for measuring bacterial numbers *in vivo* was to use PCR tracking of the dehalogenase gene itself, which would not allow individual strains to be distinguished from one another. For this reason, the five *B fibrisolvens* strains with low-level expression were excluded from the test. Although every fraction of activity would contribute to ruminal detoxification, the presence of poorly expressing strains would make measurements of plasmid numbers a questionable indication of enzyme activity. Using the four most efficient strains, detection of plasmid was expected to correlate well with levels of dehalogenase activity.

Using serial dilutions of rumen samples, the "end-point" dilution (where PCR signal was no longer detectable) would indicate when bacteria were diluted below 1 cell per microlitre (10^3 cells/ml). A single cell, containing 10-20 copies of the plasmid would yield a clear PCR product. Using a 1 µl sample for analysis, dilution below the level of 10^3 cells/ml would result in negative PCR results.

Because of the obvious limitations of this premature experiment, toxin dose schedules were designed to detect even marginal protection, by administering fluoroacetate as a cumulative series of sub-acute doses.

Experiment I

Four sheep (two inoculated test animals and two uninoculated controls) were housed in metabolism crates, with restraining collars and neck-ropes. Feed was provided at 800 g/day (oat chaff/lucerne, 3:1) and water was measured out at 5 litres per day, to allow consumption rate to be monitored. Sheep bearing recombinant bacteria were housed in a PC2 laboratory maintained with negative air-pressure, double doors, and standard decontamination practice for anyone leaving the room.

Sheep were tested for the presence of the engineered bacteria immediately prior to commencement of toxicity testing (at mid-day 4/12, and 6:30 am 5/12). Plasmid levels suggested that test-sheep 1 (T1) contained levels of the modified bacteria around 10^7 per ml of rumen contents, while test-sheep 2 (T2) had around 10^6 per ml. The decision was made to go ahead with the experiment. Control-sheep did not contain any genetically modified bacteria.

All sheep were fed doses of fluoroacetate, inoculated into snow-peas or adsorbed to feed pellets, with normal feed, as follows:

DAY 1: 5/12/96

Time	Hrs	Dose	mg/kg body wt	Accumulated mg/kg
12:00 noon	0	1	0.045	0.045
2:00 pm	2	2	0.03	0.075
4:00 pm	4	3	0.03	0.105
6:00 pm	6	4	0.03	0.135
8:00 pm	8	5	0.03	0.165
10:00 pm	10	6	0.03	0.195

At dose No 3, test-sheep T2 declined to eat the snow peas. A replacement dose of fluoroacetate was administered infused into feed pellets, placing this animal 2 hours behind the others in dosing schedule, through to the end of day 1. Control-sheep C1 was also fed further doses on feed pellets, to match this change.

DAY 2: 6/12/96

Fluoroacetate doses for all four sheep were continued:

Time	Hours	Dose	mg/kg	accumulated mg/kg
8:00 am	20	7	0.03	0.225
10:00 am	22	8	0.03	0.255*

**a lethal dose range for sheep has been estimated as 0.25 - 0.5 mg/kg. Jenson et al, (1948) Am. J. Vet. Res. 9:370-372.*

At the 8:00 am feed, sheep C2 ate only half of the fluoroacetate dose, leaving one of the two snow-peas containing the dose. The extra half-dose was provided on feed pellets. For the remaining dose, all four sheep were fed fluoroacetate infused into feed pellets.

This completed the intended dose. From this point, observations of response to the toxin were made on both sets of sheep.

Results

Five hours after the final poison dose, sheep C1 developed acute hyper-excitability. A startle response was caused by even minor, familiar sounds. C2 was also hyper-excitable. Following a sharp startle response from C1, which broke the restraining neck-rope loose, C2 underwent an extreme panic response. Panic responses from the sheep resulted in C2 breaking loose from the crate. The sheep were, fortunately, unharmed and were calmed by attendants.

All connections for monitoring heart-rate and respiration rate were torn loose during these events, and further monitoring was not possible for the control-sheep.

Both control-sheep showed hyperactive, anxious feeding behaviour, were constantly alert and in the characteristic "flight" posture (head and shoulders raised as high as possible, darting glances in all directions). C1 remained hyperactive, making occasional attempts to leap from the crate. Throughout the evening of day 2, both control-sheep were visibly agitated, remaining standing for the whole period, displaying pronounced muscular spasms and flushing of the skin, visible in the face and detectable by touch. Temperature measurements from the axilla (armpit) showed normal temperature and flushing was concluded to result from peripheral vasodilation. Respiration rate was observed visually

to be high, and continued as "panting" in C2 from 4:50 pm to at least 7:20 pm. The excitable state persisted in both control-sheep until after 10:00 pm.

Test-sheep remained calm throughout this period. T1 was the least affected by toxicity symptoms, displaying rapid respiration and elevated heart-rates from 6 pm - 7 pm. T2 displayed restlessness, alternating between standing and reclining and slight frothing was observed during rumination by T2. Neither sheep displayed any hyper-excitability. Both showed minor twitching and slight unsteadiness of stance for a short period on day 2.

DAY 3: 7/12/96

At 9 am the control-sheep both displayed hyper-excitability, with alarm responses at minor sounds. This was most pronounced with C1. Both test-sheep appeared unaffected at this stage.

Conclusions

The control-sheep showed distinct physiological and behavioural effects of fluoroacetate toxicity. Some physiological symptoms were shown by the test-sheep, but they were very mild by comparison, appeared later and subsided sooner than in the control-sheep. The sheep containing rumen bacteria with dehalogenase activity were observed to suffer considerably less than controls from the effects of the poison. These results suggest that a measurable degree of protection existed under these conditions.

