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THE ANTIBODY RESPONSE OF CATTLE TO *CLOSTRIDIUM BOTULINUM* TYPES C AND D TOXOIDS

B. C. JANSEN, P. C. KNOETZE and F. VISSER, Veterinary Research Institute, Onderstepoort

ABSTRACT

JANSEN, B. C., KNOETZE, P. C. & VISSER, F., 1976. The antibody response of cattle to *Clostridium botulinum* types C and D toxoids. *Onderstepoort Journal of Veterinary Research* 43 (4), 165–174 (1976).

The resistance of cattle with varying serum-antitoxin titres was determined by *per os* challenge. The results proved that a solid immunity can be produced against *C. botulinum* toxins C_1 and D.

The immune response of cattle to various quantities of *C. botulinum* C_1 and D toxoids, aluminium-phosphate-adsorbed and in water-in-oil emulsion was investigated. The response to antigen in water-in-oil emulsion was far superior to the other when they were used for primary and secondary stimuli.

When cattle had been given a solid basic immunity with 2 injections of antigen in water-in-oil emulsion, essentially the same booster effect was obtained with antigen in water-in-oil emulsion and in aqueous solution.

Only some of the animals injected intramuscularly with antigens in water-in-oil emulsion developed local lesions. These lesions were not large and their histological picture indicated a noticeable decline in severity within 20 weeks.

A case is thus made out for the use of *C. botulinum* C_1 and D toxoids in water-in-oil emulsion for the primary and secondary stimuli and an aqueous solution of these antigens for any booster stimulus as an improved method of protecting cattle against botulism.

Résumé

LA RÉPONSE IMMUNITAIRE EN ANTICORPS AUX TOXOÏDES DE TYPES C ET D DE CLOSTRIDIUM BOTULINUM *CHEZ LE BOVIN*

Les auteurs ont déterminé la résistance de bovins par rapport à leurs titres de sérum-antitoxine. Il leur était évident que les toxines C_1 et D de C. botulinum *administrées* per os *déterminent une forte immunité contre ceux-ci.*

La réponse immunitaire chez le bovin aux toxoïdes C_1 et D de C. botulinum, *adsorbées au phosphate d'alumine et dans une émulsion aqua-huileuse a étée recherchée. Utilisés comme stimulus primaires et secondaires, la réponse suscitée par l'émulsion aqua-huileuse a été supérieure à celle de l'autre.*

Chez bovins avec une forte immunité de base acquise à la suite de 2 injections avec l'antigène dans une émulsion aqua-huileuse, on a pu obtenir à peu près le même effet de rappel avec l'antigène dans une émulsion aqua-huileuse qu'avec l'antigène dans une solution aqueuse.

On n'a pu constater que chez certains animaux des lésions dues aux injections intra-musculaires avec l'antigène dans une émulsion aqua-huileuse. D'une étendue assez petite, l'aspect histologique de ces lésions a révélé une décroissance appréciable de leur sévérité au cours de 20 semaines.

L'utilisation des toxoïdes C_1 et D de C. botulinum *dans une émulsion aqua-huileuse destinées à l'appel primaire et secondaire et dans une solution aqueuse pour le rappel, est donc justifiée comme une meilleure méthode de protéger le bovin contre le botulisme.*

INTRODUCTION

An important relation exists between the antibody response of cattle to *Clostridium botulinum* toxoids and the protection provided against botulism. Thus far the vaccination of cattle against botulism has been done rather empirically, but recent research has made more precise investigations possible. Jansen (1971a) determined the different toxins produced by *C. botulinum* types C and D, and showed that the flocculation test can be used for determining them quantitatively (Jansen, 1971b).

Botulism in South Africa is caused by *C. botulinum* types C and D, and these two organisms produce C_1 and D respectively as their main toxins. In addition to the main toxin, type C_α also produces some D and a little C_2, while type D produces some C_1. Since there is adequate evidence that cattle protected against C_1 and D toxins are resistant to botulism, the current study is limited to an investigation of the antibody response to these two toxins.

The vaccine, issued for general use by the Veterinary Research Institute, Onderstepoort since 1950, consists of an aluminium-phosphate-adsorbed toxoid prepared according to the method described by Sterne & Wentzel (1950) from *C. botulinum* types C and D. This vaccine contains 1–2,5 flocculation units (Lf) of C_1 toxoid and 10–20 Lf of D toxoid, depending on the particular batch. No. C_2 toxoid could be detected in the type C culture filtrate used for vaccine preparation after toxoiding, and none of the experimental animals injected with this vaccine showed an antibody response to C_2. The Onderstepoort vaccine has given very satisfactory results during large-scale field use in South-Africa. Furthermore Tammemagi & Grant (1967) reported that during 1962 and 1963 the same bivalent vaccine was used in Australia on a large scale under field conditions with satisfactory results.

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MATERIALS AND METHODS

Antitoxin

According to Jansen (1971a), the International Standard* Type C antitoxin contains antibodies against toxins C_1, C_2 and D, and the International Standard* Type D antitoxin contains antibodies against toxins C_1 and D. They are consequently not suitable as laboratory standards for the exact determination of individual toxins. Separate laboratory standard antitoxins for the quantitative determination of toxins C_1, C_2 and D were prepared, therefore, for use in the flocculation test according to the method described by Jansen (1971b). The Lf was fixed at the equivalent of 1 000 LD_{50} for each of the 2 toxins. On this basis, the C_1 antitoxin contained 3 200 Lf/ml and the D antitoxin 50 000 Lf/ml in addition to concentrations of antibodies against the remaining toxins which were too low to interfere with the test.

