CS 242

NOVEL VACCINATION APPROACHES TO IMPROVE THE GROWTH RATE OF CATTLE

FINAL REPORT

Contributions by

CSIRO Division of Tropical Animal Production

Department of Primary Industries, Queensland

and

Central Queensland University

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PART I

ABSTRACT

This project aimed to develop a vaccine alternative to clenbuterol, which could be used to increase muscle growth, reduce fat deposition and improve feed efficiency in cattle. However, unlike the ß-agonist drug, the vaccine would be convenient for use in grazing livestock, and acceptable to the consumer because there would be no risk of harmful tissue residues. It has already been shown that the vaccine causes cattle to produce antibodies which have a 'clenbuterol-like' effect, when they are purified and tested on muscle samples in the laboratory. Unfortunately, the present work showed that such antibodies are not produced in sufficient strength in the live animal, to have a detectable influence on its growth or metabolism. Thus, without further investigations, there will be no immediate or long-term benefit of this work to the industry.

PART II

EXECUTIVE SUMMARY

AIMS

It is known that muscle growth can be accelerated, fat deposition reduced, and feed efficiency improved, by synthetic ß-agonist drugs such as clenbuterol. In principle, ß-agonists could be used to improve the efficiency of cattle production, but as they are feed additives, this would only be practical in intensive systems. Furthermore, the use of such compounds could present a risk to the consumer, as they are capable of leaving tissue residues which are heat-stable and orally active in humans.

MRC-funded research into the mechanism of action of ß-agonists, has identified a receptor in muscle through which the effects of drugs like clenbuterol are mediated (the $ß_2$-adrenoceptor). It was also shown that a small part of this natural ß-receptor protein can be used as the basis for a vaccine, which can be injected into cattle to cause them to produce ß-receptor antibodies. Such antibodies have been purified from the blood of treated cattle, and shown to cause 'clenbuterol-like' effects when tested in laboratory animals, or on isolated pieces of bovine muscle. Unlike ß-agonist drugs, however, the antibodies are not heat stable, not orally active, and unlikely to accumulate in animal tissues. Thus, it is conceivable to devise a vaccine treatment that would improve the efficiency of cattle growth, be convenient for use in grazing livestock, and present no risk to human health.

This project evaluated a prototype ß-receptor vaccine in live cattle, in order to determine whether ß-receptor antibodies could be generated in sufficient amounts to have 'clenbuterol-like' effects on the growth or blood chemistry of the animal. To provide an additional stimulus to the immune system, the ß-receptor antigen was given in combination with an adjuvant mixture. Several different adjuvants were tested, to identify the most potent one that would be acceptable for use in a commercial situation. Finally, because the ß-receptor antigen is difficult to make and to purify, various fragments of it were investigated in the hope of finding one that would cause a similar effect, while being easier and less expensive to make.

ACHIEVEMENTS

In the first part of the project, the vaccine was given to cull-cows and steers at pasture, and to a group of animals that were housed intensively and offered a feedlot diet. Unfortunately this attempt to test the efficacy of the vaccine failed, because no ß-receptor antibodies were produced. This was later discovered to be due to a flaw in the way the vaccine was prepared, which must be done in such a way that a stable water-in oil emulsion is obtained. No problems with emulsion stability had been found in previous experiments where a standard laboratory adjuvant was used, but the new adjuvant mixture proved to be stable only for a short period of time, if it was prepared at room temperature. Modifying the procedure so that the vaccine was prepared at 4^0C, solved this problem in the next experiment.

Six groups of cattle were used, each containing seven animals. The first group received no treatment and served as negative controls. The second group received the drug clenbuterol, and served as positive controls. The third group was given the ß-receptor vaccine mixed with the most powerful adjuvant known (Freund's adjuvant), which is used in laboratory studies as a 'gold standard', but which is unsuitable for use

in meat-producing animals. The vaccine was also administered to the remaining three groups in three different adjuvants, each containing commercially-acceptable ingredients. One of these adjuvants also contained a new experimental compound 'recombinant ovine interleukin 1-ß', which has been shown to be a potent stimulator of the bovine immune system when given in a herpes virus vaccine.

The results of this study were very clear-cut. When the animals were given Freund's adjuvant, six out of seven cattle gave a measurable antibody response, which reached a plateau about 60 days after the first treatment. An antibody response of similar magnitude was achieved with the 'commercial adjuvant', that had failed in the earlier experiments when it was made up at room temperature. Surprisingly, the antibody response was found to markedly delayed when interleukin 1-ß was added to the commercial mixture, resulting in a poorer response overall. The fourth adjuvant, which contained different ingredients, produced a very poor and erratic response.

Despite provoking a measurable antibody response in at least two groups of cattle, there was no apparent physiological response to the vaccine. In contrast to clenbuterol, the vaccine did not increase weight gain, nor did it cause any change in plasma concentrations of potassium, glucose, or urea-nitrogen. There are two possible explanations for this finding: either the antibodies were not produced in sufficient strength to cause the type of effects that have been seen previously in laboratory tests; or their effects were 'neutralised' through the production of a second type of antibody, known as 'anti-receptor-antibodies'. At this stage there is insufficient data to determine the exact reason why this technology failed, but it is clear that the vaccine is not suitable for commercial use in its present form.

The third objective from this experiment was to learn more about the way in which the vaccine interacts with the immune system to provoke the desired antibody response. Experiments that were conducted on blood samples taken from the vaccinated cattle, showed that none of the smaller fragments of the ß-receptor antigen was as effective as the full molecule in stimulating both a humoral and cellular immune response. Thus, any modification of the ß-receptor antigen to make it easier to synthesise, could be expected to affect the antibody response.

BENEFITS

In conclusion, despite promising laboratory results, the ß-receptor vaccine failed to mimic the physiological effects of the drug clenbuterol in live cattle, as it was designed to do. There is currently a lack of understanding in this area, which is unlikely to be conquered without further research. Experiments to address this issue may take some time, but could be conducted at a relatively low-cost, using small animal models. In the absence of further work, the industry is unlikely to benefit from the current finding in the near or distant future.

PART III

MAIN RESEARCH REPORT

BACKGROUND AND INDUSTRY CONTEXT OF THE PROJECT

A vaccine was invented that may have the potential to improve growth rate, feed efficiency and carcass composition in cattle grown under a variety of conditions ranging from extensive pastures to feedlots. Despite the wide range of its possible applications, the invention stemmed from an MRC-funded project (CS.108) conducted between 1989 and 1994 with the specific aim of developing a treatment to reduce dry-season weight loss in the grazing cattle of northern Australia. The steps taken to develop this vaccine are best understood in the context of this aim, and are outlined below.

Although a number of different compounds can be used to increase animal growth, most of these rely on the input of additional protein and food energy to cause their effects. Such compounds are ineffective in the dry-season, when food is restricted. Unlike other compounds, ß-agonist drugs (clenbuterol, cimaterol, ractopamine) are able to increase the efficiency with which available energy is used for protein synthesis, and can derive a large portion of this energy not from food, but by breaking down adipose tissue in the body[2]. Also, in the absence of sufficient protein in the diet, ß-agonists can mobilise protein from tissues such as the viscera, and drive this protein into skeletal muscle. Thus, regardless of whether an animal is well-fed or not, treatment with ß-agonists results in a marked drive towards muscle protein deposition, which is reflected by increased muscle mass, increased carcass weight, decreased fat mass, no change in food intake, and improved feed efficiency.

Interestingly, not all ß-agonists cause anabolic effects[3]. Through our own research[4,5], together with other published information[6], we have learnt that certain chemical properties of these drugs are important for their anabolic activity. In particular, high plasma concentrations of ß-agonists need to be maintained almost constantly. Thus, the most effective ß-agonists are those which are well absorbed from the gut, not easily metabolised, and not rapidly excreted. Unfortunately, these are the very properties that can lead to residues in the meat of treated animals. This is of particular concern because the drugs are both heat-stable and orally active in humans. Thus, drug residues could present a health risk to the consumer, if ß-agonists are used carelessly. ß-Agonist drugs have also been reported to impair meat quality in other ways, causing toughness[7]. However, their mode of action in this regard is not fully understood. Another drawback of ß-agonists is that relatively large amounts of compound need to be given, which could not easily be compressed into a slow-release implant. Instead, to be effective in farm animals, the compounds need to be given in the feed every day. Although this is practical in a feedlot, it is clearly incompatible with less intensive production systems.

The aim of project CS.108 was to develop a treatment which causes the beneficial changes in growth observed in ß-agonist-treated animals, but which is not orally active, being resistant to absorption through the gut, and easily destroyed by heat (cooking). Furthermore, the treatment should be easy to administer to the animals, perhaps being given only once or twice, and ideally should be based on the use of a natural product. These criteria would be met if the effects of clenbuterol could be mimicked by a vaccine.

Before investing in the development of such a vaccine, it was important first to identify the target receptor in muscle and adipose tissue which is responsible for the effects of ß-agonists. Having confirmed that this target is the $ß_2$-adrenoceptor[8,9], we then proceeded to develop a vaccine based on the use of a small peptide antigen derived from the bovine $ß_2$-

adrenoceptor. The planned outcome was to causes the production of antibodies which selectively activate β_2-adrenoceptors in the same way as ß-agonist drugs.

First, two variants of the vaccine (a long-peptide and a short-peptide) were tested in small animals for their ability to cause the production of antibodies which bind to bovine β_2-adrenoceptors. Both mice and rabbits produced the desired antibody response. Next, the antibodies obtained from immunised rabbits were purified and tested *in vitro* using isolated muscle tissues obtained from cattle. Antibodies raised against the short-peptide had no activity, but those raised against the long-peptide proved to be potent activators of the bovine β_2-adrenoceptor, causing their effects at concentrations which were up to 10,000 times lower than comparable ß-agonist drugs. Finally, a feasibility study was conducted to assess whether similar antibodies could be produced in cattle using the long-peptide.

Nine out of twelve vaccinated cattle produced a measurable antibody response, with the percentage of non-responders being the same as that seen in rabbits and mice. Surprisingly, in those cattle which responded to immunisation, the level of antibody production was higher than that seen using laboratory animals. Two booster injections were required to produce significant antibody titres in cattle, which appeared on about day 55 after the primary injection. The titres tended to peak on about day 70, but were still high on day 100. The antibodies were purified from the blood of these cattle, and were tested *in vitro* for their ability to activate β_2-adrenoceptors. Such antibodies have given positive results in a number of laboratory tests, demonstrating an ability to bind to β_2-adrenoceptors in the muscle of rats and cattle, to stimulate the activity of the enzyme adenylyl cyclase in skeletal muscle, and to lower blood pressure when infused into anaesthetised rats.

Although the cattle experiment was not designed to examine production characteristics, plasma urea concentrations were measured. A fall in plasma urea nitrogen is a characteristic response to ß-agonist drugs, and results when protein synthesis increases, so that extra nitrogen is being driven out of the plasma and in to muscle tissue. The cattle treated with the vaccine showed a statistically significant fall in plasma urea concentrations ($P<0.01$) when compared with their pre-treatment values, and this coincided with the production of β_2-adrenoceptor antibodies. Thus, this experiment established the feasibility of using a vaccine to mimic the effects of ß-agonist drugs in cattle.

As far as we are aware, this was the first demonstration that antibodies can be made which activate β_2-adrenoceptors, without resorting to the lengthy and difficult process involved in producing anti-idiotypes. The results were sufficiently encouraging to warrant patent protection on this invention. A preliminary patent application (PM 7084) was filed in August 1994, and renewed in July, 1995.

The present project (CS.242) was designed to measure the efficacy of the vaccine in live cattle with respect to its effects on growth rate, body composition and feed efficiency. An additional aim was to identify the specific part of the peptide antigen that is responsible for triggering the immune response and causing active antibodies to be produced. This process of 'epitope mapping' was undertaken in the hope that a variation of the peptide could be made which would be more immunogenic and less expensive to synthesise than the current version.

OBJECTIVES

In the original contract (dated 27 January, 1995) there were three objectives.

