CONCERNING ASIA I FOOT-AND-MOUTH DISEASE FORMALINIZED SAPONIZED VACCINE

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Summary

The vaccine has been studied in all its components saponin, virus, formalin.

Saponin has to be neutral, it acts by weight whatsoever its volume of injection. The minimum quantity producing on cattle the maximum adjuvant action is 25 mg.

Asia 1 virus cultivated during 24 hours produces high infectious an antigenic titers. But these parameters do not involve the real value of the vaccine and the yield of protection on cattle is only 60%. The increasing of culture duration improves its immunigenic value. Nevertheless, this change is not in relation with the appearance of the heat stable complement fixing activity, but in direct ratio with the quantity of 25 mg. immunigenic components. And these components are detected thanks to the complement fixation reaction with an arcton-treated antigen, since this fluorocarbon destros the 7 mg. component.

The formalin inactivation of Asia 1 virus needs less formalin than O or A virus. With 0.025% of formalin instead of 0.05 % the average of the vaccine protection grows from 60% to 95%.

The best Asia 1 foot-and-mouth disease vaccine requires 25 mgr saponin. Its attenuation must to be done by addition of 0.025 % formalin. The culture duration is about 30-36 hours to obtain the most significant titer in immunigenic components.

In spite of limited menans, Nong sarai foot-and-mouth disease laboratory, Thailand, has been able to produce foot-and-mouth disease vaccine since 1960 thanks to the Frenkel virus culture.

For many reasons (1) the monovalent 5 ml cattle dosis vaccine includes:

– virus (extract in the medium of 300 mg virulent epithelium)	4.70
– glycocoll buffer	0.05
– 40% formalin (adjusted according to its real titer) from 0.05 to 0.1%	P.M.
– saponin from 10 to 50 mg. in pH = 7.6	0.25
vaccinal dosis	5.00 ml.

This formula is satisfactory since during 1961 and 1962 the laboratory controls af O and A vaccine protect 88% and 92% cattle respectively.

During the same period on 102 cattle used for Asia 1 vaccine control, 41 showed various secondary lesions (control is produced with 2 intradermolingual injections of 10,000 ID 50). So the general protection average was only 60%. The same thing occurred at the beginning of 1963, see Table I. Thus it becomes necessary to study separately all the vaccine components.

The control cattle should be given special mention. These animals are very receptive to foot-and-mouth disease since they came from the Thai peninsula zone free of this disease from time immemorial. But these animals which are in a very precarious physiological state are under a strong stress due to the long railway transport, to the change of food, to the new way of living. Consequently at the end of the control period, all cattle are really in very poor condition. And this might well explain some curious reactions registered sometimes at the immunigenic controls. This could reinforce Burnet's theory (2) since immunity is under the thymus gland. This organum is very scattered on tropical calves there is no sweetbread, and stress action is the most powerful on the thymus.

We shall now study saponin, virus, formalin.

Saponin

In dispensatory of the United States of America Lippincott Cy., 1955, we find : Saponins are the most widely spread substances in plants. Saponins have been extracted from more than 500 plants. The term saponin is applicable to the glucosidic groups which have in common; to foam when mixed with water, and emulsify the small precipitates in suspension. Most of saponins are neutral, some are acid and rarely others are basic.

The producer (British Drug Houses Ltd) could provide:

a) acid saponin which is pure water extracted saponin from Quillaja saponica. This saponin is brown in 5% solution, if the solvent is distilled water pH = 4.4, if the solvent is pH = 7.5 phosphate buffer, pH is still acid = 5.5

b) basic saponin is rather a hard soap. The 5% solution is yellow and turbid, pH = 8.3 in distilled water, pH = 8.00 in phosphate buffer.

c) neutral saponin-saponinum B.P.C. which is extracted in a solvent to rid it of impurities which are soluble in water and gives a white powder. The 5% solution is yellow, pH = 6.00 in distilled water, pH = 7.33 in phosphate buffer.

We have used neutral saponins. We did not search toxic, hemolytic, abortive (3) properties, but only the inflammatory adjuvant (4,5) action.

As a preliminary, we want to state that the glucosidal complex (6) is in correlation with emulsifying properties of saponin, and does not modify the virus which can be easily dissociated. We have verified this on many occasions. On the other hand foot-and-mouth disease virus can be firstly inactivated by formalin then kept at 4°c. Saponin is only added when vaccine is manufactured. This method shows some advantages, particularly in trivalent vaccine production.

Concerning the inflammatory properties of saponin, we had first to know the action in ratio of quantity injected and the place of injection. Scientific notes did not give any information Espinet (6) or only mentioned the percentage Rivenson (7), Mackowiak et al. (8) But if the vaccinal dosis is 2 ml 0.5% i.e. 10 mg. whereas for 15 ml vaccine 75 mg. is used. In the latter, saponin is more dispersed so the effect could be the same but the problem was: is saponin acting by weight or by volume (quantity percent)?

Above all, we must notice that subcutaneous saponin injection is followed by inflammatory reactions whose volume and lasting quality can vary from one animal to another. Yes, these reactions stay within narrow limits and it is possible to measure them with calipers.

I) Inflammatory reaction according to the site of injection

Along the neck, following the spine we can recognize two places where subcutaneous tissue is loosened (Zone I an IV). In between the tissue is dense (Zone II and III). Two cattle were used, and received various quantities of saponin in 10 % solution; both sides of the neck were injected. Inflammatory reactions were measured during five days in succession. Vertical and horizontal diameters

were totalized, see Table. II. Volume of inflammatory reaction is practically not modified by the subcutaneous tissue density, in other words, the injection site is without significance.