* Microbiological Research Establishment, Porton, Nr. Salisbury, Wilts., England

The antitoxin titre of the serum of immunized animals was determined by serum-neutralization tests in white mice having an average mass of 25 g. A test dose of 100 LD_{50} of C_1 and D toxins was used and the toxins not concerned in the particular test were neutralized with monovalent sera (Jansen, 1971b). The serum was diluted serially, while the quantity of toxin was kept constant and the mixture maintained at room temperature for 1 h. Injections were done intravenously and deaths were recorded over 72 h.

The unit for each laboratory standard antitoxin was taken as equivalent to the Lf of the corresponding toxin.

Toxin production

Toxin was produced from *C. botulinum* types C_α and D by the method of Sterne & Wentzel (1950).

Adjuvants

Two types of adjuvant were used:

(1) A water-in-oil emulsion prepared by modifying the formula developed by Thomson, Batty, Thomson, Kerry, Epps & Foster, (1969) to the following:

Ondina Oil*.....50 ml
Lubrol Moa**.....8 ml
Lissapol NX**.....2 ml
Aqueous solution of toxoid.....40 ml

The Ondina oil was mixed with the lipophilic emulsifier, Lubrol Moa, and the aqueous toxoid with the hydrophilic emulsifier, Lissapol NX. Then the aqueous phase was slowly added to the oil phase with agitation.

(2) An aluminium phosphate suspension was prepared, using the following 2 solutions:

(a) $Na_3PO_4.12H_2O$.....26 g
Aqua dest.....190 ml
(b) $AlCl_3.6H_2O$.....16 g
Aqua dest.....190 ml

The 2 solutions were sterilized separately by autoclaving, then mixed, and the pH adjusted to 5,4 with sterile precautions. The toxoid was added, the pH checked and the mixture left at 4 °C for 2 weeks to allow adsorption to take place.

This adjuvant is used regularly by the Onderstepoort Veterinary Research Institute for its production of botulinus vaccine.

Experimental animals

Nine-month-old cross-bred cattle, none of which had been vaccinated against botulism prior to the experiments, were subdivided into groups of 5 for the different treatments and used while grazing on open range. Only a few were lost through intercurrent disease during the long-term experiments.

Results

The resistance of cattle to toxin dosed per os

To determine the resistance of cattle to the oral intake of toxin in relation to their serum antibody content, it was first necessary to establish the MLD for toxins C_1 and D in cattle.

* Shell Chemical Co.
** Imperial Chemical Industries Ltd

The *C. botulinum* type C_α culture filtrate used for the purpose contained about 10^5 mouse intravenous LD_{50}/ml after its D toxin content had been neutralized. Three young, fully-susceptible head of cattle were dosed with 0,1; 0,3 and 0,5 ml, respectively, *per os*. The first 2 suffered no ill effects, but the 1 which had received 0,5 ml died 3 days after. Owing to the cost of experimental animals the doses could not be worked out any finer, and 0,5 ml of this toxin was regarded as 1 MLD for a 245 kg ox, the mass of the 3rd animal. This toxic filtrate was stored at 4 °C and neutral pH.

By the same procedure, 0,05 ml of a culture filtrate of *C. botulinum* type D containing about 10^6 mouse LD_{50} per ml was determined as 1 MLD for a 247 kg ox.

Subsequently, cattle which had been vaccinated previously were exposed to varying doses of toxin after their serum antitoxin levels had been determined. The results are recorded in Table 1.

TABLE 1 The resistance of cattle with varying serum antitoxin titres to different oral doses of toxin

Toxin D

Antitoxin units/ml	Dose of toxin Cattle MLD	Result
100	10	L
50	1 000	L
33	100	L
20	500	L
0,02	500	L
<0,02	500	L

Toxin C_1

Antitoxin units/ml	Dose of toxin Cattle MLD	Result
2,0	1 000	L
2,0	50	L
0,13	10	L
0,05	200	L
0,02	100	Showed symptoms Recovered
<0,02	50	Died

L=survived.

From these results it is clear that a solid immunity against the toxins of *C. botulinum* types C and D can be produced by means of vaccination.

The immunization of cattle with aluminium-phosphate-adsorbed toxoid

The botulinus vaccine, originally developed by Sterne & Wentzel (1950) and successfully applied in the field for many years, varied in its C_1 content from 1–2,5 Lf and its D content from 10–20 Lf/2 ml dose of vaccine. Consequently, the following 3 levels of antigen were chosen to begin with for the experimental investigation of the response of cattle to aluminium-phosphate-adsorbed antigen:

(a) C_1 1 Lf
D 15 Lf
(b) C_1 5 Lf
D 60 Lf
(c) C_1 10 Lf
D 100 Lf

The dose was 2 ml injected subcutaneously.

TABLE 2 The serum-antibody levels of cattle in units/ml in response to aluminium-phosphate-adsorbed toxoid

Antigen level in Lf	Animal No.	Bleeding time in weeks													
		After 1st injection		After 2nd injection											
		6		2		4		6		8		10		12	
		C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D
C_1=1.....	1	1,0	1,25	2,0	20,0	1,4	11,0	0,5	5,0	0,17	5,0	0	5,0	0	1,67
	2	0	0	1,0	16,67	0,1	10,0	0,1	5,0	0,1	1,67	0	1,1	0	0,1
D=15.....	3	0	0	5,0	10,0	1,4	3,3	0,5	1,67	0,17	1,0	0	0,17	0	0,13
	4	0	0	0,17	20,0	0,1	10,0	0,1	2,5	0,1	2,0	0	2,0	0	1,25
C_1=5.....	1	0	0	1,0	10,0	0,25	2,0	0,1	2,0	0,1	1,0	0	0,2	0	0,1
	2	0	0	2,5	10,0	1,4	3,3	0,5	2,5	0,5	2,0	0	1,25	0	0,5
D=60.....	3	0	0	0,13	10,0	0,1	5,0	0,1	2,5	0,1	1,1	0	0,25	0	0,17
	4	0	0	1,0	10,0	0,25	2,0	0,13	2,0	0,1	1,0	0	0,25	0	0,17
C=10.....	1	0	0	1,0	2,5	0,17	2,0	0,13	1,67	0,1	1,0	0	0,25	0	0,1
	2	0	0	2,0	5,0	1,67	2,5	0,17	1,1	0,17	1,0	0	0,5	0	0,25
D=100.....	3	0	0	2,5	10,0	1,1	2,5	0,1	1,67	0,1	1,0	0	0,1	0	0,1
	4	0	0	5,0	25,0	1,67	33,0	1,1	14,3	1,0	10,0	0	5,0	0	2,0