1. To obtain quantitative data on both positive and negative effects, such as weight gain, temperament, carcass weight, carcass quality and meat quality, in grazing cattle treated with the long-peptide.

2. To obtain data on feed intake, feed conversion efficiency (measured as the ratio of feed intake to live weight gain, and feed intake to carcass weight gain) and carcass composition in vaccinated animals fed in a controlled environment. The diet provided is intended to replicate two extremes: the feedlot, and dry-season pasture conditions.

3. To investigate which amino acid portion of the long-peptide contains the epitopes which cause the desired antibody response, so that the peptide can be modified with no loss of activity, and in such a way that its immunogenicity can be improved.

A modified contract was agreed in July 1995 which included three different aims.

1. To determine why the vaccine failed in the three trials that were undertaken in part 1 of the project, and in doing so, to identify an effective adjuvant suitable for use in a commercial situation.

2. To determine the efficacy and likely duration of action of the vaccine in comparison with a synthetic ß-agonist drug, through the continual measurement of plasma metabolites such as urea, glucose and potassium.

3. To provide plasma and fresh lymphocytes from immunized cattle for the determination of likely T and B-cell epitopes on the antigen.

METHODOLOGY AND RATIONALE

1. Synthesis of the peptide and peptide fragments

The peptide that is used as a basis for this vaccine contains 24 amino acids in a sequence that corresponds to part of the bovine β_2-adrenoceptor .

H W Y R A S H K E A I N C Y A K E T C C D F F T

Due to its length and the presence of multiple cysteine (C) residues, this peptide is particularly difficult to synthesise and purify. Three different companies were contracted to perform this work during the course of the project, with varying degrees of success.

Our original supplier was the Queensland Institute of Medical research (QIMR). This was the source of the material used to treat mice, rabbits and cattle under a previous contract (CS. 108). In the first instance, QIMR had supplied a small quantity of peptide (~50 mg) in an allegedly pure form (>70% purity) for a modest price ($350). However, when contracted to supply 160 mg of pure peptide for field-testing in cattle, the material was delivered 7 weeks past the agreed date, one vial was damaged in transit with the contents unrecoverable, and analysis of the material that was received showed a high level of impurities. QIMR was unable to address the purity problem because of their limited HPLC capacity, and after independent analysis of their product confirming its poor quality, they agreed to make no charge.

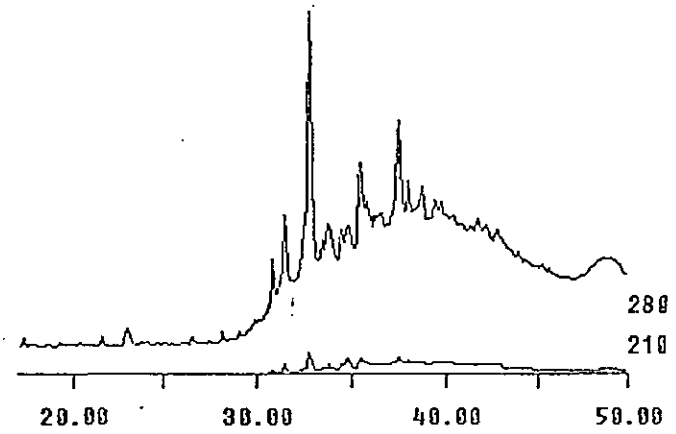


Fig 1: HPLC trace supplied by QIMR illustrating the level of purity of their product.

With one month remaining before the field trials were due to begin, inquiries were made of other peptide synthesis companies, and a written quotation of $ 3,000 was accepted from Auspep Pty. Ltd. to synthesise and supply a minimum of 100 mg peptide at 90% purity or greater within four weeks. Auspep also experienced difficulty in purifying the peptide, but managed to supply 131 mg of material at an average purity of 60 to 70%, which was deemed adequate for use in the vaccine trials. In addition, Auspep supplied 2 mg of more highly-purified material for use in the antibody assays.

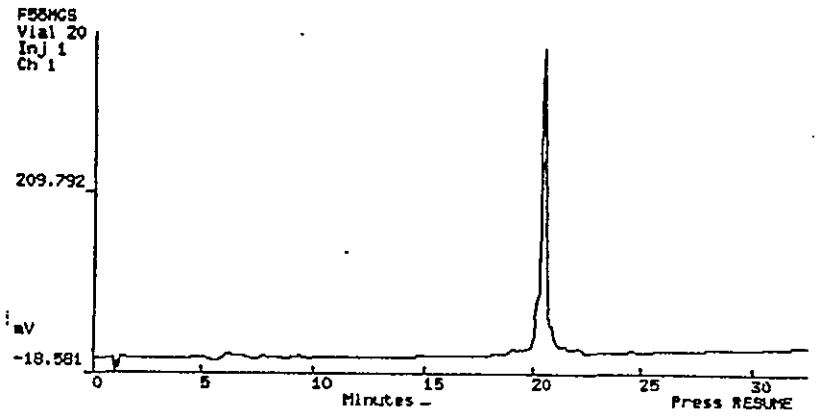


Fig. 2: HPLC trace of purified peptide supplied by Auspep and used in subsequent vaccine trials.

In addition to requiring the 24 amino acid peptide in large quantities for use in the field trials, we also needed several short fragments of the peptide in high purity, but small quantity, for use in epitope mapping experiments. Auspep offered to supply this material, but because their operations are configured for large-scale preparative work, their suggested price was unacceptably high ($10,120). Chiron Mimotopes Ltd were eventually contracted to supply the peptide fragments at a price of $4,000. The nature, quantity and quality of fragments supplied is shown below.

Fig.3:

T-epitope fragments

H17E **H W Y R A S H K E A I N C Y A K E** T C C D F F T
10 mg supplied at 86% purity

K17T H W Y R A S H **K E A I N C Y A K E T C C D F F T**
10 mg supplied at > 95% purity

B-epitope fragments*

H10A **H W Y R A S H K E A** I N C Y A K E T C C D F F T
1 mg supplied at > 80% purity

R10C H W Y **R A S H K E A I N C** Y A K E T C C D F F T
1 mg supplied at > 80% purity

H10K H W Y R A S **H K E A I N C Y A K** E T C C D F F T
1 mg supplied at > 77% purity

E10T H W Y R A S H K **E A I N C Y A K E T** C C D F F T
1 mg supplied at > 80% purity

I10D** H W Y R A S H K E A **I N C Y A K E T C C D** F F T
1 mg supplied at > 70% purity

A10T** H W Y R A S H K E A I N C Y **A K E T C C D F F T**
1 mg supplied at > 70% purity

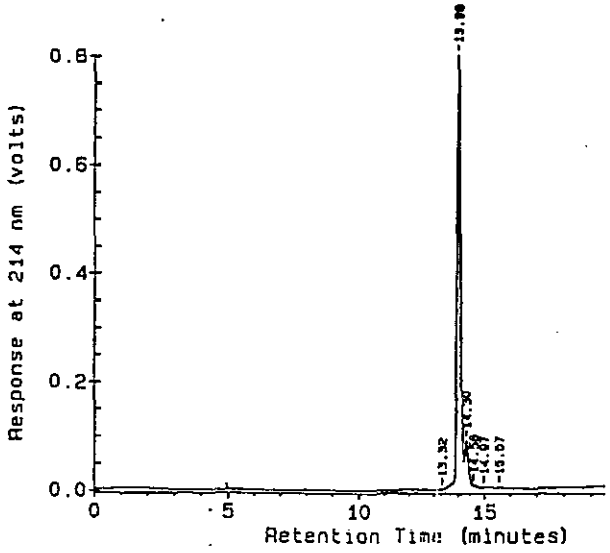
*Due to the short length of these peptides, each was synthesised commencing with the amino acid sequence S-G-S-G linked to biotin. This facilitates binding the peptides to the ELISA plates, and the spacer-arm allows enough of the desired epitope sequence to be free, allowing recognition by the antibodies.

**Due to the presence of multiple cysteine residues, these two fragments are particularly difficult to synthesise and purify. Accordingly, it was agreed with Chiron before the order was placed that 70% purity would be an acceptable target for these peptides.

Fig. 4: Sample HPLC traces showing the purity level achieved.

T-cell peptide

H17E

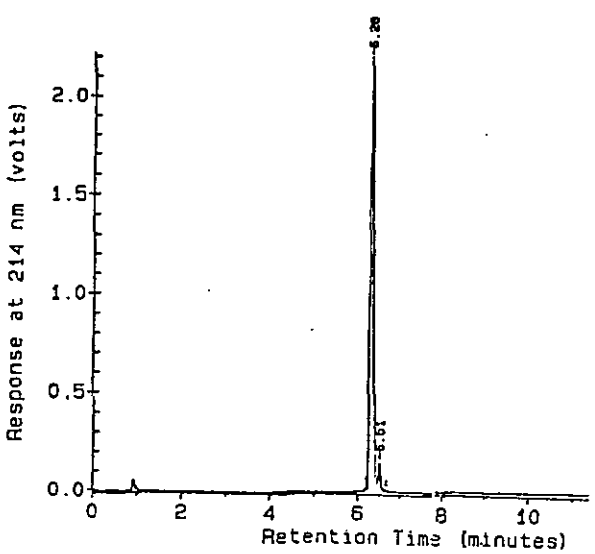


Integration Data

Retention Time (minutes)	Peak Height (microVolt)	Peak Area (microVolt.sec)	Area Percent
13.32	592	118961	1.28
13.98	801547	7975189	86.01
14.30	87913	941944	10.16
14.58	6349	104365	1.13
14.97	2868	92107	0.99
15.57	2483	40133	0.43
Total	901752	9273699	

B-cell peptide

H10A



Integration Data

Retention Time (minutes)	Peak Height (microVolt)	Peak Area (microVolt.sec)	Area Percent
6.19	20880	33154	0.62
6.28	2214426	12595590	94.35
6.51	149028	643589	4.78
6.58	7120	33455	0.25
Total	2391434	13455798	

2. Adjuvant formulation

Experiments 1 to 3

All adjuvants contained an aqueous phase and an oil phase. The adjuvant used in the first three experiments was based on the formula used in the 'Vaxtrate' vaccine, and each dose contained the following ingredients which are believed to be acceptable for use in a commercial situation.

Aqueous phase: 500 µg peptide; 2 mg Quil A; 200 µL saline; 200 µL of 15% DEAE dextran in saline.

Oil phase: 1 mL of Ondina oil/Arlacel 80 mixed in a ratio of 9:1.

The adjuvant was prepared by adding the aqueous phase dropwise to the oil phase and emulsifying the mixture with an electrical dispersing tool. Initially this procedure was carried out at room temperature.

Experiment 4

QD: The same adjuvant described above, but prepared with all reagents kept at 4^0C. This was done in order to confirm a suspicion that the failure of the adjuvant in the first series of experiments was the result of an unstable emulsion. Careful preparation of the mixture at low temperatures improves emulsion stability markedly.

IL: The same as QD, but with the addition of 100 µg recombinant ovine interleukin-1ß, which was provided and recommended for testing by Dr Paul Wood (CSIRO Div. Animal Health). Previous studies in cattle have shown that it enhances the immune response to a prototype vaccine against the bovine herpes virus.

DS: The QD adjuvant with no Quil A and with DEAE dextran replaced by dextran sulphate. DEAE dextran is a polycationic molecule, which can adsorb any smaller molecules that carry an opposite negative charge. In this case, the DEAE dextran might be attracted to the two negatively-charged glutamic acid residues found in our peptide. Unfortunately, both glutamic acid residues have adjacent lysine residues, which have a slightly longer side-chain, and which carry a positive charge. The presence of these lysines could interfere with the peptide-dextran binding, therefore, making the DEAE dextran ineffective as an adjuvant. In contrast to DEAE dextran, dextran sulphate is polyanionic, and so would be expected to attract smaller molecules that carry a positive charge. Thus, with certain peptides a stronger immune response is obtained with dextran sulphate, than with DEAE dextran. Dextran sulphate was recommended for testing by Dr Ian East (CSIRO Div. Trop. Anim. Prod.).

