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STUDIES ON THE DEVELOPMENT OF A VACCINE AGAINST BOVINE EPHEMERAL FEVER

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ABSTRACT

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Bovine ephemeral fever virus of low passage level (5th to 8th) in tissue culture has proved to be more antigenic for cattle than higher passage level (17th to 19th) or mouse-brain-adapted virus. However, the neutralizing antibody response appears to be short-lived and therefore probably provides inadequate protection.

Similar results were obtained when ephemeral fever virus of both low and high passage level was adsorbed onto aluminium hydroxide and inoculated into cattle.

Emulsions of ephemeral fever virus of both low and high passage level in Freund's incomplete mineral oil adjuvant induced high neutralizing antibody titres in vaccinated cattle. These antibodies were detectable for at least a year and the animals were found to be immune when infected with virulent bovine ephemeral fever virus.

INTRODUCTION

Ephemeral fever (EF) was described by Edmonds in 1906 (cited by Bevan, 1907) when it was first recognized in north-western Rhodesia. The following year the disease reappeared and spread rapidly over a wide area (Bevan, 1907). EF was subsequently studied in southern Africa (Theiler, 1907; Freer, 1910), Japan (Inaba, 1968), India (Meadows, 1919), Palestine (Rosen, 1931) and Australia (Mulhearn, 1937); Mackerras, Mackerras & Burnet, 1940).

Although a viral aetiology was suspected, the cause of the disease remained unknown until Van der Westhuizen (1967) succeeded in isolating and maintaining EF virus by intracerebral inoculation of suckling mice. He also adapted the virus to growth in cell culture (BHK21, clone 13) and found that passage in either tissue culture or mouse brain resulted in rapid loss of pathogenicity for cattle. Some of these virus strains were used in vaccine trials but the results indicated that the attenuated viruses had largely lost their antigenicity (Van der Westhuizen, 1967; Inaba, Tanaka, Sato, Ito, Omori & Matumoto, 1969). Consequently these studies were extended in an attempt to find a method whereby the development of immunity could be induced.

MATERIALS AND METHODS

Virus strains

(a) *Virus EF1:* The prototype EF1 virus was originally isolated from the blood of a naturally infected ox by intracerebral inoculation of infant mice (Van der Westhuizen, 1967) and stored as a 10% suspension of infective mouse brain in phosphate buffer containing 1,0% peptone and 5,0% lactose (BLP) in sealed ampoules at −70°C. Further passages of this particular virus isolate were conducted in either infant mice or tissue culture.

Serial passage in mice was performed by intracerebral injection of 0,03 ml of a 1:100 dilution of a mouse-brain suspension in BLP into a group of 1 to 3-day-old albino mice. When they showed signs of infection they were killed with ether and their brains harvested aseptically. A 10% suspension of infective brain material in BLP, stored either in sealed ampoules at −70°C or in the lyophilized form at −20°C, served as antigen for further passage.

The mouse-adapted virus strain was also passaged serially in monolayers of BHK21 cells grown in roller tubes with Eagle's medium containing 10% bovine serum which was free from EF antibodies. As soon as the cell cultures were confluent the growth medium was decanted and 1,0 ml of a 1:100 dilution of the virus in Eagle's medium plus 1% foetal calf serum added. The cultures were incubated at 37°C and were examined daily for cytopathic effects (CPE). When at least 75% of the cells showed CPE the cells were scraped from the glass with a long needle attached to a syringe and suspended in the culture fluid. This material was frozen and thawed three times and centrifuged at 600 *g* for 30 min. The supernatant fluid was mixed with an equal volume of BLP and stored either in sealed ampoules at −70°C or in lyophilized form at −20°C. The passage levels at which EF1 virus was used are indicated in the relevant experiments.

(b) *Virus EF13:* This virus was isolated in cell cultures from the leucocytes of an experimentally infected ox. The animal had been injected intravenously with virulent EF virus which had been stored as a 10% suspension of infected leucocytes in BLP at −70°C. On the 2nd day, at the height of the febrile reaction, blood was collected in sodium citrate and the leucocytes were separated according to the method described by Van der Westhuizen (1967). A 10% suspension of leucocytes in Eagle's medium was frozen and thawed three times and seeded in 0,2 ml volumes onto confluent roller tube cultures of BHK21 cells. After adsorption for 1 hour at 37°C the medium was replaced with Eagle's maintenance medium containing 1% foetal calf serum and incubated at 37°C.