Each level of antigen was injected into a group of 5 cattle but, unfortunately, 1 animal was lost adventitiously in each group. Six weeks after the 1st injection, their serum antitoxin titres were determined and a 2nd injection was given in accordance with Henning's (1956) recommendation that the optimum interval between the primary and secondary antigenic stimuli should be 6 weeks. At regular intervals after the 2nd injection, the animals were bled, their serum antitoxin titres against toxins C_1 and D determined, and the results recorded in Table 2. Sampling was stopped as soon as the antitoxin titre for 1 of the toxins in 1 or more animals of a group fell to an undetectable level in order not to upset the calculation of the mean log titre for the group.

From these results it is evident that the response to 1 injection of aluminium-phosphate-adsorbed toxoid is irregular and mostly undetectable. The response to the 2nd injection was favourable, but even with the highest dose of toxoid the C_1 antitoxin value of the sera declined to an undetectable level by the 10th week.

The immunization of cattle with antigen in water-in-oil emulsion

Three different levels of antigen in water-in-oil emulsion were prepared so that the dose to be injected was contained in 2 ml of the final mixture.

The levels were:

(a) C_1 1 Lf
D 15 Lf

(b) C_1 5 Lf
D 60 Lf

(c) C_1 10 Lf
D 100 Lf

Each level of antigen was injected subcutaneously in the dewlap in a different group of cattle. At 4 and 6 weeks after the 1st injection, their serum-antitoxin titres were determined and a 2nd identical injection was given 6 weeks after the first. At regular intervals after the 2nd injection the serum-antitoxin titres against toxins C_1 and D were determined and recorded in Table 3.

In the group which had received the lowest level of toxoid, the C_1 antitoxin titre in 1 animal declined to an undetectable level within 18 weeks; in the 2nd group, the same took place in 1 animal in 52 weeks, and in the 3rd group all animals retained a detectable C_1 antitoxin titre for more than a year. The D antitoxin titre remained appreciable in all groups for more than a year. A direct comparison of the results obtained with the lowest dose group with those obtained with aluminium-phosphate-adsorbed antigen (Table 2) convinces one of the better quality of the water-in-oil emulsion toxoid. This is borne out by a comparison of the results contained in Tables 2 and 3. In Fig. 1 and 2 the mean log titre for each group recorded in these tables is plotted separately against the sampling times for the C_1 and the D titres. The statement by Thomson *et al.* (1969) that antigens included in water-in-oil emulsion produce a longer-lasting immunity than the same antigens adsorbed on conventional adjuvants is confirmed for C_1 and D toxoids.

The rate of decline of the mean log titre for any particular dose level is less for the water-in-oil emulsion than for the aluminium-phosphate-adsorbed toxoid.

In order to develop a water-in-oil emulsion botulinus vaccine for routine field use, it became necessary to find a dose level for C_1 and D toxoids which is consistent with reasonable demands on vaccine production and yet provides protection over a sufficiently long period. In respect of C_1 toxoid it was decided to investigate the immune response to 5 Lf and 10 Lf because, according to Fig. 1, 1 Lf in water-in-oil emulsion is better than 10 Lf adsorbed on aluminium phosphate, while Sterne & Wentzel's vaccine, as issued by the Onderstepoort Veterinary Research Institute, has a C_1 content of 1–2,5 Lf. Furthermore, the inclusion of 10 Lf C_1 toxoid seemed feasible from a production point of view. In respect of D toxoid it was decided to investigate the response to 5 Lf and 10 Lf because, according to Fig. 2, the response to 15 Lf in water-in-oil emulsion is better than 100 Lf on aluminium phosphate adjuvant, and Sterne & Wentzel's vaccine contained only 10–20 Lf of D toxoid.

TABLE 3 The serum-antibody levels of cattle in units/ml in response to antigen in water-in-oil emulsion