FR: A commercial preparation of Freund's adjuvant, the most potent adjuvant known, and one which had been shown to be effective with our ß-adrenoceptor peptide in previous studies. This was selected as our benchmark formula.

Vaccine preparation

As done in the first experiments, the peptide was prepared as a suspension in saline, such that each dose of vaccine contained 0.5 mg. The Quil A, DEAE dextran and dextran sulphate were also prepared in saline, at concentrations of 0.5%, 7.5% and 5% respectively. All vaccines were prepared as a water-in-oil emulsion; at a ratio of 1:1 for Freund's Adjuvant, and 3:7 for the remaining adjuvants. All emulsions were prepared at 4^0C, fresh on the morning of each vaccination. The volume of emulsion given to each animal was 1.4 mL.

3. Animals and treatments

Four experiments were conducted to examine the effects of the vaccine in cattle under different field conditions. Experiments 1 to 3 were performed using the original Quil A/DEAE dextran-based adjuvant prepared at room temperature as described above. Experiment 4 compared the effects of clenbuterol with those of the vaccine given in a number of different adjuvants, all prepared at 4^0C.

Experiment 1: Cull Cows

Fifty four cows were used. The animals were obtained from Belmont Field Station, and were run on pasture there during the course of the experiment. The cattle were weighed following a 48 h fast, then divided into three groups of 18 animals per group, with each group having a similar mean fasted liveweight. One of the groups was treated with the ß-adrenoceptor vaccine in the form of a free peptide presented in a Quil-A-based adjuvant. A second group was treated with the adjuvant alone, while the third group received no treatment and served as controls.

Immunizations were given by subcutaneous injection into 2 sites on the neck of each animal on days 0, 28 and 56 of the experiment. The cattle were weighed every 2 weeks, and liveweight gain was calculated between days 14 (after the cattle had re-alimented) and day 98. The animals were slaughtered at a commercial abattoir (Teys Bros. Biloela) on day 106. AQIS approval was obtained for products from these cattle to be used for human consumption.

Experiment 2: Feedlot Steers

In experiment 2, 30 steers were obtained from a local sale yard. The cattle were housed in pens at the Tropical Beef Centre and fed lucerne chaff for 4 weeks to allow them to become accustomed to their new environment, and to train then for sampling procedures. On day 0 of the experiment, liveweight was recorded following a 48 hour fast. Six cattle that were representative of the group were then slaughtered to obtain comparative carcass data. The remaining 24 cattle were assigned to two groups of 12 animals per group, having similar mean fasted liveweight. One group was treated with ß-adrenoceptor vaccine while the second group received adjuvant alone, according to the protocol described in

experiment 1. The animals were then treated as they would be in a commercial feedlot. A diet of high-fibre feedlot starter mix (Farmstock) with decreasing rations of Rhodes grass hay was presented *ad libitum* for the first 14 days. Then the animals were fed feedlot #3 finisher ration *ad libitum* with 500g of hay twice weekly for the remainder of the study. Feed intake was recorded for individual animals twice-weekly, and feed conversion efficiency (FCE) was calculated as liveweight gain / feed intake. One animal in the ß-adrenoceptor vaccine group had to be removed from the study on day 60 due to foot problems unrelated to the treatment. On days 100 to 102 the remaining 23 cattle were slaughtered for chemical determination of carcass composition.

Experiment 3: Grass-Fed Steers

This experiment was identical to experiment 1, except that 60 steers were used, and run on Brian Pastures Research Station, Gayndah. One animal in the group treated with adjuvant only was destroyed during the experiment due to a severe case of three-day sickness. The animals were slaughtered at South Burnett Meat Works on days 102 and 103, and chiller assessment data were obtained together with the weights of individual retail cuts.

Experiment 4: Pen trial

Forty-two young steers were obtained from a local sale yard, and housed in pens in the Tropical Beef Centre Animal House. Initially, the cattle were fed an unrestricted amount of lucerne hay, and this was gradually replaced by a diet of grassy hay plus weaner mix in a ratio of 2:1, given at a rate of 6 kg per animal per day.

After one month of becoming accustomed to their new diet and environment, the cattle were fasted for 48 hours to obtain a reliable estimate of their liveweight. The cattle were then allocated to one of six groups, with seven animals per group, and each group having a similar mean liveweight (155 kg). Each of the six groups received one of the following treatments.

Group 1 (CN):	Untreated controls.
Group 2 (CL):	Untreated to day 63. Clenbuterol (4 mg/day) included in the diet from day 63 to day 98.
Group 3 (FR):	Peptide vaccine in Freund's adjuvant injected on days 0, 28 and 56.
Group 4 (DS):	Peptide vaccine in dextran sulphate-based adjuvant injected on days 0, 28 and 56.
Group 5 (QD):	Peptide vaccine in Quil A / DEAE dextran-based adjuvant injected on days 0, 28 and 56.
Group 6 (IL):	Peptide vaccine in Quil A / DEAE dextran, plus interleukin-1ß given on days 0, 28 and 56.

Group 2 (cattle treated with clenbuterol) was included in this experiment in order to provide benchmark data on the physiological effects of a ß-agonist drug. The rationale behind the use of the different adjuvants is discussed above under the heading 'adjuvant formulation'.

Because the anabolic effects of ß-agonists in muscle can be counterbalanced by a loss of fat and viscera, there is often no net weight gain response. This caused us to examine the blood chemistry of treated cattle. After the animals were allocated to their treatment groups (day 0), they were weighed and blood samples were collected every fortnight to day 56, then weekly to day 98. The blood samples were analysed for the presence of antibodies to the peptide, and to determine plasma concentrations of urea-N, glucose and potassium. Blood was also taken from selected animals on day 70 for use in T-cell epitope analysis.

4. Antibody titres

Antibody concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) using the 24 amino acid peptide as the test antigen. Microtitre plates were coated by adding 100 µL of a buffer solution containing 0.2 M sodium acetate (pH 2.5) and 4 µg of peptide/mL to each well. The plates with the coating buffer were stored in a refrigerator until required, then washed four times with a second buffer (wash buffer) which contained 9 g/L sodium chloride and .55 g/L Tween 20.

Serum samples were diluted 1:100 in assay buffer, which contained 10 phosphate-buffered saline tablets (Oxoid)/L and .55 g Tween 20/L. Further serial dilutions were made using the same buffer, so that the final serum concentrations were 1:100; 1:200; 1:400; 1:800 etc. up to 1:25,600. Each sample (100 µL) was added to a well and allowed to incubate at room temperature for 60 min. The plates were rinsed with washing buffer as described above, before the addition of 100 µL of horseradish peroxidase-conjugated anti-bovine antibody (Silenus Laboratories) at a dilution of 1:8000 in assay buffer. The plates were left at room temperature for a further 60 min, then washed again as described above. Finally, 100 µL of a solution which contained 10 µL H_2O_2 and 55 mg ABTS dissolved in 100 mL of substrate buffer (10.5 g/L citric acid; 9.6 g/L Na_2HPO_4, pH 4.0 accurately with citric acid or Na_2HPO_4) was added to each well. The plates were left for 20 min, then the absorbency was read at a wavelength of 405 nm using an ELISA plate reader. Antibody titres were interpolated from a titration curve of absorbance versus serum dilution. The titre was defined as the serum dilution value which gave an absorbance reading 0.1 units higher than that of a negative control sample.

5. Epitope analysis

As described above, the vaccine is based on a 24 amino acid peptide derived from the sequence of the bovine $ß_2$-adrenoceptor, and contains multiple cysteine residues which make it expensive to synthesise and difficult to purify. The peptide is also virtually insoluble, which prevents it from being conjugated to a larger protein molecule to enhance its immunogenicity. By identifying the T- and B-cell epitopes on the peptide, we aimed to determine which amino acids are important in eliciting the desired antibody response. The remaining amino acids could then be deleted from the sequence, and a shorter version of the peptide could be designed that is easier to make, less expensive, and amenable to conjugation

T-cell epitopes

The role of the T-helper cells in the humoral immune response is vital, as activated T-helper cells are involved in antigen presentation to the antibody-producing B-cells, and in the production of interleukins which recruit and activate several cell types in the immune response.

Animals from the adjuvant trial (experiment 4) were selected for a T-cell proliferation assay on the following basis. Most cattle that showed a positive antibody response to the full peptide on day 56 (ELISA titre > 1000) were selected, and these included one steer from the clenbuterol-treated group. Five animals from the untreated group were tested also, to provide negative control data. Fourteen animals were assayed in total. A heparinised blood sample was taken from these animals on either day 70, or day 84. Lymphocytes were purified from whole blood using a density gradient centrifugation method. They were cultured in quadruplicate in the presence of various concentrations of peptides (0 to 100 mg/mL); or with concanavalin A (2 mg/mL), a potent T-cell mitogen used to assess cell viability. The cells were incubated for five days. For the final 16 hours, tritiated thymidine (1 mCi) was present in each well. The cells were harvested onto glass-fibre filters, and radioactivity was measured by liquid scintillation counting. The results are expressed as stimulation indices (SI), where: SI = mean cpm of stimulated wells/mean cpm of control wells. An SI of greater than 2 was considered to represent a positive response.

As T-cell epitopes are typically from 13 to 32 amino acids in length, two peptides were selected for testing, each containing 17 amino acids, and corresponding to the N-terminal and C-terminal regions of the full 24 amino acid peptide (Fig. 3).

B-cell epitopes

It is known that when an animal is vaccinated with a large peptide, the peptide is degraded into many smaller fragments before being presented to the antibody-producing B-cells. Each fragment may provoke an antibody response in a particular type of B-cell, with the result that polyclonal antibodies are produced. However, because different fragments cause reactions of different strength, it is not unusual to find that polyclonal antiserum is comprised of one or two major populations of antibodies, that are directed towards a few specific fragments or 'dominant epitopes'. The B-cell epitopes may be quite small, containing as few as four or five amino acids in a key sequence, with additional amino aids in the flanking regions acting to present the epitope in a particular conformation.

Before attempting to modify our ß-adrenoceptor vaccine, we tried to identify a dominant B-cell epitope within the 24 amino acid peptide. This was done by studying the reaction of the polyclonal antiserum to six overlapping fragments of the peptide, each containing 10 amino acids (Figure 3). Each of these fragments formed the basis of an individual ELISA assay. Analysis of possible B-cell epitopes was performed using the same technique described for antibody titre analysis, except that different ELISA plates were each coated with one of the 6 amino acid fragments of the full peptide. A large quantity of blood was collected from each animal used in experiment 4, on day 70. Serum was obtained from the clotted blood and stored frozen until assayed. Of the 28 cattle that were immunized, 26 gave an antibody response to the full peptide, and so the serum from all of these animals

was screened initially at a dilution of 1:400 to test for reactivity with the peptide fragments. The serum obtained from six control animals was also screened, for comparison.

Of the samples taken from treated animals, 13 showed a strong reaction with the peptide fragments, and so these were tested further at dilutions of 1:400, 1:800, 1:1600 and 1:6400 to obtain more quantitative information. The non-reacting serum from one of the control animal was also included in further tests to provide some baseline data.

6. Blood chemistry

Plasma concentrations of potassium, urea-N and glucose were measured in experiment 4. Potassium is used by pharmacologists as a standard measure of ß-adrenoceptor activity *in vivo*. There is a constant exchange of Na^+ and K^+ ions across cell membranes in the body, and skeletal muscle is one of the major contributors to this ion pumping activity by virtue of its large mass. This is particularly relevant, as skeletal muscle is the tissue targeted by our vaccine. When the $ß_2$-adrenoceptors in muscle are activated, the Na^+/K^+ pump is inhibited, so that there is less efflux of K^+ from the muscle, and a consequent reduction in plasma potassium concentrations.

Plasma urea-N concentrations reflect whole-body protein turnover, and provide a classical test for agents that are designed to increase protein synthesis, or reduce protein breakdown. It has been shown previously that clenbuterol can cause marked reductions in plasma urea-N, and that such an effect can be accounted for solely by changes in skeletal muscle. Based on our previous study in under-fed heifers, plasma urea-N concentrations were expected to reach minimum levels in clenbuterol-treated cattle within 7 to 14 days of treatment, then to return close to control values after 5 weeks of treatment.