Although the cultures were examined daily no CPE were evident. On the 3rd day the cells were suspended in the culture fluid as described above, frozen and thawed three times and seeded into new cultures. Similar material was passaged every 3rd day and CPE was first observed in cultures of the third passage. After five passages in BHK21 cells, further passages were made in cultures of primary hamster kidney cells. When at least 75% of the cells showed CPE, the detached cells and culture fluids were harvested, frozen

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and thawed three times and centrifuged at 600*g* for 30 min.

The supernatant fluid was mixed with an equal volume of BLP and lyophilized. The passage levels at which this virus isolate was used are indicated in the relevant experiments.

(c) *Challenge virus:* Virulent EF virus obtained at the height of the febrile reaction from an animal infected by inoculation of blood from a natural case was stored in sealed ampoules at −70°C in the form of a 10% suspension of leucocytes in BLP. This strain was not related to EF1 and EF13 and regularly reproduced the disease in susceptible cattle.

Virus assay

Mouse-brain-adapted EF1 virus was assayed for infectivity by inoculating randomized families of seven suckling mice intracerebrally with decreasing ten-fold dilutions of virus in BLP, using 0,03 ml per mouse. The mice were observed for 7 days and the virus titre, calculated according to the method of Reed & Muench (1938), expressed as $\log_{10}$ mouse LD_{50}/ml.

Mouse-adapted EF1 virus passaged in tissue culture as well as the virus isolate EF13 were assayed by preparing tenfold dilutions of the virus in Eagle's medium and seeding 1,0 ml of each dilution onto at least two roller tube cultures of BHK21 cells. The cultures were observed for CPE for 7 days and titres calculated as above, expressed as $\log_{10}$ $TCID_{50}$/ml.

Serum-virus neutralization tests

Serum was collected from all experimental cattle prior to and at various intervals after vaccination. Those that were challenged were also bled for serum 4 weeks after administration of the challenge virus.

The neutralization test was carried out in suckling mice using the constant serum-virus dilution procedure. Mouse-brain-adapted EF1 virus of between the sixth and ninth passage level was employed throughout the study. Serial ten-fold dilutions of the virus were prepared in BLP. A fixed volume of each virus dilution was mixed with an equal volume of undiluted serum and incubated at 37°C for 45 minutes. Each serum-virus mixture was injected into a family of seven suckling mice, each mouse receiving 0,03 ml intracerebrally. Deaths of mice were recorded from the 2nd to 7th days. The neutralizing indices are expressed as $\log_{10}$ mouse LD_{50} of virus neutralized.

Adjuvants

The following adjuvants were employed:

(a) Aluminium hydroxide. Vaccine was prepared by mixing equal volumes of the virus suspension and Alhydrogel*.

(b) Freund's incomplete adjuvant was made as follows: A mixture of mineral oil (9 parts) and Arlacel A** (1 part) was thoroughly emulsified with an equal volume of aqueous virus suspension.

Experimental animals

Susceptible cross-bred Afrikaner cattle ranging from 18 months to 3 years in age were used in all the experiments except that in which the effect of the vaccine on milk production was determined. Susceptible adult lactating Friesland cows were used for the latter. To avoid natural infection most experiments were conducted during the winter months. When experiments extended over long periods the animals were stabled under insect-free conditions. Rectal temperatures were recorded daily and the animals were observed for clinical symptoms after vaccination or challenge.

RESULTS

The effect of route of administration on the antibody response to EF1 virus

The results are summarized in Table 1. There was no appreciable antibody response in cattle inoculated either intramuscularly or intravenously with 1,0 ml. of mouse-brain-adapted and attenuated EF1 virus. Although the 5 ml dose of tissue culture-propagated EF1 virus appeared to stimulate low level neutralizing antibodies, the route of administration did not influence the response sufficiently to justify a repetition of the experiment with a larger number of animals in each group. In general, the results showed that the immunogenicity of EF1 virus, administered as a single injection in the aqueous form, was unsatisfactory.

*Danish Sulphuric and Superphosphate Works Ltd.
**Atlas Chemical Industries Inc., U.S.A.