Experiment II

A follow-up experiment involved feeding small doses of fluoroacetate to the same sheep, to discover whether the toxic effects would continue to accumulate. The schedule was as follows:

Day 3

Time	Hours	Dose	mg/kg	Accumulated mg/kg
9:00 am	45	9	0.015	0.270
3:15 pm	51.25	10	0.012	0.282
8:45 pm	56.45	11	0.015	0.297

The final total accumulated dose is theoretically a lethal dose of fluoroacetate for sheep.

Results

From 46 hours after the first fluoroacetate dose, the control-sheep appeared to improve in condition. During day 3, the test-sheep showed no obvious signs of toxicity. All four sheep displayed more frequent rumination than usual and reduced feed intake, during days 3 and 4. During the toxicity test period, faeces and urine production was less than 50% of normal, for all four sheep. A strong preference for the lucerne component of the diet was always observed, but on days 3 and 4 the oat chaff component was only partly consumed. Lucerne was eaten avidly. C1 and T1 were the least affected in terms of appetite.

Conclusion

The recovery of normal physiology and behaviour during the low-dose section of this work suggested that the sheep were able to clear fluoroacetate from their systems at a rate greater than the input rate of 3 micrograms/kg/hour. Using this measurement, it can be estimated that to clear a potentially lethal dose (0.3 mg/kg) would require approximately 4 days.

Experiment III

The proceedure for Experiment I was repeated with minor modifications, using the same sheep. On day 7, fluoroacetate was administered as hourly doses of 0.015 mg/kg body weight, beginning at 8 a.m. It was not certain at this stage, what effect the residual symptoms from Experiments I and II would exert upon Experiment III. As with Experiment I, fluoroacetate doses for the first period were discontinued at a cumulative level of 0.195 mg/kg, and sheep were observed for 5 hours. This brought the total dose to 0.492 mg/kg, which is above the maximum theoretical lethal dose for sheep.

Day 7: 11/12/96

Time	Hours	Dose	mg/kg	Cumulated mg/kg
8:00 am	140	12	0.015	0.312
9:00 am	141	13	0.015	0.327
10:00 am	142	14	0.015	0.342
11:00 am	143	15	0.015	0.357
12:00 am	144	16	0.015	0.372
1:00 pm	145	17	0.015	0.387
2:00 pm	146	18	0.015	0.402
3:00 pm	147	19	0.015	0.417
4:00 pm	148	20	0.015	0.432
5:00 pm	149	21	0.015	0.447
6:00 pm	150	22	0.015	0.462
7:00 pm	151	23	0.015	0.487
8:00 pm	152	24	0.015	0.492

Results

From Experiment I it was observed that symptoms of fluoroacetate toxicity were seen as acute (nervous system) effects and more chronic (cardio-pulmonary) effects. In Experiment III, as with Experiment I, both control-sheep exhibited nervous system effects. They showed extreme nervousness, with panic responses to minor, familiar, noises.

After monitoring sheep through to 157.25 hours (1:15 am, day 8), the test-sheep showed no outward signs of toxicity. The control-sheep had experienced several episodes of severe agitation and had to be physically restrained from breaking loose from their containers by the research staff. By 157 hours, the control-sheep appeared to be settling down. Both test-sheep displayed a slight increase in nervousness during the later stages of Experiment III, showing an enhanced wariness of incidental noises. This was a minor feature compared with the response of the control-sheep and neither test-sheep showed any inclination to panic.

At approximately 5 am (161 hrs), the control-sheep suffered acute toxicity symptoms which led to a panic response, and died. At 8.00 am, both test-sheep showed quiet disposition, with slightly elevated heart rate and normal respiration rate. Test-sheep 2, however, died without nervous or muscular spasms at 165 hours (8:53 am, day 8). Autopsy confirmed that death was due to chronic fluoroacetate poisoning. Test-sheep 1 was monitored for several hours, and showed a return to normal heart and respiration rates. Feeding behaviour was normal and no signs of increased nervousness were observed.

Conclusions

Throughout the series of experiments, the inoculated test-sheep showed increased tolerance to fluoroacetate poisoning when compared to the uninoculated control-sheep. At no stage did the test-sheep exhibit the intense behavioural symptoms characteristic of acute fluoroacetate poisoning. However, the control-sheep displayed such symptoms for a large part of days 2 and 7. Owing to the somewhat inadequate restraining systems, the control-sheep had to be calmed by staff for prolonged periods of the experiment, to prevent mechanical damage from their panic responses. Test-sheep did not require any calming treatment at any stage. The most pronounced symptoms, in the test-sheep, were shown by the animal which appeared to contain the lower levels of detoxifying bacteria. Toxicity effects observed during Experiment III suggested that a significant proportion (>30%) of the systemic effect of previous doses was still present, as a residual toxicity effect after 3 days recovery.

We conclude that toxicity effects were proportional to the number of modified bacteria present in the sheep. However, the statistical significance of this preliminary test is limited by the small number of animals used. To establish that the modified bacteria were the direct cause of protection, with a high degree of certainty, these results should

be corroborated with a repeat experiment. Repetition will involve improved housing and restraining systems for the animals, to reduce the need for intervention by research staff. In other respects, the repeat experiment will use a protocol similar to that of Experiment I, with progression to Experiment III protocol if necessary. Preparations are now in progress for the repeat experiment.