Finally, $ß_2$-adrenoceptors inhibit glucose uptake in muscle and liver, so that treatment with ß-agonists often results in an increase in plasma glucose concentrations. In earlier studies where clenbuterol was infused into steers over a 3 hour period, we observed a marked rise in plasma glucose, consistent with this effect. Compared with potassium and urea-N concentrations, plasma glucose is the least reliable indicator of a drug or vaccine response, because of the rapid, marked, and short-term changes that can occur in response to the stress of blood sampling. Nevertheless, glucose concentrations are easy to measure, and may have provided some useful data in the present study.

Plasma concentrations of potassium were measured by flame photometry using KCl standards. Plasma glucose and Urea-N concentrations were measured using commercially available assay kits (Sigma Chemical Co.).

RESULTS AND DISCUSSION

Experiment 1

TABLE 1. Production Responses of Grass-Fed Cows

	Control Only	Adjuvant Vaccine	Peptide	PSE*
Initial Fasted Liveweight (Day 0), kg	425	426	425	11.0
Liveweight Day 14, kg 497	496	497	11.4	
Final Liveweight (Day 98), kg	542	539	542	11.3
Liveweight gain (Days 14 to 98), kg/day	0.54	0.51	0.54	0.04
Hot carcass wt, kg	276	274	275	6.0
Fat Depth (P8), mm	23.4	19.4	21.6	1.44
Eye muscle area, cm^2	69.3	68.0	70.4	1.98
Strip loin wt (trimmed), kg	6.39	6.22	6.18	0.17
D-Rump wt (trimmed), kg	5.93	5.76	5.84	0.16

Values are means for all animals.
*PSE Pooled standard Error.
No significant differences among treatment groups as determined by analysis of variance at 5% probability level

Antibody Responses of Grass-Fed Cows

In marked contrast to earlier studies, of the 18 animals immunized in this trial none achieved antibody titres in excess of 1000 during the course of the experiment and could be classed as a good responders. Five cows showed low antibody titres in the range 200-1000, and 13 had no detectable antibodies.

Experiment 2

TABLE 2. Production Responses of Lot-Fed Steers

	Adjuvant Only	Peptide Vaccine	PSE*
Initial Fasted Liveweight (Day 0), kg	348	349	8.1
Liveweight Day 14, kg 387	384	9.5	
Final Liveweight (Day 98), kg	453	453	11.8
Liveweight gain (Days 14 to 98), kg/day	0.85	0.83	0.07
Feed intake, kg/day	9.41	9.08	0.52
Feed/gain	12.3	11.2	1.0
Hot carcass wt, kg	260	262	5.0
Estimated gain in carcass wt., kg	65	68	3.4
Hide wt, kg	37.0	34.7	1.1
Heart wt, kg	1.48	1.50	0.04
Liver wt, kg	4.60	4.60	0.1
Kidney fat wt, kg	4.61	4.56	0.23
Eye muscle area, cm^2	93.3	96.5	6.6
Semitendinosus muscle wt, kg	2.0	2.0	0.1
Biceps femoris muscle wt, kg	6.0	6.0	0.1
Longissimus dorsi. muscle wt, kg	2.59	2.66	0.08
Gastrocnemius muscle wt, kg	1.67	1.64	0.03
Soleus muscle wt, kg	0.29	0.30	0.01

Values are means for all animals.
*PSE Pooled standard Error.
No significant differences between treatment groups as determined by analysis of variance at 5% probability level

Antibody Responses of Lot-Fed Steers

Of the 12 animals immunized, none achieved an antibody titre in excess of 1000 during the course of the experiment. Two steers produced antibodies in the titre range of 200 to 1000, while 10 produced no detectable response.

Experiment 3

TABLE 3. Production Responses of Grass-Fed Steers

	Control	Adjuvant Only	Peptide Vaccine	PSE*
Initial Fasted Liveweight (Day 0), kg	467	468	469	7.5
Liveweight Day 14, kg 533	525	528	9.1	
Final Liveweight (Day 98), kg	622	610	610	9.5
Liveweight gain (Days 14 to 98), kg/day	1.07	1.01	0.97	0.05
Hot carcass wt, kg	332	327	327	5.0
Fat depth (P8), mm	17.6	18.1	16.3	1.0
Eye muscle area, cm^2	68.8	67.7	71.5	1.5
Point end brisket (deckle on), kg	4.89	4.86	4.85	0.11
Navel end brisket, kg	5.21	5.06	5.07	0.10
Cube roll (5 rib), kg	2.74	2.68	2.92	0.10
Short rib (bone in), kg	2.83	2.81	2.69	0.07
Chuck roll, kg	7.96	7.97	7.97	0.22
Tenderloin (strap on), kg	2.77	2.74	2.71	0.07
D-Rump, kg	6.67	6.71	6.46	0.13
Striploin (3 rib), kg	6.25	6.32	6.31	0.12
Silverside, kg	12.3	11.9	11.9	0.24
Topside, kg	10.1	9.95	9.81	0.18
Fat wt, kg	16.8	16.5	16.8	0.49
Bone wt, kg	32.3	31.1	31.3	0.66

Values are means for all animals.
*PSE Pooled standard Error.
No significant differences among treatment groups as determined by analysis of variance at 5% probability level

Antibody Responses of Grass-Fed Steers

Of the 20 animals immunized, only 1 achieved an antibody titre in excess of 1000 during the course of the experiment, and could be classed as a good responder. Thirteen steers produced antibodies in the titre range of 200 to 1000, and 6 had no detectable antibodies.

Summary of Experiments 1 to 3

The vaccine caused no significant effects on growth or carcass composition, but more importantly, it failed to elicit an antibody response. This result was unexpected in view of our previous experiments (CS.108) in which 9 out of 12 cattle showed a good antibody response. All the cattle treated previously had titres over 1000, and values as high as 12000 were observed in several animals.

Before the project could proceed any further to evaluate the possible physiological effects of the vaccine, it was necessary to identify the cause of the poor immune response. Several factors might have been responsible.

- The source of the peptide was changed from a relatively crude peptide mixture originally supplied by QIMR, to a more pure peptide supplied by Auspep. This was both unavoidable, in light of QIMR's inability to meet our order, and desirable, as it is important to know that it is the designer peptide which elicits the response, and not an impurity in the preparation.

- The vehicle was changed from Freund's adjuvant, a potent and reliable mixture that is recognised as a 'gold standard' in immunology, to one containing Quil A / DEAE dextran. The latter adjuvant is commercially acceptable (as evidenced by AQIS granting approval for the meat from treated cattle to be sold for human consumption) and has been shown previously to be effective with other peptide antigens. However, it's efficacy was unproven with the current peptide. The decision to make this change was a calculated risk, but one judged to be relatively minor, in comparison with the cost savings made through not having to condemn the treated carcasses.

- The third factor was that the adjuvant emulsion obtained with the Quil A / DEAE mix was unstable. The oil and water phased were prone to separate, and this was particularly evident when the vaccine was transported to our field stations, and administered in the high summer temperatures that were experienced at the time of the trials.

With these possible factors in mind, the experimental objectives were renegotiated mid-way through the project and a fourth cattle experiment was designed. Experiment 4 was to achieve several aims as listed above (see OBJECTIVES). Briefly, the cause of the vaccine failure was to be identified, its potency at causing a physiological response was to be determined, and serum would be provided for epitope analysis.

Experiment 4

Weight gain

The six groups of cattle were initially balanced according to their fasted liveweights, but repeated fasting of the animals to obtain accurate weight gain figures would have disrupted our measurements of blood chemistry. Therefore, the gains reported in Figure 5 are based on fed liveweights, taken from the first such measurement made on day 14.

Unexpectedly, there was an immediate and marked increase in weight gain in response to clenbuterol treatment. Although clenbuterol is known to increase muscle mass, a corresponding decrease in the mass of fat and viscera often results in no net increase in liveweight gain, and sometimes no increase in carcass weight. The net effects on body weight are dependant on the age, sex and diet of the animal, and are difficult to predict. Nevertheless, in the present experiment clenbuterol-treated steers showed a marked and significant growth response by gaining 6.9 kg more weight than controls over the first week of treatment (days 63 to 70), with this difference increasing to 10.1 kg after 3 weeks, and 12.5 kg after 5 weeks ($P<0.01$). By contrast, there was no apparent effect on weight gain in any of the vaccinated cattle, except in the group treated with Freund's adjuvant, which showed a trend towards reduced weight gain in the latter part of the experiment. This amounted to a difference between control and treated cattle of -9.8 kg by day 98.

Plasma Potassium Concentrations

Potassium concentrations are shown in Figure 6. It can be seen in the first panel that over the first 56 days of the experiment, there was no difference in potassium concentrations between untreated cattle and those destined to be treated with clenbuterol. However, as soon as clenbuterol treatment started, there was a marked rise in potassium concentrations that was sustained until day 98. A statistical comparison was made between the clenbuterol-treated and control groups by determining the area under each plot of potassium concentration versus time, over the clenbuterol treatment period. A significant difference was found at the level of $P < 0.01$.

This result was unexpected, as ß-agonists are reputed to *decrease* potassium concentrations as discussed above. Several published articles support this, including our own recent work in cattle where clenbuterol was infused over a 3 hour period. Thus, it appears that the short-term and long-term effects of clenbuterol on potassium concentrations are opposite. Nevertheless, this significant result supports our earlier conclusion made from observations on weight gain, that clenbuterol was given at an appropriate and effective dose in the present experiment.

In contrast to clenbuterol, the vaccine caused no changes in plasma potassium concentrations in any of the treated groups. This observation supports our earlier conclusion that the ß-adrenoceptor vaccine failed to mimic the effects of the ß-agonist drug.

Fig. 5: Weight gain in steers (experiment 4)

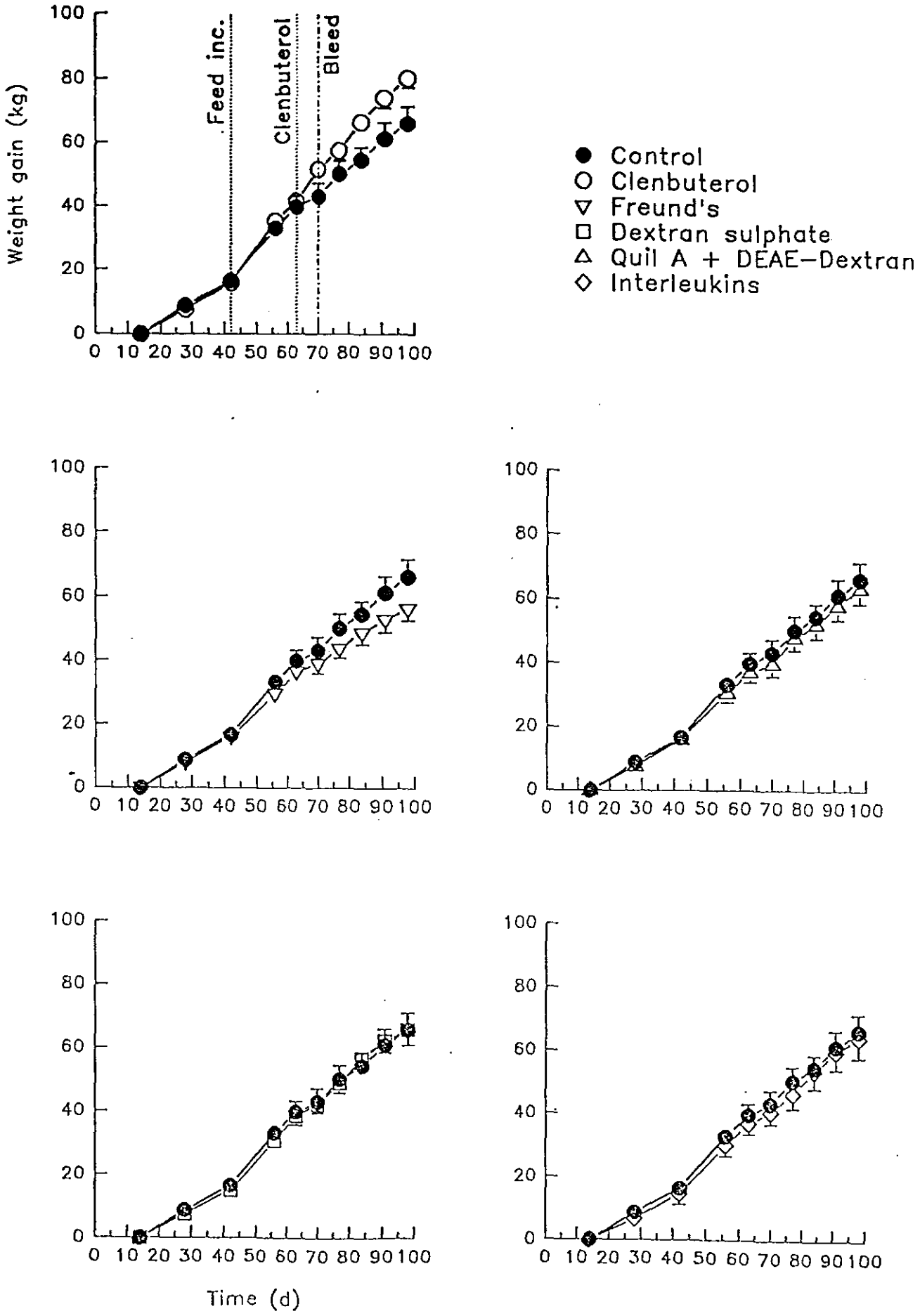


Fig. 6: Plasma potassium concentrations in steers (experiment 4)