Antigen level in Lf	Animal No.	Bleeding time in weeks																	
		After 1st injection				After 2nd injection													
		4		6		2		6		10		18		26		36		52	
		C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D
C_1=1.....	1	0	1,0	0	1,0	11,0	100,0	5,0	25,0	2,5	14,3	1,0	5,0	0,1	5,0	0	5,0	0	1,7
	2	0	1,0	0	10,0	25,0	125,0	16,7	25,0	11,0	16,7	1,0	11,0	1,0	10,0	0,5	10,0	0,17	2,5
D=15.....	3	1,0	1,0	10,0	50,0	14,0	100,0	5,0	20,0	2,5	14,3	1,0	14,3	1,0	11,0	0,25	10,0	0,17	5,0
	4	0	0,1	0	10,0	11,0	100,0	2,5	20,0	1,25	12,5	0,25	10,0	0,25	10,0	0,17	5,0	0,13	1,7
	5	0	1,0	0	10,0	12,5	100,0	5,0	25,0	1,25	16,7	0	3,3	0	2,5	0	2,5	0	1,0
C_1=5.....	1	1,0	10,0	10,0	10,0	20,0	125,0	10,0	33,0	2,5	33,0	1,7	14,3	0,5	5,0	0,25	5,0	0,17	1,0
	2	1,0	10,0	10,0	50,0	25,0	125,0	16,7	25,0	10,0	20,0	1,25	10,0	0,17	5,0	0,1	5,0	0	2,5
D=60.....	3	0	1,0	0,1	10,0	33,0	100,0	14,3	25,0	10,0	16,7	2,5	10,0	1,0	10,0	1,0	10,0	0,5	5,0
	4	0	1,0	1,0	10,0	25,0	100,0	14,3	25,0	10,0	20,0	1,25	10,0	1,0	10,0	1,0	10,0	0,25	5,0
	5	0	0,1	0,1	12,5	20,0	125,0	10,0	50,0	5,0	50,0	3,3	20,0	1,0	11,0	0,5	10,0	0,25	1,0
C_1=10.....	1	0	1,0	0,1	10,0	100,0	1 000,0	25,0	125,0	12,5	125,0	1,7	16,7	0,5	10,0	0,5	10,0	0,25	5,0
	2	10,0	10,0	10,0	10,0	11,0	250,0	10,0	125,0	10,0	125,0	10,0	33,0	0,25	16,7	0,1	12,5	0,1	3,3
D=100.....	3	0	1,0	0,1	10,0	50,0	1 000,0	20,0	125,0	12,5	100,0	1,7	100,0	1,0	50,0	1,0	16,7	0,5	10,0
	4	0	1,0	10,0	10,0	33,0	125,0	14,3	33,0	11,0	25,0	1,25	16,7	1,0	10,0	0,5	10,0	0,5	10,0
	5	1,0	10,0	10,0	50,0	11,0	50,0	5,0	20,0	2,5	10,0	10,0	10,0	1,0	10,0	1,0	10,0	0,5	5,0