TABLE 1 Antibody response of cattle to mouse brain or tissue culture adapted EF1 virus administered by various routes

Cow No.	Inoculum	Virus titre	Route of inoculation	Dosage	Neutralizing antibody indices	
					Pre-inoculation	4 weeks after inoculation
5136	*Mouse brain EF1 M8	$10^{6,5}$	**i.v.	1 ml	< 0,5	0,5
5147	Mouse brain EF1 M8	$10^{6,5}$	i.v.	1 ml	< 0,5	0,5
5142	Mouse brain EF1 M8	$10^{6,5}$	i.m.	1 ml	< 0,5	1,0
5127	Mouse brain EF1 M8	$10^{6,5}$	i.m.	1 ml	< 0,5	0,6
4962	Tissue culture EF 1 M8 TC 14	$10^{5,5}$	s.c.	5 ml	< 0,5	1,5
4971	Tissue culture EF 1 M8 TC 14	$10^{5,5}$	i.m.	5 ml	< 0,5	1,8
2483	Tissue culture EF1 M8 TC 14	$10^{5,5}$	i.v.	5 ml	< 0,5	2,4

*EF1 virus isolate after 8 intracerebral passages in mice and 14 passages in tissue culture
**i.v. = intravenously; i.m. = intramuscularly; s.c. = subcutaneously

TABLE 2 Antibody response of cattle vaccinated with 2 doses of EF1 or EF13 virus

Cow No.	Inoculum	Neutralizing antibody indices			4 weeks after 2nd inoculation
		Pre-inoculation	4 weeks after 1st inoculation	6 weeks after 1st inoculation	
6026	EF1	<0,5	1,7	<0,5	1,1
6049	,,	<0,5	0,5	<0,5	0,8
5980	,,	0,7	1,5	1,2	2,8
5921	,,	0,7	1,2	0,6	0,8
6063	,,	<0,5	1,5	<0,5	0,8
8118	EF13	<0,5	1,3	<0,5	2,4
8196	,,	<0,5	1,0	<0,5	2,0
8109	,,	<0,5	2,3	<0,5	4,0
8233	,,	<0,5	1,3	<0,5	2,8
8181	,,	<0,5	1,4	<0,5	2,8

The effect of two injections on the antibody response to EF1 and EF13 virus

Five cattle were each inoculated subcutaneously with 1,0 ml of the 18th tissue culture passage level of lyophilized EF1 virus. These injections were repeated 6 weeks later. The titre of the inoculum was $10^{6,0}$ $TCID_{50}$/ml on both occasions.

The eighth tissue culture passage level of lyophilized EF13 virus was similarly used to inoculate 10 cattle subcutaneously. Each 2,0 ml dose, which was repeated, contained $10^{6,0}$ $TCID_{50}$ of virus/ml.

The results presented in Table 2 show that two such injections of an aqueous suspension of EF1 virus, at a fairly high tissue culture passage level, failed to elicit a marked antibody response.

Whereas a single injection of EF13 virus at a relatively low level of passage in culture failed to elicit much response, a second injection 6 weeks later resulted in markedly elevated antibody levels (Table 2).

The antigenicity of live EF virus strains incorporated in aluminium hydroxide

1. *The antibody response to two injections of EF1 virus given at different intervals.* Lyophilized EF1 virus of the 14th tissue culture passage (titre $10^{5,5}$ $TCID_{50}$/ml) was reconstituted in water, mixed with an equal volume of aluminium hydroxide gel and 2,0 ml doses were injected subcutaneously into each of six susceptible cows. At 2, 4 and 6-week intervals two animals were respectively reinoculated with the same dose of antigen-adjuvant mixture.

Sera obtained from the animals were tested for neutralizing antibodies as indicated in Table 3. From the results it is evident that all the animals developed high antibody titres against EF1 virus and that the interval of 2 to 6 weeks that elapsed between the first and second inoculation had no appreciable influence on the eventual titres.

2. *The antibody response and effect on lactation of milk cows to two injections of EF1 virus.* Nine Friesland cows, at various stages of lactation, were vaccinated with lyophilized, mouse-brain-adapted EF1 virus. The inoculum had a titre of $10^{6,5}$ mouse LD_{50}/ml and was mixed with an equal volume of aluminium hydroxide. All the animals received 2,0 ml of the virus-adjuvant mixture subcutaneously and the injection was repeated 14 days later.