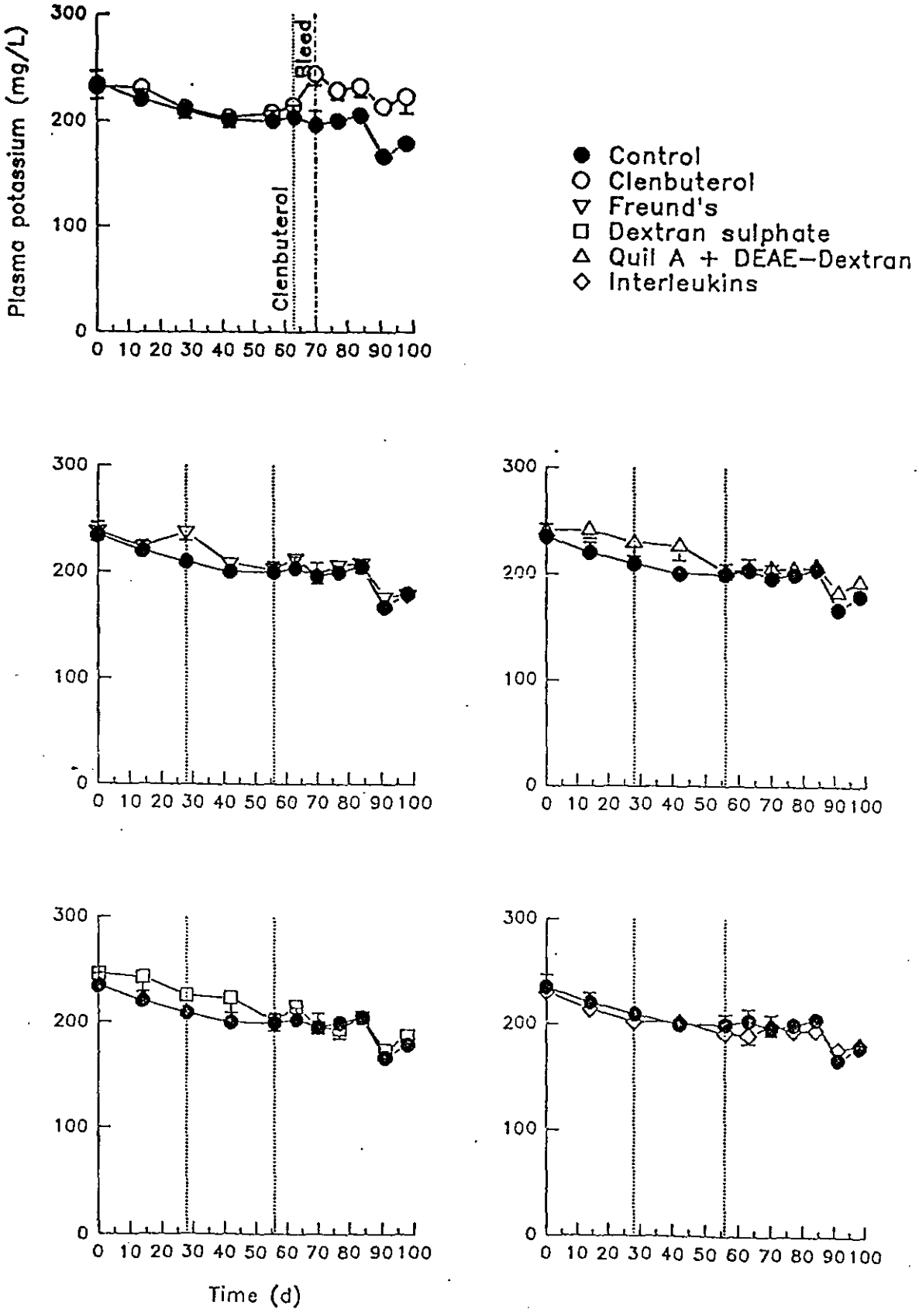


Fig. 7: Plasma urea-nitrogen concentrations in steers (experiment 4)

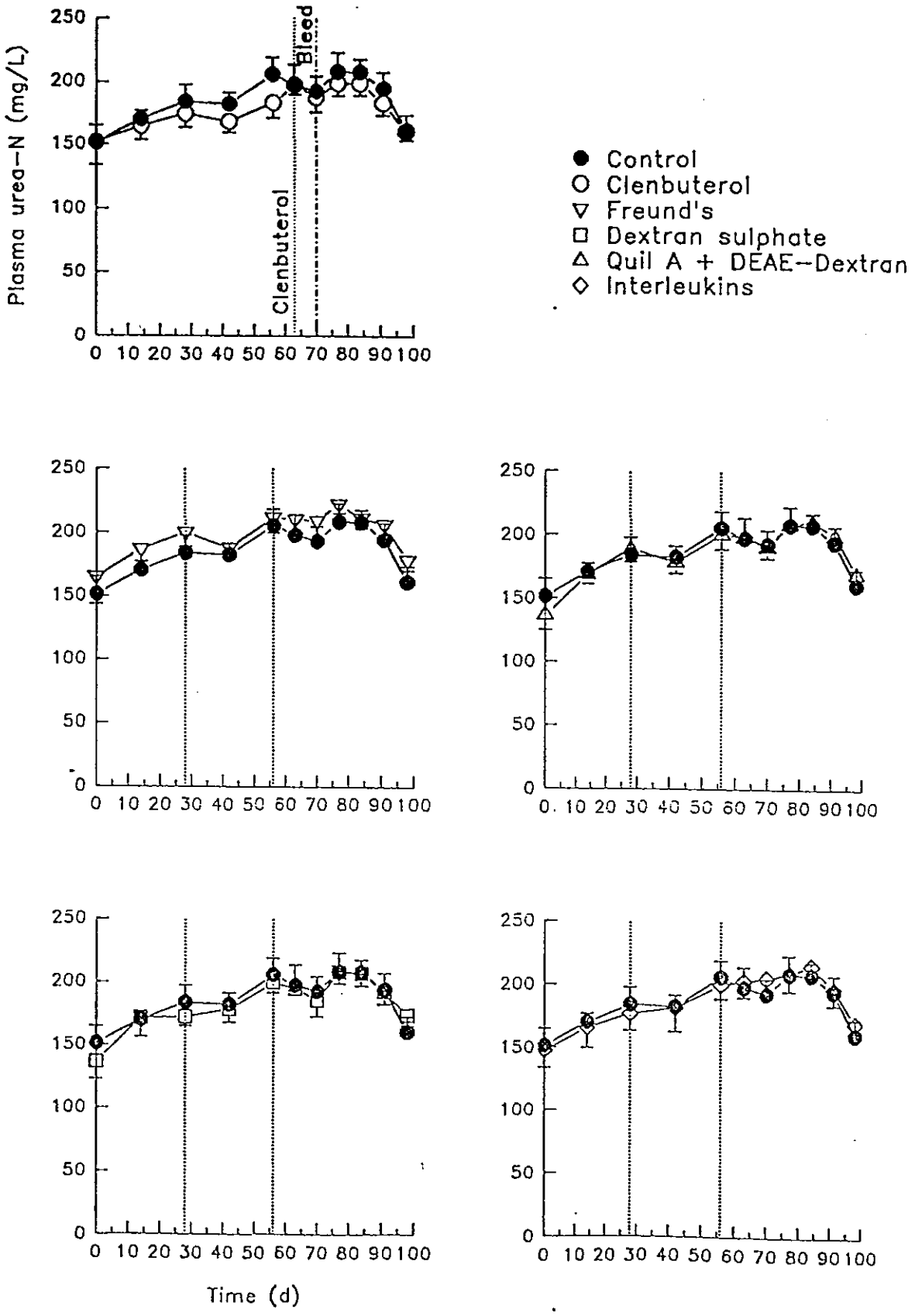
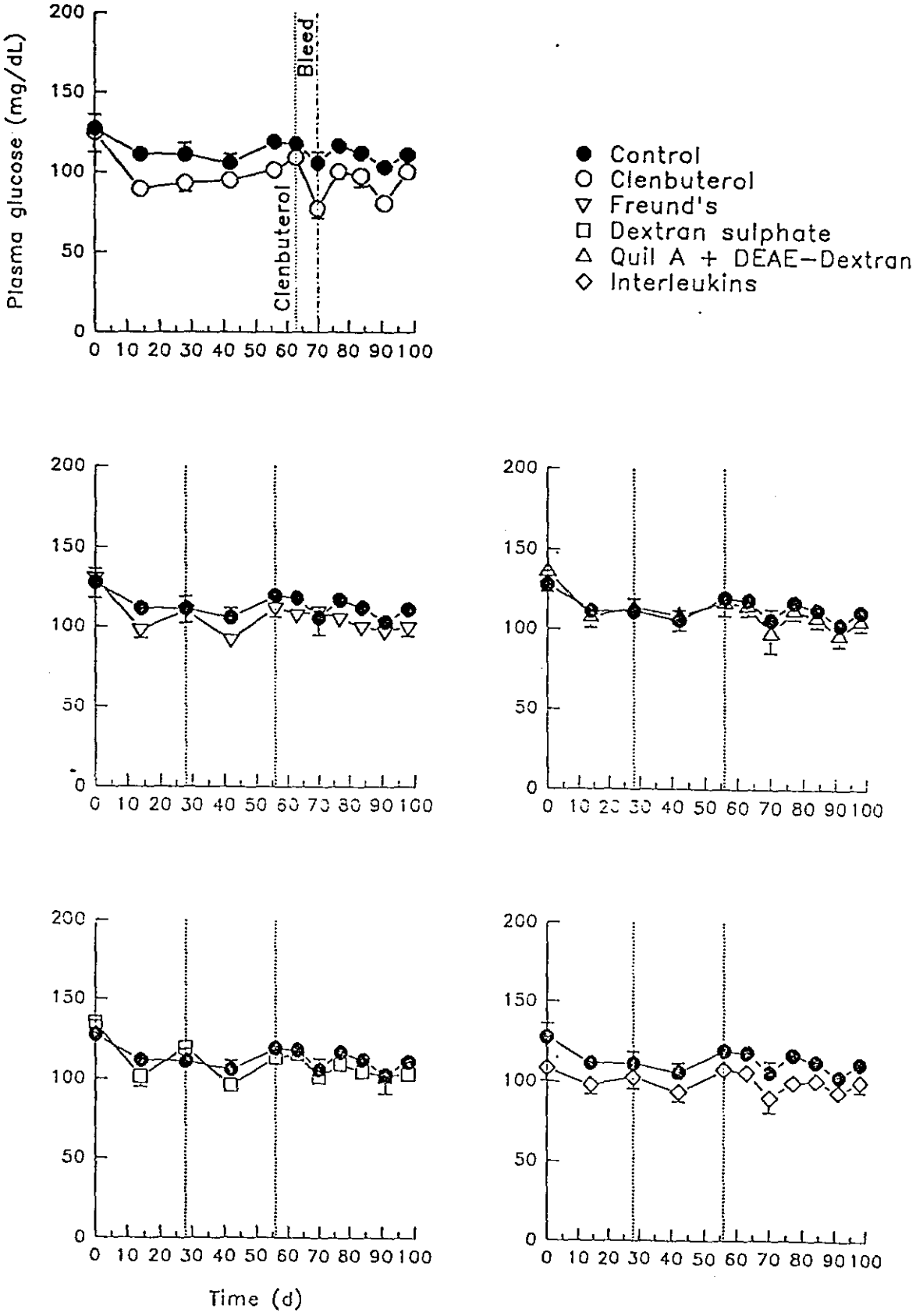


Fig. 8: Plasma glucose concentrations in steers (experiment 4)



Plasma Urea Nitrogen Concentrations

Despite the fact that we observed marked changes in plasma urea concentrations in earlier studies of under-fed heifers, in the present study which used well-fed steers, no differences in plasma urea-N were observed between the untreated animals and those treated with clenbuterol, or the ß-adrenoceptor vaccine (Figure 7).