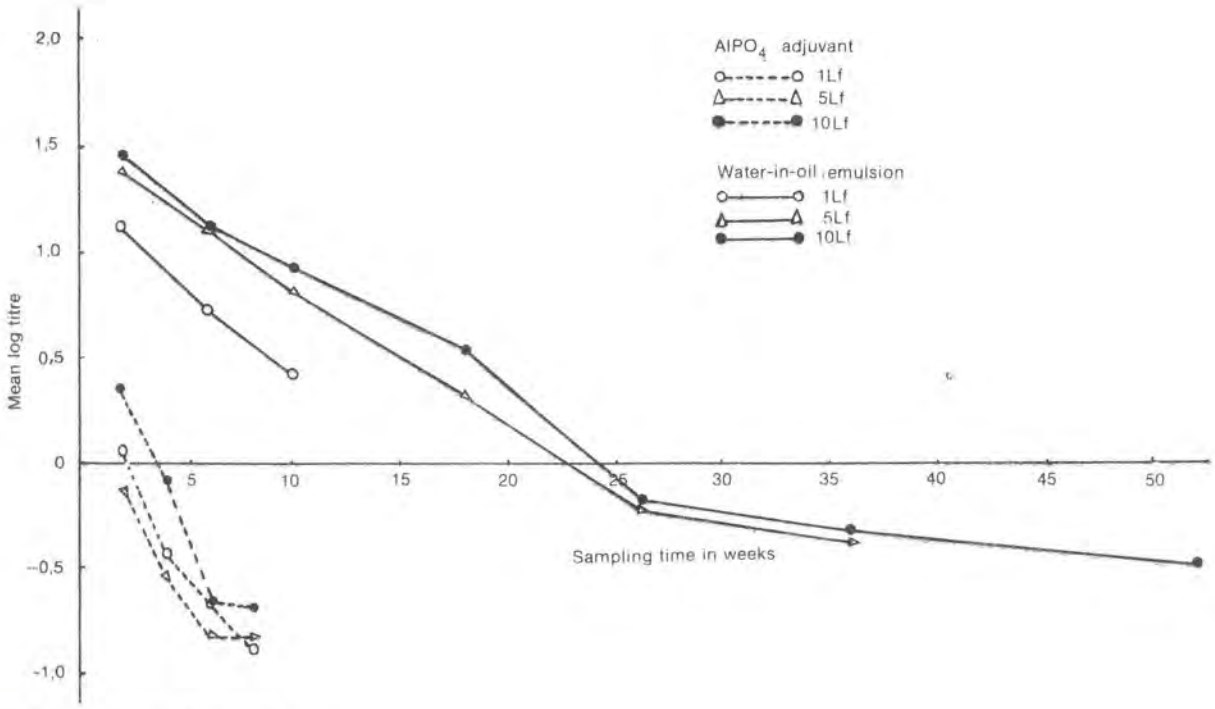

FIG. 1 Response to C_1 toxoid

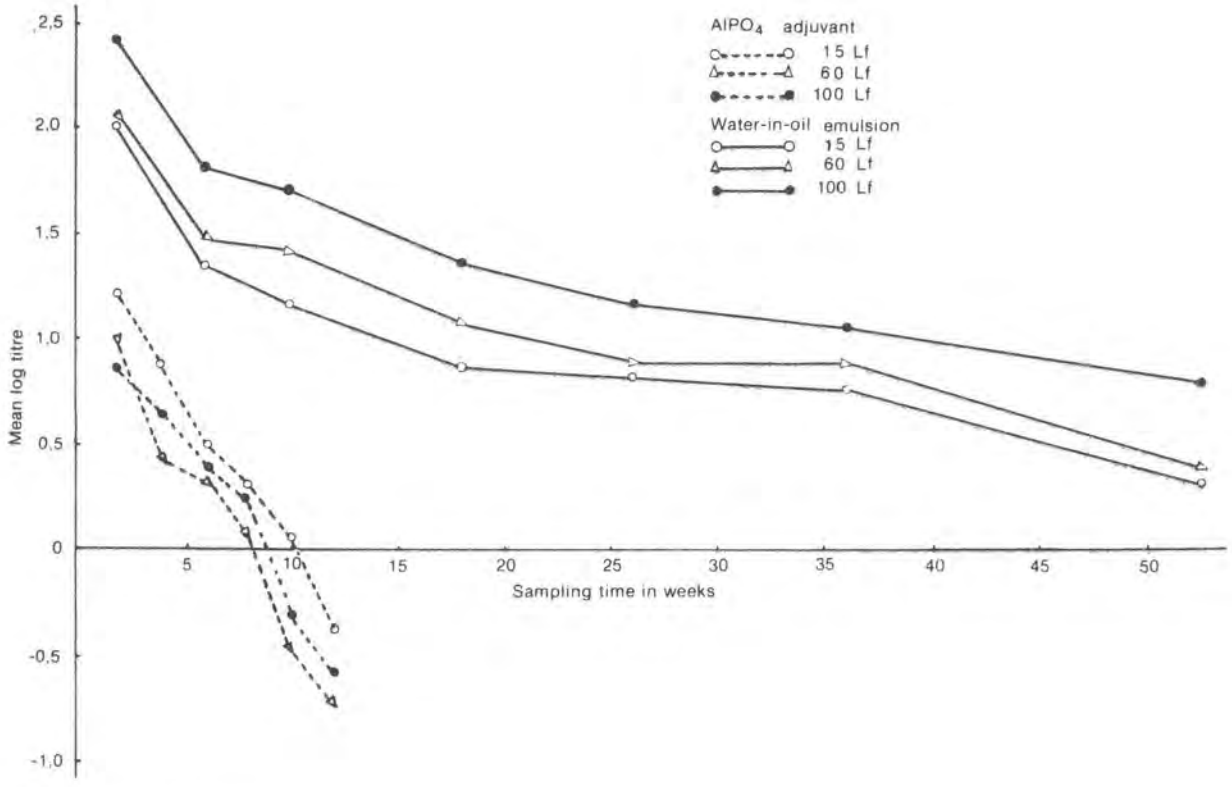

FIG. 2 Response to D toxoid

TABLE 4 The serum-antibody levels of cattle in units/ml in response to water-in-oil emulsion vaccine at two different dose levels

Antigen level in Lf	Animal No.	Bleeding time in weeks after second injection																											
		2		4		6		10		14		18		22		26		30		34		38		42		46		50	
		C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D
	1	33,0	20,0	20,0	14,0	14,0	10,0	1,4	3,3	1,0	3,3	1,0	1,4	1,0	0,14	1,0	0,1	0,5	0,1	0,5	0,1	0,5	0,1	0,5	0,1	0,5	0,1	0,5	0,1
$C_1=5$.....	2	10,0	3,3	5,0	2,0	4,0	1,4	2,0	0,4	2,0	0,4	1,4	0,1	0,4	0,04	0,33	0,03	0,33	0,02	0,33		0,33		0,33		0,14		0,1	
$D=5$.....	3	33,0	20,0	14,0	14,0	14,0	14,0	4,0	10,0	4,0	10,0	2,0	5,0	1,4	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	0,5
$C_2=1$.....	4	14,0	4,0	10,0	4,0	5,0	3,3	1,4	2,0	1,4	2,0	0,33	1,0	0,33	0,1	0,14	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,04
	5	10,0	4,0	5,0	3,3	4,0	1,4	1,4	0,2	1,4	0,2	0,33	0,1	0,33	0,1	0,14	0,04	0,02	0,02										
	1	50,0	20,0	40,0	20,0	20,0	14,0	10,0	10,0	10,0	10,0	10,0	4,0	5,0	1,0	5,0	1,0	5,0	1,0	3,3	0,4	2,0	0,4	2,0	0,4	2,0	0,4	1,0	0,1
$C_1=10$.....	2	33,0	33,0	20,0	20,0	10,0	20,0	3,3	4,0	1,0	3,3	1,0	2,0	1,0	1,0	1,0	0,33	0,5	0,33	0,5	0,33	0,5	0,33	0,5	0,33	0,33	0,2	0,33	0,2
$D=10$.....	3	33,0	14,0	20,0	10,0	14,0	5,0	10,0	4,0	5,0	4,0	4,0	1,4	2,0	0,14	3,3	0,05	2,0	0,05	1,0	0,05	1,0	0,05	1,0	0,05	1,0	0,05	0,5	0,04
$C_2=1$.....	4	33,0	14,0	14,0	5,0	10,0	3,3	1,4	1,4	1,4	1,4	1,0	0,33	1,0	0,2	0,33	0,1	0,14	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
	5	10,0	3,3	10,0	3,3	5,0	3,3	2,0	1,4	2,0	1,0	1,0	0,14	0,2	0,14	0,2	0,04	0,2	0,04	0,2	0,04	0,14	0,04	0,14	0,04	0,14	0,03	0,14	0,03

TABLE 5 The response of cattle to a booster dose of antigen in water-in-oil emulsion

