

# Formalin-Treated Foot-and-Mouth Disease Virus: Comparison of Two Adjuvants in Cattle

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Use of colloidal aluminas as adjuvants in foot-and-mouth disease virus (FMDV) vaccines has provided the only practical vaccine for control of FMDV epizootics in cattle. Experimentally, it is possible to immunize cattle against FMDV without use of adjuvants, but multiple inoculations are required. Thus, the potency of practical FMDV vaccines not only depends on the mass of antigen involved but it also depends on the efficiency of the adjuvant used in its preparation. Unfortunately, certain properties of colloidal alumina present features which can be detrimental to vaccine potency (1). Recent publications describing use of other compounds (2-5) reflects a growing interest in more effective adjuvants for FMDV vaccines.

The extraordinary adjuvant activity of emulsified antigens as described by Freund, *et al.* (6) aroused interest in several research programs (7-12) and led to the present investigations with FMDV antigen.

This report describes limited studies which compared antibody response and immunity in cattle after inoculations with formalin-treated FMDV either adsorbed to colloidal alumina or emulsified in mineral oil for adjuvant effect.

## Materials and Methods

### ANTIGEN PREPARATION:

FMDV, type A, sub-type 119 (FMDV A-119) obtained from the fourth passage in primary outgrowths of bovine kidney

cells, was used as the source of antigen (13). Serum-free Hanks' balanced salt solution was used as tissue culture media for virus production. Harvested fluids were clarified by centrifugation at 1,000 g for 30 minutes at 4°C. in an International PR-2 apparatus using a No. 384 head. Infectivity of the clarified suspension was  $1.3 \times 10^6$  PFU/ml. when assayed with the method described by Bachrach, *et al.* (14).

This preparation was treated with 1:5,000 formalin at 37°F. for 192 hours. Although specific tests for innocuity were not carried out, the combined effect of time, temperature, and formalin concentration is comparable to those ordinarily applied to FMDV vaccines.

The colloidal alumina<sup>1</sup> (gel) preparation was adjusted to pH 7.4 with N/1 NaOH, and its concentration was adjusted to 2.5 per cent as determined by dry weight analysis of Al<sub>2</sub>O<sub>3</sub>. Equal volumes of gel and formalin-treated virus were combined and stored at 4°C. until used.

The oil phase for emulsified antigen was prepared by mixing 1 part emulsifier<sup>2</sup> with 9 parts light mineral oil<sup>3</sup>. This mixture was sterilized in an autoclave. Equal volumes of antigen and oil phase were mixed by recycling between two syringes joined by a double-hubbed coupler until a water-in-oil emulsion formed (15).

Relative kinematic emulsion viscosity was determined with the procedure described by Berlin (15). Briefly, this value was obtained by measuring the average flow time in seconds for 0.1 ml. from a vertically mounted 1.0 ml. serological pipette previously calibrated with reagent grade glycerol. All measurements were made at 22°C. and the value for emulsion viscosity was derived from the ratio: emulsion flow time/glycerol flow time.

To characterize emulsion stability, a Wintrobe hematocrit tube was filled to the 100-mm. mark and centrifuged at 1,000 g for 1 hour at 22°C., in an International Model U Centrifuge using a No. 240 head. With this method it was possible to parti-

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<sup>1</sup>"Alhydrogel" is a form of colloidal aluminium hydroxide commonly used in European FMDV vaccines. Obtained from Dansk Svovlsyre—o.g., Superphosphate Fabrik, Denmark.

<sup>2</sup>"Arlacel A" (mannide mono-oleate). Obtained from the Atlas Powder Co., Wilmington, Delaware.

<sup>3</sup>Mineral oil was ESSO Bayol F, obtained from the ESSO Standard Oil Co., Linden, N.J.

tion and measure percentage of aqueous phase, percentage of emulsion, and percentage of excess oil phase.

#### IMMUNIZATION OF CATTLE:

Sixteen grade Hereford steers weighing 600-700 pounds were used. Subcutaneous (SC) and intramuscular (IM) routes of inoculation were used to determine the effect each route had on antibody response and immunity. With each adjuvant-antigen preparation, four steers were inoculated IM in the right gluteal region and four SC on the right side just anterior to the point of the shoulder. The dose with each preparation was 2.0 ml.

#### ANTIBODY ASSAYS

Blood samples were collected weekly for the first month after inoculation and bi-weekly thereafter. After samples were allowed to clot at room temperature, the serum was poured off and clarified by centrifugation. All samples were heated at 56°C. for 30 minutes, then stored in rubber-capped vials at -15°C. until used.