Although plasma urea-N values are often used a screen for anabolic agents, our results show that they do not always reflect an improved weight gain response. Furthermore, because of their lack of response to clenbuterol, urea-N values are not useful for judging the efficacy of the ß-adrenoceptor vaccine in the present study.

Plasma Glucose concentrations

Unfortunately, no marked differences in glucose concentration were observed that could be attributed to treatment with clenbuterol, or the ß-adrenoceptor vaccine (Figure 8). The values observed for glucose concentration are at the high end of the normal range for cattle, and this could have masked any small increase that might have occurred.

Antibody response

As described above, the antibody response was measured using the ELISA technique, which detects any antibodies that recognise the 24 amino acid peptide. Antibody titres for individual cattle are shown in Figure 9.

The response variables are discussed in terms of the number of responders (those that produced antibody titres > 1000), and the number of injections required to achieve that response (see Figure 10). The magnitude of the response (i.e. the highest titre reached), and whether the response was sustained, are also considered to be important factors. These were judged by calculating the area under the plot of antibody titre versus time, over the last four weeks of the experiment. Figure 11 summarises these values.

As expected, no antibodies were detected in the sera of control animals, but the sera from four animals in the clenbuterol-treated group tested positive for the presence of antibodies on at least one occasion, and before clenbuterol treatment commenced. As these cattle were not presented with the vaccine, the antibodies detected could be regarded as 'autoantibodies', produced against the animals' native ß-adrenoceptors. At present we have no particular explanation for this, except that it is known that a certain percentage of the human population has been found to test positive for ß-adrenoceptor autoantibodies. The functional relevance of autoantibodies detected in these animals is uncertain, as their appearance was intermittent, and those tested in the T-cell assay were found to be inactive.

Freund's Adjuvant: It can be seen from Figure 10 that none of vaccines was effective after a single injection, but that two injections produced a response in the majority of cattle (5 out of 7) treated with Freund's adjuvant. The third injection caused no further increase in titre in these animals, and may, or may not have contributed to sustaining the response. Overall, the cattle given Freund's adjuvant had the highest antibody titres over

Fig. 9: Antibody titres for individual cattle (experiment 4)

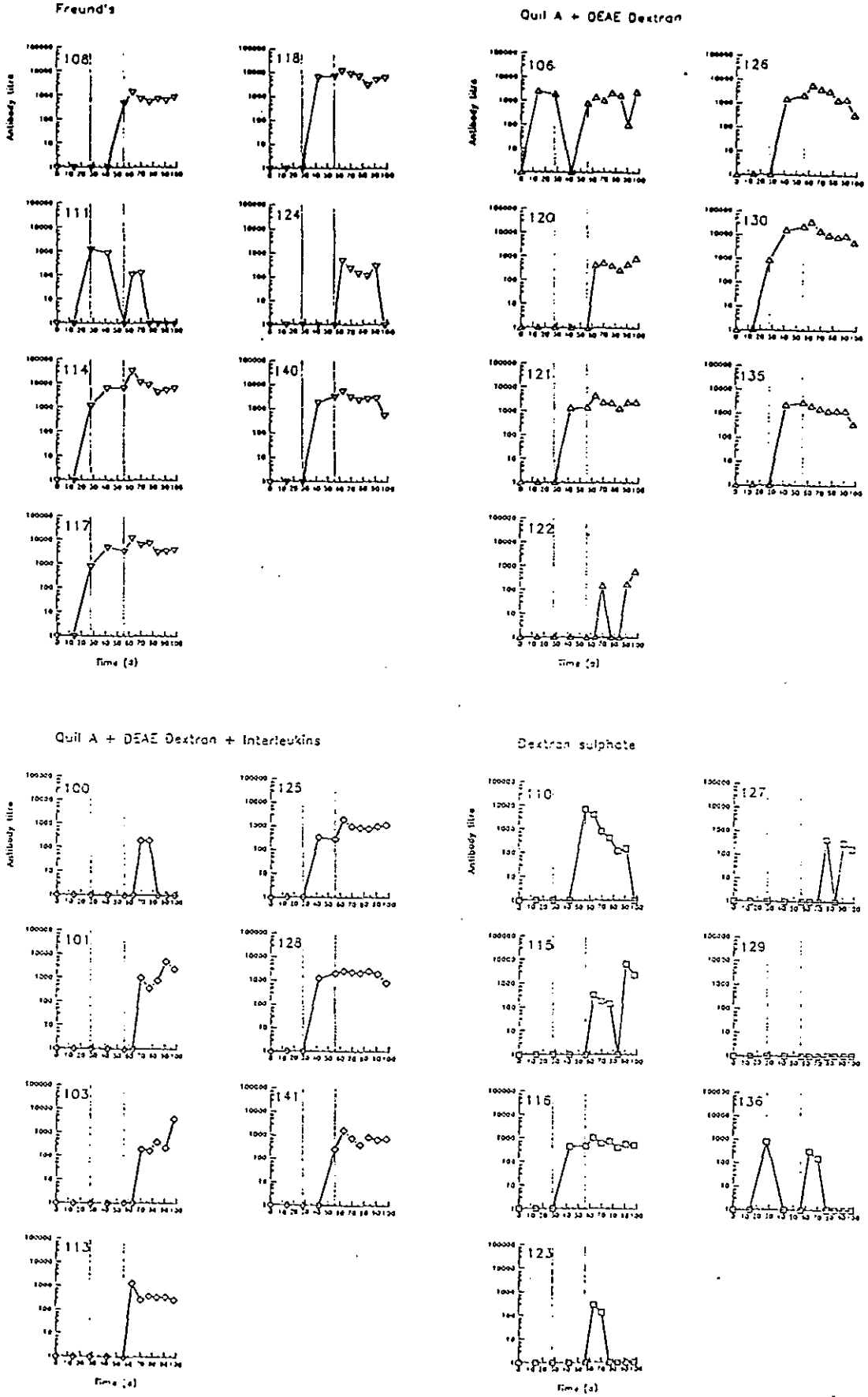
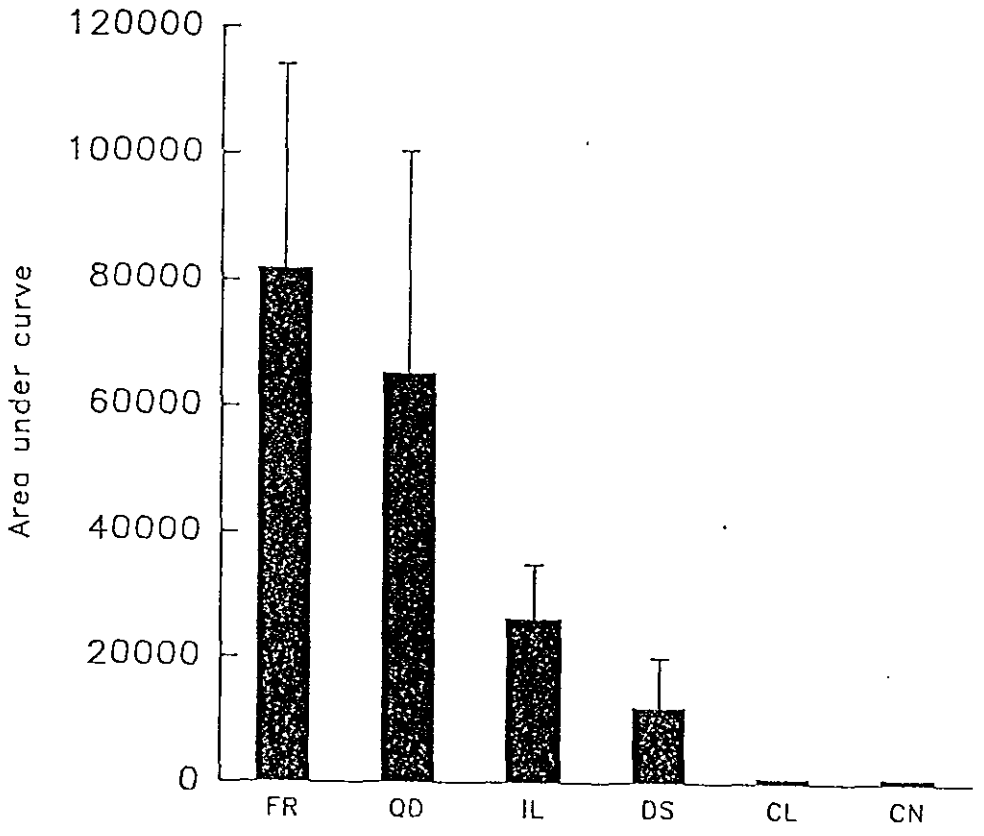


Fig 10: Number of cattle with antibody titre > 1000

Number of injections	Freunds	Dextran sulphate	Quil A + DEAE-Dextran	Interleukins
1 inj.	2	0	1	0
1or 2 inj.	5	1	5	1
1,2 or 3 inj.	6	3	5	6
non-Responders	1 @ < 1000	3 @ < 1000 1 @ no response	2 @ < 1000	1 @ < 1000

Fig. 11: Integrated values for antibody titre over last 4 weeks of experiment 4.



the last four weeks of the experiment (Figure 11). These data confirm that Freund's is a potent adjuvant in cattle, and that a non-response rate of between 15 and 30 % can be expected.

Quil A / DEAE dextran: The response to this adjuvant compares favourably with that to Freund's. Figure 10 shows that five out of seven cattle responded after two injections, and that the third injection gave no apparent advantage. The integrated values for antibody titre over the last four weeks were slightly less than for Freund's adjuvant, but not significantly so. These data confirm our hypothesis that the most likely reason this adjuvant failed in the previous field trial, was inadequate emulsification.

Interleukin-1ß: When this compound was added to the Quil A / DEAE dextran formula, the antibody response was both delayed and attenuated. Only one animal responded to two injections, although six were classed as responders after the third injection (Figure 10). Furthermore, the integrated value for antibody titre over the last four weeks was less than half that observed in the group treated with Quil A / DEAE dextran alone (Figure 11). This result was unexpected, as IL-1ß has been shown previously to enhance the immune response of cattle.

Dextran sulphate: This was the least successful adjuvant, producing a response in only three animals, and requiring three injections to do so (Figure 10). Furthermore, the integrated value for antibody titre was less than half that seen in the group treated with interleukin-1ß (Figure 11). These data are consistent with the peptide carrying a negative charge, and not being attracted by this polyanionic molecule.