The results presented in Table 4 show that the animals developed substantial antibody titres to EF and that there was no deleterious effect on the milk yield. The slight decline was regarded as normal because it did not exceed that of the controls.

3. *The antibody response to two injections of EF 13 virus.* Lyophilized EF13 virus of the sixth serial passage in BHK21 and primary hamster kidney cells (titre $10^{5,0}$ $TCID_{50}$/ml) was used. Each animal was inoculated with 2,0 ml of virus-adjuvant mixture, which represented 0,25 ml of the original virus material suspended in water to 1 ml volume.

Six weeks after vaccination all the animals received a second injection of freshly constituted antigen-adjuvant mixture. The serum was assayed for neutralizing antibodies at weekly intervals for 12 weeks after the first injection.

The neutralizing antibody indices are presented in Table 5. Four weeks after the first inoculation only 10 animals (40%) had developed detectable antibody levels and these gradually declined. During the 1st and 2nd week after the second vaccination there was a marked increase in antibody concentration which was comparable to titres obtained after natural infection (Van der Westhuizen, 1967; Snowdon, 1970). The titres had decreased substantially in most animals by the 6th week following the second vaccination (Table 5).

The antigenicity of EF1 and EF13 virus strains incorporated in Freund's incomplete adjuvant

Lyophilized EF1 virus of the 14th passage in BHK21 cells was emulsified in Freund's incomplete oil adjuvant. The virus titre was $10^{5,0}$ $TCID_{50}$/ml and the 2,0 ml inoculum injected subcutaneously into each of two cows represented 0,25 ml of the original virus material suspended in water to 1 ml volume. After an interval of 2 weeks the animals were given an identical booster dose.

The results listed in Table 6 indicate that both animals retained a substantial antibody titre over a period of 17 months.

Lyophilized EF13 virus of the sixth passage in BHK21 and primary hamster kidney cells was similarly emulsified in Freund's incomplete adjuvant. The virus titre and dose of vaccine used to inject the four cows listed in Table 7 were identical to those mentioned above.

The animals received only one injection subcutaneously and the sera were tested for neutralizing antibodies for a period of 24 weeks.

The results presented in Table 7 show that substantial antibody levels appeared during the 2nd week, reached their highest titres by the 6th week and persisted in high concentration for at least 24 weeks.

TABLE 3 Immune response of cows to vaccination at 2, 4 and 6 weeks intervals with 2 doses of EF1 virus adsorbed onto aluminium hydroxide

Cow No.	Titre of inoculum	Pre-inoculation antibody level	Antibody level before 2nd inoculation	Interval between 1st and 2nd inoculation	Antibody level 4 weeks after 2nd inoculation
5204	$1 \times 10^{5,5}$	<0,5	3,1	2 weeks	3,1
5191	,,	<0,5	4,0	,,	6,0
5167	,,	0,9	5,0	4 weeks	6,0
5280	,,	<0,5	1,9	,,	5,0
5264	,,	<0,5	1,8	6 weeks	6,0
5267	,,	<0,5	2,0	,,	4,0

TABLE 4 Effect of 2 injections of aluminium hydroxide-adsorbed EF1 virus on the antibody response and milk yield of cows

Cow No.	Pre-inoculation antibody level	Post-inoculation antibody level	Average daily milk production over 14 days before vaccination (kg)	Average milk production over 14 days after vaccination (kg)
4649	<0,5	3,8	10,3	9,7
4729	<0,5	3,8	9,6	8,6
2489	0,7	2,9	14,6	12,6
4664	<0,5	5,4	13,5	12,3
2066	<0,5	3,6	10,3	8,0
2358	1,3	4,4	21,2	18,5
2154	1,5	4,0	10,1	9,2
3913	<0,5	4,1	19,2	13,1
2454	0,7	2,3	18,8	16,6
Mean			14,2	12,6
Controls				
2830	n.d.	n.d.	20,6	18,7
4645	,,	,,	18,0	16,4
2980	,,	,,	18,3	16,8
2548	,,	,,	11,1	9,0
4019	,,	,,	8,5	8,3

TABLE 5 Antibody response of cattle to vaccination with 2 doses of EF13 virus adsorbed onto aluminium hydroxide