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Serum antibody titers were determined using a constant virus-fivefold serum dilution virus neutralization test. Virus for the test was tissue-cultured FMDV A-119 diluted so that each 0.03 ml. dose of serum-virus mixture contained 100 mouse LD<sub>50</sub>. Ten 6- to 9-day-old Rockefeller H strain mice were used as indicator hosts for each test mixture. Mice were inoculated intraperitoneally and serum endpoints, based on survival numbers, were calculated with the method of Reed and Muench (16). Antibody titers were expressed as 50 per cent protective dilutions (PD<sub>50</sub>) derived as the logarithm (base 10) of the reciprocal serum dilution protecting half the mice against 100 mouse LD<sub>50</sub>, FMDV A-119.

Analysis of variance of all PD<sub>50</sub> values was determined as described by Snedecor (17). Variation was partitioned into effects that were due to adjuvants, individual cattle, routes of inoculation, days after inoculation, and the interactions between these components.<sup>4</sup>

#### IMMUNITY STUDIES

At 224 days after inoculation, the immune status of the test cattle was challenged. Half the cattle in each adjuvant group were exposed by direct contact with

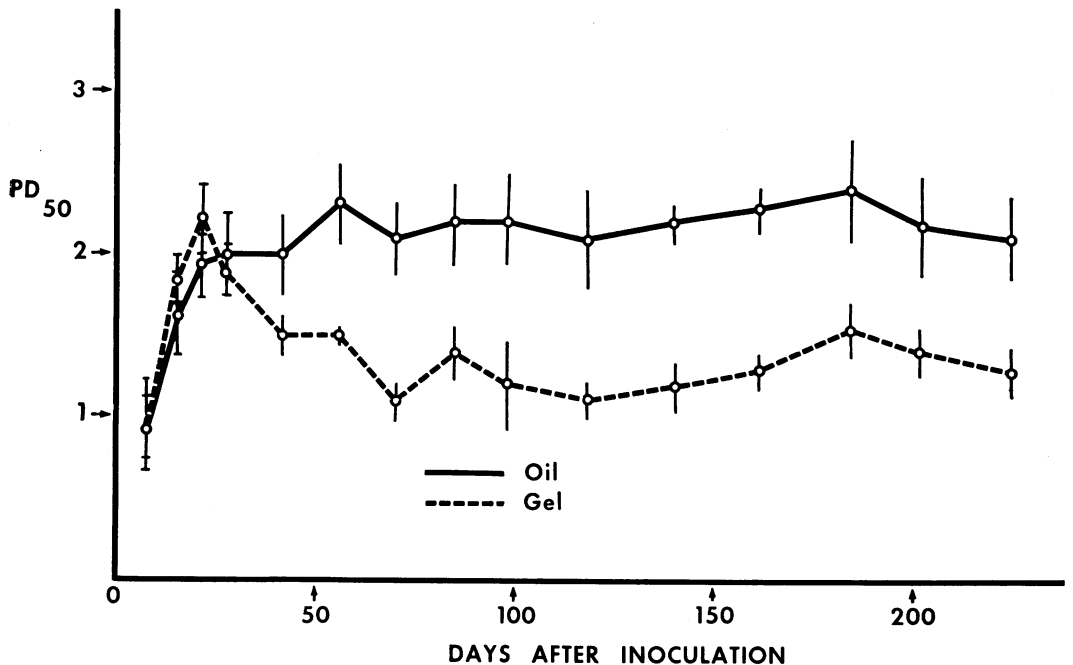


Fig. 1.—Geometric Mean Antibody Titers for Cattle Inoculated with Formalin-Treated FMDV Adsorbed to Alumina Gel or Emulsified in Oil. (PD<sub>50</sub> endpoints expressed as the logarithm of the reciprocal serum dilution protecting half the mice inoculated with 100 LD<sub>50</sub> of mouse-adapted FMDV. Vertical lines through each sampling date represent the standard error of the day's mean.)

an equal number of infected cattle and the other half by direct inoculation of homologous virulent cattle virus.

*A. Inoculation Challenge.* Two cattle from each adjuvant and route group were randomly selected and moved into isolation pens. Each animal was inoculated with 7,000 bovine ID<sub>50</sub>, FMDV A-119 using a single intra-epithelial injection on the lingual dorsum. Injection of the challenge dose (.03 ml.) was performed without anaesthesia. Infectivity of the challenge virus was predetermined by titrations in cattle as described by Henderson (18). During the next 14 days, each animal was examined twice daily for signs of foot-and-mouth disease.