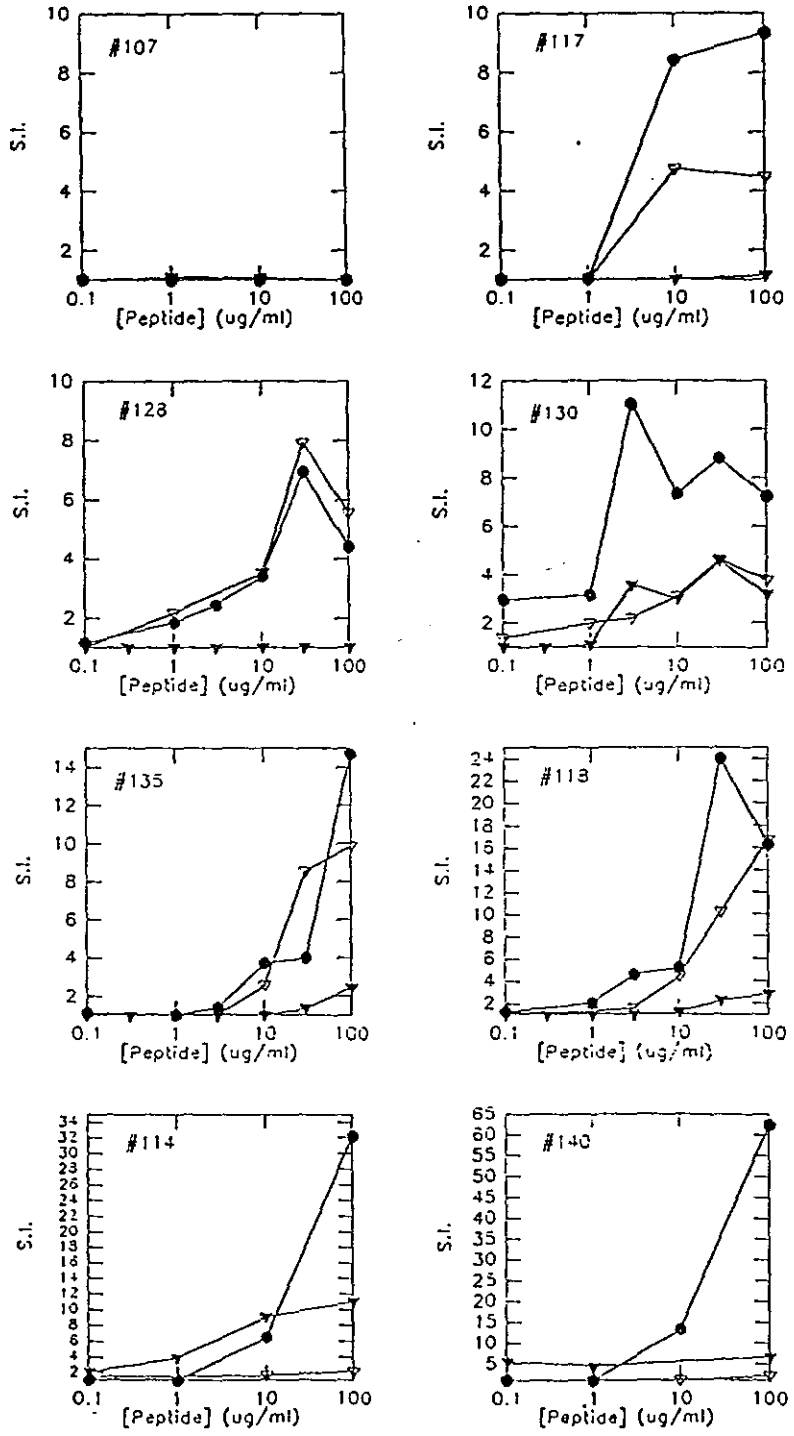
T-Cell epitope mapping

Figure 12 shows the responses of animals in the T-cell proliferation assay. None of the five negative control animals (ELISA negative), nor the clenbuterol-treated animal, gave a response in the T-cell assay. The results for one non-responding animal (#107) are shown in Figure 12 for comparison. For one responding animal, the isolated T-cells failed to be stimulated by concanavalin A, and were judged to be non-viable. In the remaining seven ELISA-positive animals, the greatest proliferative response was shown against the full 24 amino acid peptide, indicating that a greater population of T-cells in each animal responds to this sequence. For the two shorter peptides, there was considerable variation in the response between animals.

When compared with the response to the full sequence, peptide H17E showed a good response in three animals (#128, #135 and #118), an intermediate response in two animals (#117 and #130), but little or no response in the remaining two cattle (#114 and #140). Similarly, peptide K17T showed a good response in one animal (#114), an intermediate response in two animals (#130 and #140), but little or no response in the remaining four (#117, #128, #135 and #118).

Thus, the results show that there is variability between individual cattle with respect to the T-cell epitopes they recognise on the peptide H24T. The N-terminal sequence is perhaps more important than the C-terminal in the majority of treated animals. In theory, it would be possible to shorten the peptide by removing some amino acids at the

Fig. 12:
Results of T-cell epitope mapping. Each graph shows the rate of lymphocyte proliferation (expressed as the stimulation index: SI) in serum harvested from an individual animal, and incubated in the presence of various concentrations of three test peptides. Filled circles represent the full peptide (H24E), open triangles represent H17E, filled triangles represent K17T.



C-terminal end, and still retain activity, provided this did not adversely affect any B-cell epitopes that may be present.

B-Cell epitope mapping

None of the control samples gave a significant response to any of the peptide fragments. Figure 13 shows the outcome of screening the antisera from the vaccinated cattle. Thirteen of the cattle that showed a measurable, but poor response to the full peptide (mean titre 593), also failed to respond to any of the fragments. This result was not unexpected, and in part reflects the poor immunogenicity of some of the adjuvants used, such as dextran sulphate. In contrast, serum from the 13 cattle that responded well to the full peptide (mean titre 4192), also reacted with the fragments. It was evident that these reactions varied in strength, both between animals and across different peptides. At this stage, none of the peptide fragments could be identified as causing a positive response in the majority of cattle, and so the 13 reactive samples were tested further to obtain more quantitative information. The non-reacting serum from one of the control animals was also included in further tests, to provide baseline data.

Each sample was prepared at dilutions of 1:400, 1:800, 1:1600 and 1:6400, and tested again against each of the 6 peptide fragments. The results are shown in Figures 14a and 14b Each panel represents the reaction of an individual animal to the 6 peptide fragments, which are denoted by different symbols. The serum dilution is shown on the horizontal axis, using a Log scale for convenience. The mean response of the control serum is shown as a dotted line on each graph for comparison. The mean response of the vaccinated cattle is shown in the last panel of Figure 14b.

Although there was some variation in the size of the response between different cattle, there was no apparent correlation between this and the type of adjuvant formulation used. Furthermore, qualitatively, the results were very consistent. Five of the six peptides were recognised equally well in all the cattle, with the remaining peptide (H10A), being poorly recognised in most cases.

Conclusions from epitope testing

- The 24 amino acid peptide may contain multiple B-cell epitopes (at least two) over the 19 or so amino acids which form the C-terminal end of the molecule.

- Although the deletion or substitution of five or six amino acids at the N-terminal end of the molecule would not be expected to affect the B-cell response adversely, this strategy to reduce the size of the peptide is not recommended, on the basis that an important T-cell epitope may reside in this region. Furthermore, such a modification would not eliminate any of the cysteine residues that cause the problems with the peptide's synthesis.

Fig. 13: Results of B-cell epitope mapping.

Results of screening the serum from 26 cattle at a 1:400 dilution for reactivity with six fragments of a 24-amino acid peptide immunogen. The parent peptide was given in four adjuvants: Freund's (FR); dextran sulphate (DS); Quil A plus DEAE dextran (QD); or Quil A, DEAE dextran plus interleukin-1ß (IL). Columns headed #1 to #6 denote six different overlapping fragments of the parent peptide, each consisting of 10 amino acids. The tests were performed by ELISA. A result was judged as negative (-) when the absorbance of the sample was not greater than 0.1 unit above control values. Positive responses were classified as low (+), medium (++), high (+++), or very high (++++) depending on whether absorbance readings fell in the range of 0.1 to 0.4; 0.4 to 0.6; 0.6 to 0.8; or greater than 0.8 units above control values respectively.

Animal	Treatment	#1	#2	#3	#4	#5	#6
108	FR	-	-	-	-	-	-
111	FR	-	-	-	-	-	-
114	FR	-	+	+	+	++	++
117	FR	+	++	++	+	+	++
118	FR	++	+	+	-	-	++
124	FR	-	-	-	-	-	-
140	FR	-	-	-	-	-	-
110	DS	-	+	+	-	-	-
115	DS	-	-	-	-	-	-
116	DS	-	-	-	-	-	-
123	DS	-	-	-	-	-	-
136	DS	-	++	++	+	++	+
106	QD	-	-	+	-	-	-
120	QD	-	-	-	-	-	-
121	QD	+	++	+++	+	++	++
122	QD	-	-	-	-	-	-
126	QD	+	++	++	++	+++	++
130	QD	++	+++	+++	+	++	++++
135	QD	-	+	+	-	-	-
100	IL	-	-	-	-	-	-
101	IL	-	+++	+++	+++	+++	+++
103	IL	-	+++	+++	+++	++++	++++
113	IL	-	-	-	-	-	-
125	IL	-	-	-	-	-	-
128	IL	-	++	+++	++	++	++
141	IL	-	-	-	-	-	-

Fig 14a: Results of B-cell epitope mapping. Testing was performed by ELISA. Each graph shows the absorbance reading for various dilutions of serum taken from a single animal, and tested against six different peptide fragments.