Animal Numbers	Neutralizing antibody indices											
	Pre-inoculation	Weeks following vaccination											
		1	2	3	4	5	6	7	8	9	10	11	12
8110	<0,5	<0,5	1,2	<0,5	1,2	1,1	1,6	5,5	5,7	4,7	5,5	5,5	5,1
8291	0,6	<0,5	1,2	1,4	0,9	<0,5	0,9	5,5	5,7	4,7	5,5	5,5	3,7
8284	<0,5	1,2	3,9	3,4	+3,7	3,1	3,3	4,2	5,7	3,2	4,0	4,3	3,9
8243	<0,5	<0,5	0,6	0,5	1,0	1,2	1,0	4,1	4,4	3,2	4,1	4,4	3,6
8268	<0,5	1,3	1,0	2,2	+2,8	1,0	0,6	4,1	5,7	1,1	4,2	4,0	3,2
8269	<0,5	<0,5	0,9	<0,5	<1,7	1,7	1,4	5,5	5,7	4,7	5,5	5,5	5,1
8225	<0,5	<0,5	0,5	<0,5	1,1	<0,5	0,5	3,0	4,1	3,1	4,1	4,1	2,6
8236	<0,5	<0,5	2,0	1,3	+2,2	1,7	0,5	3,8	4,2	2,1	3,7	3,8	2,7
8218	<0,5	0,9	1,5	1,6	+1,8	0,5	5,5	4,0	4,7	5,5	5,5	5,5	5,1
8242	<0,5	0,8	1,2	0,7	1,0	1,0	0,8	4,2	4,1	2,4	3,3	4,4	2,5
8172	<0,5	<0,5	1,0	1,2	<0,5	1,2	0,9	3,0	4,2	3,6	4,1	3,7	3,2
8146	<0,5	<0,5	1,8	0,6	1,0	0,7	0,6	4,3	5,7	3,4	3,2	4,0	3,2
8240	<0,5	<0,5	0,6	0,9	+1,9	1,3	0,6	3,4	5,7	3,9	5,5	5,5	3,9
8232	<0,5	<0,5	0,5	1,0	1,5	2,2	1,3	1,8	3,2	2,2	3,2	13,5	2,6
8226	<0,5	<0,5	0,5	<0,5	<0,5	1,1	0,8	3,7	5,7	3,8	3,9	4,1	3,6
8124	1,1	0,6	0,7	1,2	+2,7	1,1	0,5	2,5	4,1	2,8	3,9	4,1	2,8
8207	<0,5	<0,5	1,6	1,3	+1,9	0,9	1,3	3,5	4,6	3,8	4,2	4,1	3,4
8163	<0,5	1,4	2,2	0,6	1,0	<0,5	0,5	3,6	5,7	4,9	4,0	5,5	4,0
8139	<0,5	0,8	1,7	0,8	+2,0	2,2	2,3	5,5	4,5	3,3	5,5	3,9	3,6
8282	1,0	<0,5	1,3	1,5	<0,5	<0,5	0,5	5,5	4,2	4,7	3,9	4,1	3,7
8292	<0,5	<0,5	1,0	0,9	0,8	3,2	2,4	5,5	5,7	4,7	5,5	5,5	5,1
8241	0,7	0,9	0,6	2,0	1,0	1,5	0,8	4,0	4,7	3,3	4,2	4,0	3,1
8294	<0,5	<0,5	1,4	2,2	+2,5	2,4	0,8	2,1	4,5	4,4	5,5	4,2	3,8
8222	<0,5	<0,5	2,1	0,7	1,0	2,2	0,5	2,4	5,7	4,7	4,4	4,4	3,5

TABLE 6 Antibody response of cows to vaccination with 2 doses of EF1 virus incorporated in Freund's incomplete adjuvant

Cow No.	Neutralizing antibody indices				
	Pre-inoculation	Months after 2nd inoculation			
		1	12	17	20
5208	<0,5	4,5	4,1	3,1	5,0
4821	<0,5	3,6	3,5	3,0	n.d.