*B. Contact Challenge.* The remaining 8 cattle were moved into a large isolation pen containing 9 normal control steers. One control steer was selected as a virus donor and inoculated with the same procedure and virus dose used for challenge by direct inoculation. For the next 17 days, this "experimental herd" was observed for signs of FMDV contagion.

## Results

Cattle inoculated with the adjuvant-antigen preparations as described in this report did not develop fever or lesions referable to virulent FMDV as a result of the inoculation.

The oil adjuvant preparation was mixed with antigen until all the aqueous phase was stabilized. At the time of inoculation, this preparation contained 78 per cent emulsion, 22 per cent excess oil phase, and its relative viscosity was 0.86.

### ANTIBODY RESPONSE:

Analysis of variance indicated a nonsignificant difference in routes of inoculation which permitted the individual PD<sub>50</sub> values for each adjuvant group to be pooled. Geometric mean PD<sub>50</sub> values for each group, and the standard error for each mean, are shown in Figure 1. As illustrated by Figure 1, the antibody response pattern for the gel group is qualitatively similar to that obtained with FMDV vaccines currently used (19). In this group, antibody appears by 7 days after inoculation, rises to peak levels between 14-28 days, and then declines to a plateau. Although both adjuvant groups reacted similarly during the first 28 days, antibody levels in the oil group

thereafter were sustained near peak levels while those in the gel group fell to a plateau. In the plateau region, a six- to ten-fold difference was maintained between the two adjuvant groups for the remainder of the test period.

### IMMUNITY STUDIES

*A. Inoculation Challenge.* Within 24 hours, each animal had a primary lesion at the site of inoculation, but there was a striking difference in the size and appearance of lesions between the two adjuvant groups. Cattle in the oil adjuvant group had dry, sessile lesions 1-3 cm. in diameter, and there was no further development of mouth lesions after 24 hours. At 24 hours, cattle in the gel group had turgid vesicles 3-6 cm. in diameter which showed continuous enlargement for 72 hours. In one steer (Table I, No. 3021), the entire lingual dorsum was affected by the third day. Within 72 hours after inoculation, all animals in both groups developed fevers above 103°F.

Individual antibody titers and challenge reactions are summarized for this group in Table I. Each animal reacted with a marked antibody response as evidenced by differences in PD<sub>50</sub> values between 7-14 days after challenge. Where generalization of lesions occurred, it was completed by the third day except in steer No. 3074 where lesions were found on the sixth day. Three of the four cattle in the oil adjuvant group resisted challenge while all cattle in the gel group developed generalized lesions.

*B. Contact challenge.* The morning after inoculation of the donor steer, lesions were found in the mouth and on the feet of this animal. After 4 days, 3 control steers had similar lesions; an additional 4 on the sixth day, and the last control steer had lesions 8 days after the donor was inoculated. Febrile response in the control cattle was noted the same day or one day prior to lesion development.

Serum samples from cattle in both adjuvant groups were assayed for antibody 7 and 14 days after exposure. Marked antibody response in any steer not having lesions was accepted as proof of exposure to FMDV sometime during the 17-day observation period. The results of this experiment are also shown in Table I. In every instance where cattle in either adjuvant group developed lesions, fever above 103°F. was noted 1-3 days before the lesions were observed. Steer No. 3040 was

**TABLE I — Resistance of 12 Cattle 224 Days after Inoculation with Formalin -Treated FMDV**

Steer No.	Adjuvant + Route <sup>1</sup>	Challenge Method	Lesions <sup>2</sup>		Antibody Titers <sup>3</sup> Days after Challenge		
			Mouth	Feet	0	7	14
3021	Gel — IM	Inoculated	+	+	0.9	4.8	4.3
3080	Gel — IM	Contact	+	+	0.9	2.4	4.4
3081	Gel — IM	Inoculated	+	+	1.1	5.0	4.9
3074	Gel — SC	Inoculated	+	+	1.3	4.9	4.5
3050	Gel — SC	Contact	+	0	1.4	1.1	4.1
3071	Gel — IM	Contact	+	+	1.5	1.8	4.0
3045	Gel — SC	Inoculated	+	+	1.7	5.1	4.3
3077	Gel — SC	Contact	0	0	2.1	1.8	3.6
3076	Oil — IM	Contact	+	+	0.5	<0.3	4.2
3090	Oil — IM	Contact	+	+	2.0	2.1	4.1
3070	Oil — IM	Inoculated	0	+	2.0	4.8	4.3
3040	Oil — SC	Contact	0	0	2.1	2.0	4.0
3075	Oil — IM	Inoculated	0	0	2.5	3.7	4.2
3018	Oil — SC	Contact	0	0	2.5	2.6	3.4
3038	Oil — SC	Inoculated	0	0	2.6	3.7	4.2
3026	Oil — SC	Inoculated	0	0	2.9	4.4	4.8