Group	Animal No.	Serum-antitoxin value in units/ml given in columns under weeks after booster injection																							
		1		2		3		7		10		15		20		26		32		38		44		50	
		C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D
1	1	10,0	33,0	33,0	100,0	20,0	100,0	10,0	33,0	10,0	33,0	5,0	14,0	5,0	14,0	5,0	14,0	3,3	14,0	3,3	14,0	3,3	14,0	3,3	10,0
	2	14,0	10,0	14,0	20,0	14,0	20,0	5,0	5,0	2,0	3,3	2,0	1,4	2,0	1,4	2,0	1,4	2,0	1,4	2,0	1,4	2,0	1,4	1,4	1,0
	3	20,0	20,0	20,0	50,0	20,0	50,0	10,0	50,0	10,0	40,0	10,0	20,0	10,0	20,0	10,0	20,0	10,0	20,0	10,0	20,0	10,0	20,0	5,0	14,0
	4	4,0	14,0	10,0	14,0	3,3	20,0	1,4	10,0	1,0	3,3	0,33	1,4	0,33	1,4	0,33	1,0	0,33	0,33	0,33	0,33	0,33	0,33	0,33	0,33
	5	4,0	4,0	5,0	3,3	10,0	10,0	5,0	3,0	0,4	1,0	0,33	0,2	0,33	0,14	0,14	0,1	0,14	0,1	0,14	0,1	0,14	0,1	0,1	0,1
2	1	10,0	10,0	10,0	14,0	10,0	14,0	5,0	10,0	5,0	3,3	5,0	3,3	5,0	3,3	5,0	3,3	5,0	3,3	5,0	3,3	5,0	3,3	5,0	3,3
	2	3,3	10,0	3,3	10,0	2,0	14,0	1,4	5,0	1,0	3,3	1,0	3,3	1,0	3,3	1,0	3,3	1,0	3,3	1,0	3,3	1,0	3,3	1,0	3,3
	3	14,0	10,0	20,0	10,0	14,0	3,3	5,0	3,3	5,0	3,3	4,0	2,0	4,0	2,0	4,0	2,0	4,0	2,0	4,0	2,0	4,0	2,0	4,0	2,0
	4	2,0	10,0	2,0	10,0	2,0	10,0	1,0	5,0	0,33	1,4	0,33	0,4	0,33	0,4	0,33	0,4	0,33	0,33	0,33	0,33	0,33	0,33	0,33	0,33
	5	10,0	10,0	10,0	14,0	5,0	14,0	3,3	5,0	2,0	2,0	1,4	1,4	1,4	1,0	1,4	1,0	1,4	0,4	1,4	0,4	1,4	0,4	1,4	0,4

TABLE 6 The response of cattle to a booster dose of aluminium-phosphate-adsorbed antigen

Group	Animal No.	Serum-antitoxin value in units/ml given in columns under weeks after booster injection											
		2		4		6		8		12		16	
		C_1	D	C_1	D	C_1	D	C_1	D	C_1	D	C_1	D
3	1	3,3	10,0	2,0	4,0	1,4	2,0	1,0	1,4	0,14	0,14	0,03	0,1
	2	0,2	4,0	0,14	3,3	0,1	2,0	0,07	1,0	0,02	0,1	—	0,02
	3	3,3	4,0	2,0	2,0	1,0	0,5	0,2	0,1	0,1	0,02	0,02	—
	4	4,0	4,0	2,0	2,0	1,4	1,0	1,4	0,2	0,1	0,03	0,07	0,03
	5	3,3	10,9	1,4	4,0	0,5	2,0	0,1	1,0	0,03	0,2	0,02	0,1
	6	0,4	4,0	0,2	2,0	0,1	0,4	0,07	0,1	0,02	0,04	—	0,02
	7	10,0	3,3	4,0	2,0	2,0	0,5	1,0	0,14	0,33	0,03	0,2	0,02
	8	4,0	10,0	3,3	3,3	1,0	1,4	0,5	1,0	0,33	0,2	0,14	0,1

Hence the final dosage levels tested in separate groups of cattle were as follows:

Group I: C_1 5 Lf
D 5 Lf

Group II: C_1 10 Lf
D 10 Lf

The vaccine was injected subcutaneously in a volume of 2 ml and the injection repeated 6 weeks later. Sampling was carried out at varying intervals after the 2nd injection and the results recorded in Table 4.

The results show that, 2 weeks after the secondary stimulus, all animals had very high antitoxin titres against C_1 and D toxins. The C_1 and D titres in the 5 Lf group lasted well and only after 34 weeks some of the members of the group lost their titres. At 50 weeks all animals in the 10 Lf group still had detectable antitoxin levels against C_1 and D toxins.

The effect of a booster injection of toxoid

To complete the current study, the effect of a booster injection given 12 months after the primary injection was investigated. For this purpose the cattle used in the previous experiments were available. Because of the irritant effect of water-in-oil emulsion antigens, it was decided to include a group in which the antigen for the booster dose was given in aqueous solution without adjuvant. A group in which the primary, secondary and booster injections consisted of the routine aluminium-phosphate-adsorbed vaccine issued by the Onderstepoort Veterinary Research Institute was included for comparison. Unfortunately, the number of suitable animals was insufficient for further variations which could possibly amplify the results recorded in Tables 5 and 6. The final groups were then as follows:

Group	Primary and Secondary doses	Booster dose
1	C_1 5 Lf Water-in-oil D 5 Lf emulsion	C_1 5 Lf Water-in-oil D 5 Lf emulsion
2	C_1 10 Lf Water-in-oil D 10 Lf Emulsion	C_1 5 Lf Aqueous D 5 Lf solution
3	Aluminium-phosphate-adsorbed vaccine	Aluminium-phosphate-adsorbed vaccine

The cattle which had received 10 Lf of C_1 and D each for the primary and secondary dose were selected for the aqueous booster dose in preference to the 5 Lf group on the assumption that an aqueous booster dose would be inferior to one in water-in-oil emulsion. If this proved to be so, a lower grade basic immunity could not be blamed as a possible reason. The results are given in Tables 5 and 6.

All the animals in Groups 1 and 2 retained appreciable serum antibody titres for at least 50 weeks after the booster dose.

When the results recorded in Tables 5 and 6 are represented graphically by plotting separately the mean log titre against the sampling times for the C_1 and the D toxoids, Fig. 3 and 4 are obtained.

From Fig. 3 it can be seen that the response to a booster injection of C_1 toxoid in aqueous solution was slightly lower than the response to the same antigen in water-in-oil emulsion for about 21 weeks but thereafter continued at a higher level. An analysis of variance revealed no significant difference in the group means at any of the 12 times of sampling. When the primary and secondary injections consisted of aluminium-phosphate-adsorbed toxoid, the reaction to a booster dose of aluminium-phosphate-adsorbed toxoid was much smaller and declined rather rapidly to an undetectable level within 12 weeks.