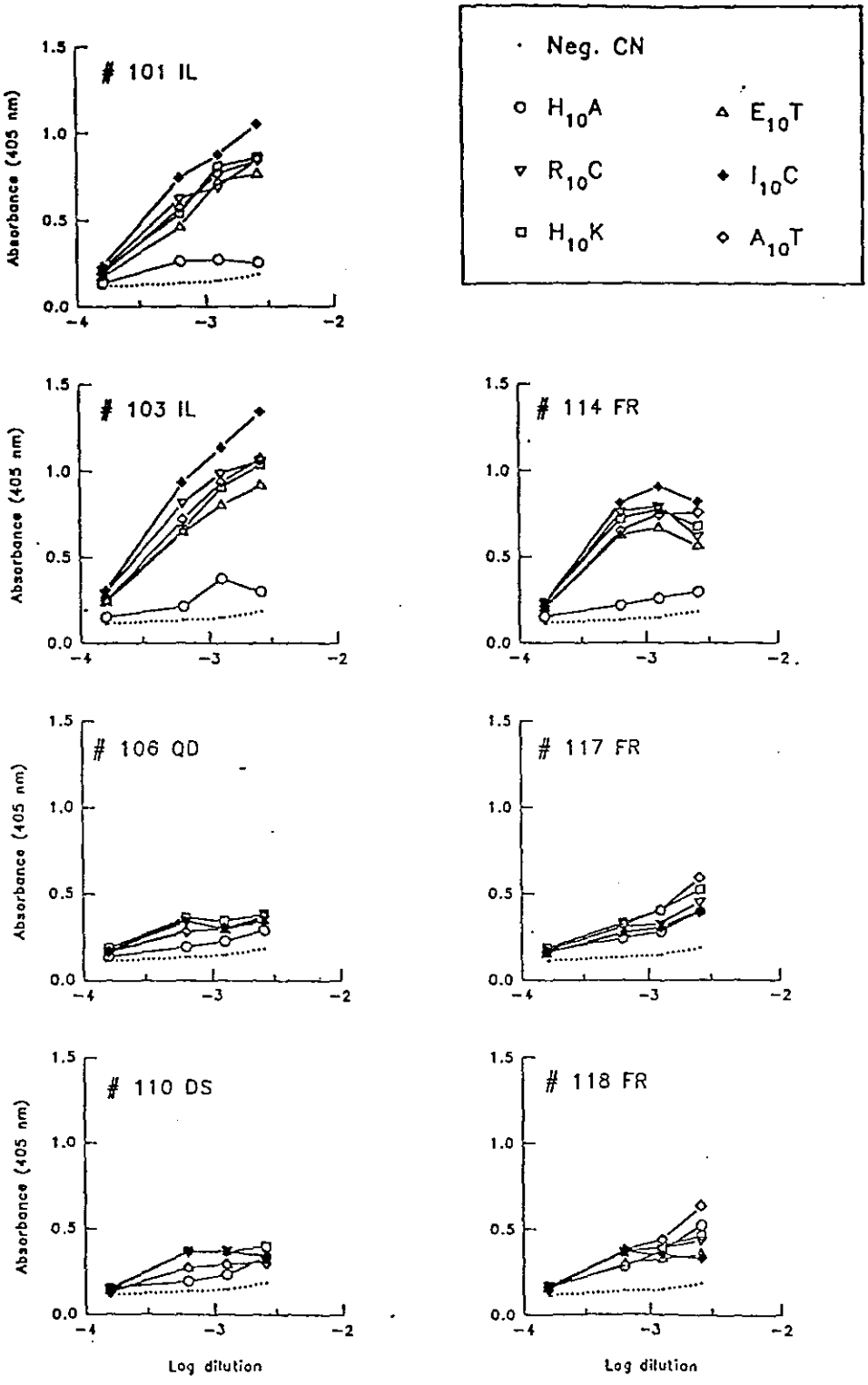
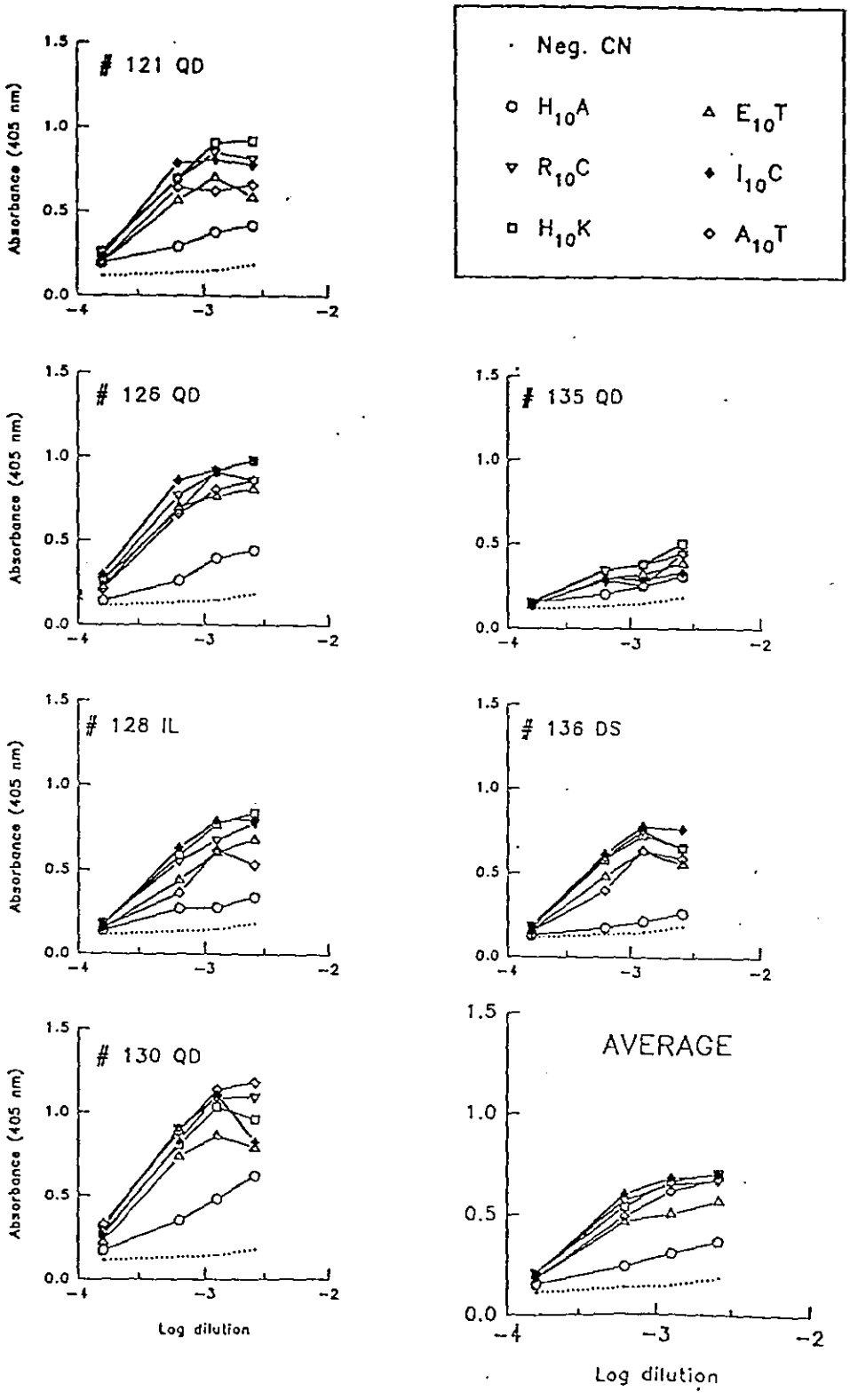


Fig 14b: Results of B-cell epitope mapping. Testing was performed by ELISA. Each graph shows the absorbance reading for various dilutions of serum taken from a single animal, and tested against six different peptide fragments.



Summary

Experiments 1 to 3 failed to reveal whether the vaccine was capable of causing any physiological effects, because no antibody response was elicited. The lack of antibody response was accounted for by an unstable adjuvant emulsion, and this was overcome in experiment 4 by preparing the vaccine at 4^0C.

From the standpoint of comparing different adjuvants for use in cattle, experiment 4 produced very clear results. We have confirmed the efficacy of Freund's adjuvant, and shown that Quil A/DEAE dextran has comparable potency when prepared under appropriate conditions. It is clear that interleukin-1ß delays and attenuates the response to this peptide when presented in a Quil A-based vaccine, although the reason for this unexpected observation is not known. The failure of the dextran sulphate adjuvant in comparison to the response obtained to Quil A/DEAE dextran, suggests strongly that either the peptide carries a predominant negative charge, or that Quil A is the main ingredient which provokes the response to the former adjuvant.

The unexpected but marked weight gain response to clenbuterol, contrasted with the lack of an anabolic response to the peptide. This indicates that the ß-adrenoceptor vaccine did not mimic the physiological effects of the ß-agonist drug, as it was designed to do. This conclusion is supported by the blood chemistry results, specifically those relating to potassium.

Epitope mapping has resulted in a better understanding of how the ß-adrenoceptor vaccine interacts with the immune system, but has not afforded the opportunity to modify the peptide through the substitution or deletion of troublesome amino acids. Instead, the results suggest that improving the physical properties of the molecule may only be achieved if the peptide is lengthened, and this would doubtless add to its cost.

SUCCESS IN ACHIEVING THE OBJECTIVES

Three large-scale trials were conducted concurrently, involving the treatment of 144 cattle over 100 days, in an attempt to meet the original objectives 1 and 2. The experiments were well-coordinated and executed, and a considerable amount of data was collected. Unfortunately, however, objectives 1 and 2 were not achieved because of a problem that occurred with the vaccine formulation. The vaccinated cattle produced no ß-receptor antibodies, and so the effect of such antibodies on growth and body composition could not be assessed.

The objectives were modified in agreement with the Corporation. First it was necessary to identify the cause of the vaccine failure, and to gather more information about suitable adjuvants. Through data collected in cattle, and confirmed in parallel studies using pigs, it was established that the original formula designed for commercial use was highly effective, provided that extreme care was taken in its preparation, so that a stable emulsion was obtained. It was also discovered that various modifications of this formula, which might have been expected to improve its efficacy, had the opposite effect. The data were clear and conclusive, and so this new objective was achieved.

The next objective was to determine the efficacy of the vaccine in causing a physiological response *in vivo*. This objective was also achieved, albeit through a negative result. Several variables were measured in vaccinated cattle and in cattle treated with the ß-agonist drug clenbuterol. Whereas the drug caused marked changes in weight gain and plasma potassium concentrations, neither of these variables, nor any others measured, showed any response to the vaccine. It was concluded that whereas the vaccine could elicit the production of antibodies which can mimic the effects of clenbuterol *in vitro*, such antibodies have no clenbuterol-like effects at concentrations found *in vivo*.

The third objective, to perform T and B-cell epitope analysis with a view to improving the vaccine, was also achieved, again with a discouraging result. These experiments afforded a better understanding of how the peptide antigen interacts with the immune system, but did not reveal a way in which troublesome amino acids could be removed or replaced without the loss of significant antigenic activity. Thus, as well as being ineffective *in vivo*, the current vaccine remains expensive and difficult to produce.

IMPACT ON MEAT AND LIVESTOCK INDUSTRY

The results of this project are unlikely to have any direct impact on the Meat and Livestock Industry - now, or in five years time. If further research can identify a way in which the positive results obtained *in vitro* can be translated into a production response *in vivo*, then there would be great potential benefits, particularly to the grazing Livestock Industry. Abandoning research to invent a production vaccine altogether, may conceivably disadvantage the industry, if such a product were to be developed by overseas competitors.

CONCLUSIONS AND RECOMMENDATIONS

Vaccine Potential

The experiment was designed to determine the potential of the vaccine to enhance production in beef cattle. The results are disappointing, but clear-cut. Despite eliciting an antibody response, the vaccine caused neither the change in growth, or plasma potassium concentration, seen with the ß-agonist drug clenbuterol. If there had been a modest response in any of the variables measured, we might argue that a commercial product is feasible, through modification of the peptide to enhance its immunogenicity and/or physiological efficacy. However, this can not be argued from the present results.

It should be noted that the present data are not consistent with those from our earlier experiments, in which we found the antibodies to be potent ß-agonists *in vitro*. This points to a lack of knowledge of an important part of the system we are trying to manipulate. Although we could speculate about possible reasons for the vaccine failure, we have no certain knowledge of this, and so it is difficult, if not impossible, to evaluate this type of technology fully. Nevertheless, it is reasonable to conclude that we can see no commercial potential for this invention in its present form.

Based on the high costs of the vaccine itself, together with the expense of conducting cattle experiments, it is recommended that if work were to proceed, then these studies should be conducted on a lesser-scale in animal models. Sheep would be an ideal species for such work, but the achievement of an anabolic response *in vivo*, even in a species such as the rat, following either active or passive immunization, would be a considerable advance from the current position.

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PART IV

ADMINISTRATIVE DETAILS REPORT

CS242: NOVEL VACCINATION APPROACHES TO IMPROVE THE GROWTH RATE OF CATTLE

EQUITY POSITION

	94/95	95/96	TOTAL	PERCENT
CSIRO	227051	263875	490926	64.86%
MRC	130517	135432	265949	35.14%
TOTAL	357568	399307	756875	100.00%

Ratio of Investment per year			
CSIRO	63.50%	66.08%	64.86%
MRC	36.50%	33.92%	35.14%
	100.00%	100.00%	100.00%

Intellectual Property Arising

To date, all decisions regarding the protection of this invention have been made in consultation with its owners, who are the MRC and the three core partners involved in the TBC joint venture. A provisional patent application was lodged in 1994, and was rolled over in 1995. Costs for maintaining patent protection in 1996 and thereafter would be considerable, and impossible to justify based on the present results. Therefore, it is recommended that the current provisional patent be allowed to lapse.

If further studies in this area were undertaken, and resulted in an effective vaccine, then there would need to be an additional 'inventive step', which at this stage is 'not obvious' , and therefore, should be patentable. In view of this, allowing the present results to enter the public domain should not disadvantage the owners of this intellectual property.

Commercial Exploitation of the Project - Report on Progress

In view of the present results, it is not considered appropriate to develop a commercialisation plan.