1. IM = intramuscular route; SC = subcutaneous route.
2. Oral lesions were marked positive if vesicles or erosions appeared anywhere in the mouth, except in the immediate vicinity of injection site, after challenge inoculation; feet were marked positive if vesicles appeared on one or more feet.
3. PD<sup>50</sup> endpoints expressed as the logarithm of the reciprocal serum dilution protecting half the mice inoculated with 100 LD<sup>50</sup> of mouse-adapted FMDV.

febrile on the 8th day after exposure, but subsequently did not develop lesions. Two of four cattle in the oil adjuvant group resisted this challenge while 1 of 4 in the gel group was resistant. Anamnestic antibody response found in the cattle not having lesions is evidence that their immunity was challenged.

One might note in Table I that no generalized lesions occurred in cattle with PD<sub>50</sub> values approximating 2.5 or higher. However, cattle with PD<sub>50</sub> values approximating 2.0 gave variable results suggesting this level of serum antibody does not indicate dependable immunity. These data are in general agreement with data reported by Borgen, *et al* (20), where immunized cattle with antibody titers of 1:256 resisted a contact challenge.

### Discussion

The results of this study have shown that use of oil adjuvant involved a more efficient antigen stimulus and a more sustained antibody response than did use of gel adjuvant with the same antigen mass. Immunity studies have also shown that cattle in the oil adjuvant group were more resistant to re-infection indicating that duration of immunity from a single dose of emulsified antigen may extend well beyond that ordinarily obtained with FMDV

vaccines in current use. These results indicate the potential value of oil adjuvants with FMDV vaccines.

However, it must be recognized that general use of emulsified antigens could be a hazardous procedure until more extensive studies resolve questions regarding certain disadvantages associated with Freund-type adjuvants. These disadvantages, which tend to restrict application of emulsified antigens to research problems, are related to the occasional pyogenic, cystogenic, and allergic reactions attributed to their use. Indeed, ingredients known to cause these reactions have been deliberately used to induce allergic phenomena in animals for comparison with certain human disease syndromes (21, 22). However, it is also known (23) that antigen emulsions prepared without these noxious agents, still retain remarkable adjuvant activity. The oil adjuvant used in this study was prepared with materials free from known noxious agents. Throughout this study, no signs related to allergic reactions were seen in test cattle inoculated with oil adjuvant. Similarly, post mortem inspection of these cattle gave no gross evidence of abnormal tissue reactions.

Another disadvantage with oil adjuvants is a tendency for phase separation when inadequately emulsified. Berlin (15) first recognized that emulsion stability was not

only related to storage quality but also to adjuvant potency. Stability of vaccine emulsions has been difficult to evaluate unless samples are stored and observed over long periods of time. Characterization of stability as described in this report appears to give a good practical reference to stability since no aqueous phase separation occurred after 7 months in sterile samples held at 4°C. Unstable samples prepared for comparison did show marked separation on storage when the initial test showed any degree of aqueous phase separation.