TABLE 7 Antibody response of cattle to a single injection of EF13 virus incorporated in Freund's incomplete adjuvant

Cow No.	Pre-vaccination	Neutralizing antibody indices as determined at weekly intervals														
		1	2	3	4	5	6	7	8	9	10	11	12	16	20	24
7995	1,0	<0,5	3,6	2,7	2,6	3,2	4,2	<5,1	4,9	<5,1	<5,1	<5,1	<5,0	3,3	<4,0	2,9
7996	1,0	<0,5	4,2	4,7	4,7	<5,5	<5,5	<5,1	5,1	<5,1	<5,1	<5,1	<5,0	4,8	<4,0	<4,0
8014	0,9	1,3	1,8	2,1	2,5	3,9	<5,5	<5,1	4,9	<5,1	<5,1	<5,1	3,9	3,8	2,8	2,5
8024	<0,5	0,6	2,3	3,1	4,7	<5,5	<5,5	<5,1	4,9	<5,1	<5,1	<5,1	<5,0	4,0	<4,0	<4,0

Immunity to challenge

To evaluate vaccine-induced immunity six cattle which were vaccinated with EF13 virus incorporated in aluminium hydroxide, two cattle vaccinated with EF13 virus in Freund's incomplete adjuvant and one animal vaccinated with EF1 virus in Freund's incomplete adjuvant were randomly selected and challenged with virulent EF virus. The neutralizing antibody indices of sera before and after challenge, the time interval between vaccination and challenge and the reaction to challenge are presented in Table 8.

Cows 8014 and 7995 possessed high antibody titres and did not react. Although the cattle of the aluminium hydroxide group had low antibody titres, only one of the six animals developed fever and mild EF symptoms, which included fever, salivation, nasal discharge and swollen lymph nodes but no stiffness.

Cow 4821 had a high antibody titre and did not react to the challenge.

The three controls all developed typical EF manifested by fever, inappetence, salivation, nasal discharge, enlarged lymph nodes and general stiffness.

TABLE 8 The relationship between neutralizing antibody indices and immunity to challenge

Cow No.	Vaccine	Time interval to challenge (weeks)	Antibody level before challenge	Antibody level 4 weeks after challenge	Clinical symptoms following challenge
8014	EF13 + F.A.	24	3,9	4,4	0
7995	,,	,,	5,6	5,4	0
8110	EF13 + A.H.	24	1,9	5,4	*
8218	,,	,,	2,0	1,0	0
8292	,,	,,	5,6	5,4	0
8225	,,	,,	1,1	5,4	0
8236	,,	,,	1,1	5,4	0
8242	,,	,,	1,9	2,0	0
4821	EF1 + F.A.	70	3,5	5,5	0
Controls					
8215	—	—	0,5	5,5	**
6859	—	—	0,5	5,6	**
6101	—	—	0,5	5,6	**

F.A. = Freund's incomplete adjuvant
A.H. = Aluminium hydroxide adjuvant
* = Mild EF symptoms
** = Severe EF symptoms

Discussion and Conclusions

Administration of EF1 virus of the 14th passage level in BHK21 cells by various routes into susceptible cattle failed to induce satisfactory levels of neutralizing antibodies. Two inoculations of EF1 virus of the 18th passage level in BHK21 cells gave similar results. This indicated that EF1 virus was poorly antigenic at these particular passage levels. The situation was very different when the same antigen was incorporated into Freund's incomplete adjuvant. The vaccinated animals developed high antibody titres and retained them for long periods. In an attempt to find a more immunogenic virus a new tissue culture isolate of low passage level was employed. Although the antibody concentration was low after the first injection of the new strain, EF13, the response to the second injection was satisfactory. The fact that the anti-body concentration rapidly declined within a few weeks was disappointing, but nevertheless this experiment proved that low passage virus was more antigenic than higher passage levels.

The experiments conducted with viruses of higher and lower tissue culture passage levels adsorbed onto aluminium hydroxide showed that though the animals developed appreciable antibody titres, these diminished within a short period of time and would probably not protect the animals over the entire summer season. It is conceded, however, that it is difficult to determine the concentration of neutralizing antibodies required to protect an animal to challenge of virulent EF virus.

The most satisfactory results were obtained with virus strain EF13 incorporated in Freund's incomplete adjuvant. A single injection evoked higher antibody titres of longer duration than any of the other preparations. It would seem as if the immunity induced thus should persist long enough to protect animals through a summer season. The prospects for developing an efficient vaccine on this basis are therefore favourable.

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