Fig. 4 shows that for D toxoid, the response to the aqueous antigen was slightly lower than the response to the same antigen in water-in-oil emulsion throughout the whole test period. From an analysis of variance however, no significant statistical difference could be found between these 2 responses. The primary, secondary and booster injections of aluminium-phosphate-adsorbed D toxoid gave results comparable to those obtained with aluminium-phosphate-adsorbed C_1 toxoid.

The local changes brought about by the toxoids in water-in-oil emulsion

It is generally known that the subcutaneous injection of an antigen in water-in-oil emulsion often leads to unsightly granulomatous swellings. Although in our experience the intramuscular injection of such emulsions did not result in any detectable swellings, it was necessary to investigate further its effect on the muscle, particularly in slaughter animals. To this end 2 ml of *C. botulinum* toxoid, suspended in water-in-oil emulsion according to the formula given under Materials and Methods, was used to inject 5 cattle intramuscularly along the side of the neck from which the hair had been clipped. At varying intervals up to 29 weeks an animal was slaughtered and the muscle at the injection site removed and cut into thin layers to examine for traces of any lesion.

Whenever a lesion was found it was examined histologically. The results are recorded in Table 7.

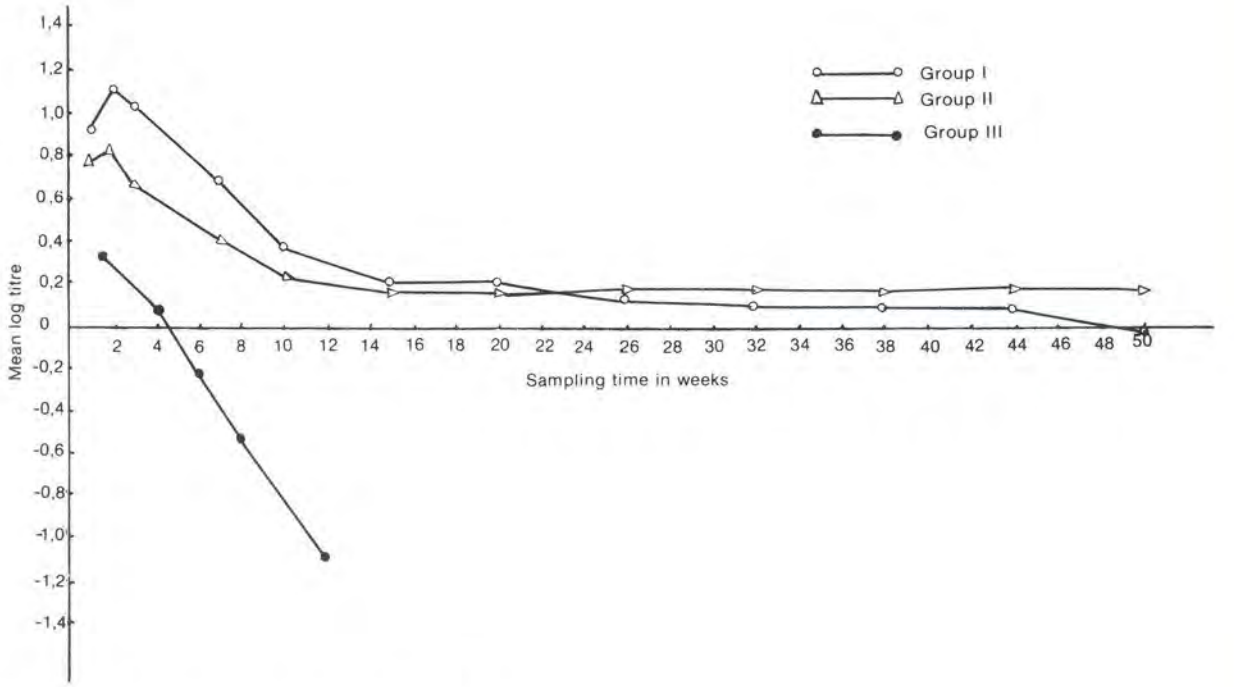

FIG. 3 Response to booster injection of C_1 toxoid

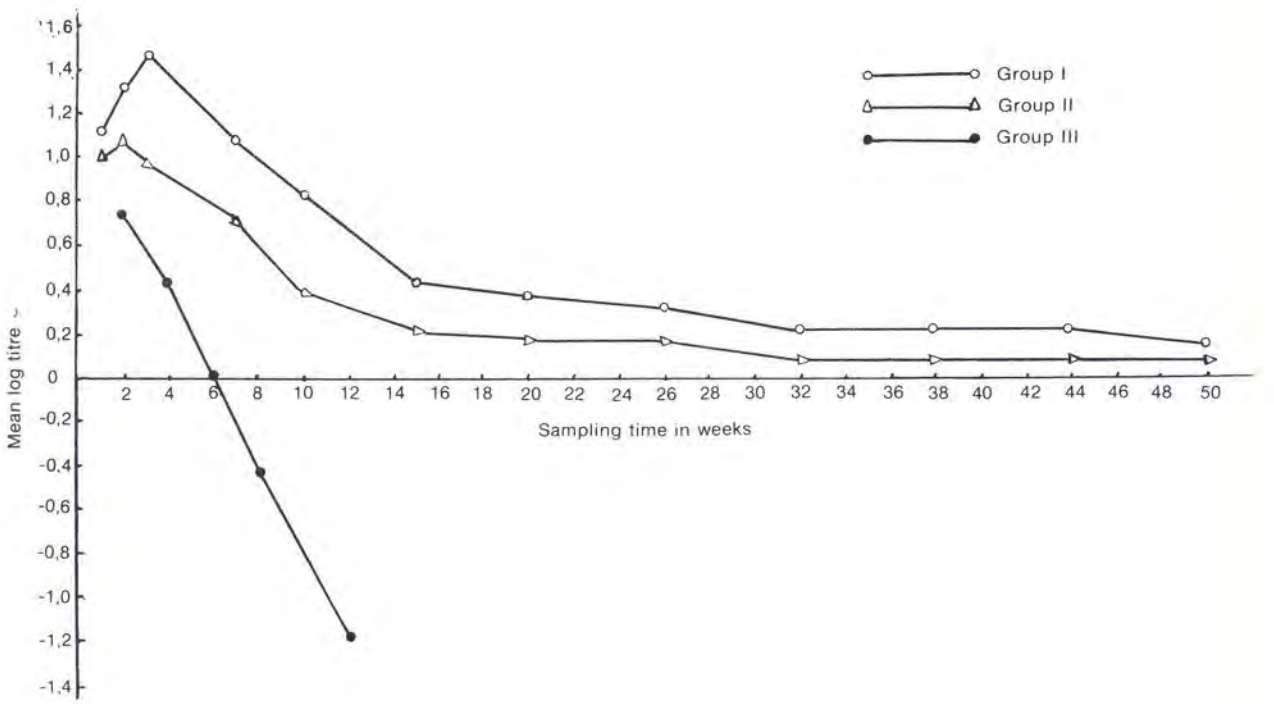

FIG. 4 Response to booster injection of D toxoid

TABLE 7 The local effect of *C. botulinum* toxoid in water-in-oil emulsion injected intramuscularly

Animal No.	Week after injection	Lesion
1	4	Present
2	8	Present
3	16	None
4	20	Present
5	29	None

In animal No. 1 the lesion appeared as a light grey area, 4 mm² in size, which could be described histologically as a focal, chronic, interstitial myositis with a foreign body reaction against lipids. A few giant cells were present and there was only a slight loss of muscle fibres.

The lesion in animal No. 4 consisted of a few grey, branched streaks among seemingly normal muscle bundles over an area of 4 mm². Histologically, this was similar to the 1st lesion except that it appeared more chronic, the collagen fibres were more prominent, and the cell reaction, having decreased, consisted mostly of lymphocytes. A fair quantity of lipid material was still present.

From these results it is clear that only some of the cattle injected intramuscularly with toxoid in water-in-oil emulsion developed detectable lesions. These lesions were not large and their histological picture indicated a progressive decline in severity in the course of time.

Discussion and Conclusions

There is no definite information on the quantity of botulinus toxin ingested by cattle on open range, although, judging by the degree of toxicity attained by carcass debris and the mass eaten by cattle, it could be substantial. In the circumstances the level of immunity required by cattle exposed to toxic material cannot be known and in practice one has to resort to providing them with as high a level of immunity as possible. Table 1 shows that it is possible to protect an animal against a relatively large oral dose of toxin even after its serum antitoxin content has dropped to a low level. Fortunately, the protection afforded by a vaccine in animals with a sound basic immunity is augmented in nature by the booster effect following on a sublethal oral dose of toxin (Jansen, Knoetze & Visser, 1970).