#### REFERENCES

1. MOEHLMAN, H. The present state of research on the virus of foot-and-mouth disease. *Archiv. Exp. Vet. Med.* 8: 316, 1951.
2. PYL, G. and HEINIG, A. Aluminium salts of organic acids replacing aluminium hydroxide in foot-and-mouth disease vaccine. *Archiv. Exp. Vet. Med.* 9: 724-731, 1955.
3. RIVENSON, S. Foot-and-mouth disease: Adjuvant vaccines. *Gac. Vet. B. Aires.* 18: 140-158, 1956.
4. ESPINET, R. G. Foot-and-mouth disease immunization using intradermal saponin vaccines. *Gac. Vet. B. Aires.* 18: 217-222, 1956.
5. MACKOWIAK, C., PETERMANN, H. B., CAMAND, R., and LANG, R. Concentrated trivalent saponin vaccine against foot-and-mouth disease prepared from culture virus. *Bull. Acad. Vet. Fr.* 32: 313-318, 1959.
6. FREUND, J., CASALS, J., and HOSMER, E. P. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc. Soc. Exper. Biol. & Med.*, 37: 509, 1937.
7. FREUND, J., THOMSON, K. J., HOUGH, H. B., SOMNER, H. E., and PISANTI, T. Antibody formation and sensitization with the aid of adjuvants. *J. Immunol.* 60: 383, 1948.
8. HENLE, W. and HENLE, G. Effect of adjuvants on vaccination of human beings against influenza. *Proc. Soc. Exper. Biol. & Med.*, 59: 179, 1945.
9. SALK, J. E., BAILEY, M. L., and LAURENT, A. M. The use of adjuvants in studies on influenza immunization. II. Increased antibody formation in human subjects inoculated with influenza virus vaccine in a water-in-oil emulsion. *Am. J. Hyg.*, 55: 439, 1952.
10. WOODHOUR, A. R., JENSEN, K. E., and WARREN, J. Development and application of new parenteral adjuvants. V. Comparative potencies of influenza vaccines emulsified in various oils. *J. Immunol.*, 86: 681, 1961.
11. MCKINNEY, R. W., and DAVENPORT, F. M. Studies on the mechanism of action of emulsified vaccines. *J. Immunol.*, 86: 91, 1961.
12. BINN, L. M., and HILLEMANN, M. R. A guinea pig potency test for adenovirus vaccine. *J. Immunol.* 84: 20, 1960.
13. GRAVES, J. H. Foot-and-mouth disease vaccine—The production of antigen in cultured cells in the absence of serum. *Am. J. Vet. Res.* 24: 183-186, 1963.
14. BACHRACH, H. L., CALLIS, J. J., HESS, W. R., and PATTY, R. E. A plaque assay for foot-and-mouth disease virus and kinetic of virus reproduction. *Virology* 4: 224-235, 1957.
15. BERLIN, B. S. Gross physical properties of emulsified influenza virus vaccines and the adjuvant response. *J. Immunol.* 85: 81-89, 1960.
16. REED, L. J. and MUENCH, H. A. A simple method for estimating fifty per cent endpoints. *Am. J. Hyg.*, 27: 493, 1938.
17. SNEDECOR, G. W. *Statistical methods.* 5th Ed. Iowa State College Press. 1957: 329-393.
18. HENDERSON, W. M. The quantitative study of foot-and-mouth disease virus. *Agri. Res. Coun. Rept. No. 8.* Her Majesty's Stationery Office. 1949.
19. VAN BEKKUM, J. G. Neutralizing antibodies of sera of cattle vaccinated against foot-and-mouth disease. Doctorate Thesis. Univ. Utrecht, 18 June, 1959.
20. BORGES, H. C., DAL PRATO, A., and DINTER, Z. Antikörperreaktion und immunität bei rindern, vakziniert gegen Maul-und-Klauensuche. *Acta Vet. Scand.* 3: 1-12, 1962.
21. LEE, J. M., and OLITSKY, P. K. Simple method for enhancing development of acute disseminated encephalomyelitis in mice. *Proc. Soc. Exp. Biol. & Med.* 89: 263, 1955.
22. LEE, J. M., and SCHNEIDER, H. A. Critical relationships between constituents of the antigen-adjuvant emulsion affecting experimental allergic encephalomyelitis in a completely susceptible mouse genotype. *J. Exp. Med.* 115: 157-167, 1962.
23. SALK, J. E. Use of adjuvants in studies on influenza immunization. III. Degree of persistence of antibody in human subjects two years after vaccination. *JAMA.* 151: 169, 1953.

### Collection of blood samples from caudal vessels of cattle.

The collection of blood samples from the caudal vessels of cattle is described. This technique requires little restraint and provokes little or no excitement in most cattle. The site of injection is at the second or third coccygeal intervertebral space posterior to the junction of the tail with the body, and exactly on the ventral midline, as determined by the hair pattern. The needle used is either a California bleeding needle or an 18 gauge, 3/4 inch hypodermic needle. After cleaning the site and elevating the tail the needle is inserted at right angles to the tail to a depth of about

1/2 an inch. The needle enters the intervertebral space and is then slowly withdrawn until blood flows from it. If only a few drops of blood appear the tail is lowered slightly so as to allow of a freer blood flow. After withdrawal of the needle and lowering of the tail a small hematoma usually forms which is quickly absorbed. If the initial attempt is unsuccessful one of the other coccygeal spaces should be tried. The technique can also be used for injection of drugs into the bloodstream.

*New Zealand Vet. J.* 10:41, 1952.