The aluminium-phosphate-adsorbed vaccine prepared by the Onderstepoort Veterinary Research Institute has given good protection to a very large percentage of animals when injected shortly before the period of highest incidence of botulism. In this study, however, it has been proved that *C. botulinum* C_1 and D toxoids in water-in-oil emulsion stimulate a much higher and more persistent level of basic immunity than when adsorbed on aluminium phosphate. Also, according to Fig. 3 and 4, the form in which the booster injection is given is less important when a solid basic immunity exists.

A water-in-oil emulsion vaccine would solve the problem of maintaining a higher degree of immunity throughout the year, and this has the advantage that the injections can be done at a time coinciding with routine farm practices and the cattle will be protected against higher doses of toxin. A distinct disadvantage, however, is the undesirable granulomatous swelling resulting from subcutaneous injection in a large percentage of animals. Even the focus of chronic interstitial myositis caused by intramuscular injection of the emulsion could raise objections in the case of slaughter stock.

The fact that intramuscular lesions are not formed in all animals injected and that the lesions that do form virtually disappear after some months, makes feasible the injecting of calves at the age of 5–6 months, the stage at which they are weaned and become subject to botulism. Two intramuscular injections at an interval of 6 weeks would establish a sound basic immunity and protect them for at least 34 weeks. In cattle kept for breeding or milking, the temporary lesions are of no consequence and, in those destined for slaughter, the extent of the lesions should be negligible by the age of 18 months which is the earliest likely age of slaughter for cattle reared on natural pasture.

An annual booster dose could be given with toxoid in aqueous solution on the strength of the results recorded in Table 5. This would prevent the development of undesirable side-effects.

For studying the effect of a booster dose, the toxoid content of the water-in-oil emulsion and aqueous vaccine was 5 Lf for both the C_1 and D toxoids. All animals showed a titre that may be regarded as protective for at least 50 weeks after the injection. The use of this quantity of toxoid in a vaccine for booster purposes would, therefore, be justified.

When, however, the toxoids were used at the 5 Lf level for the establishment of a basic immunity, the titre in some members of the group fell to below the detectable level after 34 weeks. The use of the vaccine at this strength in a water-in-oil emulsion for the primary and secondary stimuli could be acceptable on the strength of the conclusion, based on a comparison of Tables 2 and 3, that it is a vast improvement on the aluminium-phosphate-adsorbed vaccine. Only if it proves in practice that some animals vaccinated with vaccine at 5 Lf per dose die of botulism within the first year of vaccination, would one have to consider giving a booster dose after 34 weeks. The alternative would be to increase the toxoid content to 10 Lf per dose. This would result in an appreciable serum-antibody titre lasting for at least 50 weeks after a primary and secondary injection.

A system of improved protection of cattle against botulism has thus been evolved by the employment of a water-in-oil emulsion vaccine in combination with an aqueous vaccine.

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