II) Inflammatory reactions according to quantity and dilution

From the preceeding experiment, it was easy to conclude that inflammatory reaction was in direct ratio with the weight of saponin. With 10 mg. average reaction: 6.5 cm, with 25 mg: 9.9 cm, with 50 mg: 11.4 cm, with 100 mg: 16.7 cm. The second problem was to know the inflammatory reactions when the same quantity of saponin is diluted, this fact was important for trivalent vaccine production. Four cattle were experimented on. Each of them received a fixed quantity of saponin respectively diluted in 1, 5, 10 and 50 ml. Once again individual reactions were noted, some of them were no longer measurable after 3 days. However they were concordant, see Table III. Undoubtedly it seemed normal to think that saponin diluted in large volume: 50 ml, provokes a bigger reaction since more spread than the same quantity under a small volume: 1 ml, this fact is barely evident. On the contrary, volume increasing, whatsoever the dilution is in direct ratio with the quantity in weight. Consequently, when we prepare trivalent vaccine we can mix the 3 monovalent viruses but in this case we must only add one dosis of saponin.

III) Adjuvant action of Saponin

When Dresser (9) studied the adjuvant action of lipodophillic substances he reminded Freund's statements. Adjuvants act 1) in keeping antigen at the injection site and then diffusing it slowly. 2) by stimulation of macrophages and lymphocytes, 3) by producing antibodies by unelucidated reaction. To demonstrate this third point Dresser injected separately adjuvant and antigen. We did the same. Three guinea pig groups received respectively 1) inactivated foot-and-mouth disease virus, 2) the same + 2.5 mg. saponin, 3) separately inactivated virus and saponin. Infections were repeated four times at eight day intervals. Guinea pigs were bled one month after the last injection. Serum titers were established by complement fixing test, see Table IV. Saponin has no action on a distant site.

Adjuvant action of saponin foot-and-mouth disease vaccination in cattle was studied with O and Asia 1 virus. With fixed antigen dosis but in unsufficient

quantity to produce full protection, we respectively added 10, 25, 50 mg. saponin. 90 cattle were used 36 with 10 mg. saponin, 30 with 25 mg., 24 with 50 mg., 25 cattle out of 36 showed foot-and-mouth disease lesions, also 9 out of 30 with 25 mg. and 8 out of 24 with 50 mg., see Table V. So, the minimum quantity of saponin producing the biggest adjuvant effect is 25 mg. per animal. (Laboratories using hydroxyde + saponin generally limit saponin dosis to 5–10 mg,)

However Richou and al. (10) recently showed that the adjuvant power of saponin of the same origin is variable. And to this effect they recommened a new method of saponin titration using staphylococic anatoxin as antigen and rabbit as reactor.

Finally it is worthwhile noticing that the respective susceptibility to saponin varied according to the animals. For instance 25 mg. provokes in the guinea pig a fatal necrotic escharre and with this animal we cannot overpass 2.5 mgr. Rabbit is more tolerant. On the pig 50 mgr. produces necrotic abcess, with elimination of the antigen, then without protective action. 25 mg. is well tolerated but has to be injected deeply by the subcutaneous route. On goat and sheep G. Bory (11) showed that the toxic dosis is 300 mg., 10–15 mg. is often necrotic, 3–5 mg. is well accepted.

Virus

Clinical evolution of Asia 1 foot-and-mouth diesease virus is slow to act comparatively to foot-and-mouth disease produced by viruses of other types. It is the same for contagiousness and Asia 1 outbreaks never look as explosive as O or A epizootics.

In Frenkel culture, Asia 1 virus needs a longer time for growing. Its infectious peak is never reached before 20–24 hours. For vaccine production generally we used cultures harvested at the 24 th hour.

Here a parenthesis is required. When Frenkel (12) was successful with virus culture on epithelium in survival he recommended the use of the infectious titer as a marker for virus production. In Girard and Mackowiak's hands, this criterion was a failure, particulary when virus gave a very high titre that is to say when vaccine contained very little virus. So people returned to the old empirical method. (Berne OIE meeting 1947) in weighing the virulent epithelium and only considered infectious and antigenic titers as parameters to ensure the existance of virus in the culture. With the exception of Henderson and Galloway (15)

this opinion was shared by all vaccine producers, Moosbrugger (16), Ubertini (17), Willems (18). These insisted, apparently not without reason, on the importance of obtaining a very high complement fixing titer.

As far as Asia 1 vaccine is concerned these tests were not in direct correlation with the protective value of the vaccines (see Table VI). Recently Graves (19) confirmed that virus infectivity was no more a factor of immunigenicity.

Thus, we should have to increase the virus culture duration. This process was before recommended by Fogedby and Johnson (20) though Henderson (21) showed that this property depended above all on the virus strain. Besides, sellers (22) with virus cultivated on pig kidney cells showed that vaccine prepared with virus harvested after 42 hours gave a better protective effect than vaccine produced from 18 hour old culture. Whereas the infectious titer was in both cases the same, it was higher for culture harvested at the 66th and 90th hour. On our sides, a preliminary trial, at a laboratory scale, gave a similar result. Starting from the same virus we produced three different culture lines, the first one of 18 hours duration, the second of 24 hours, the third of 48 hours. After respectively 9 passages for each (see Table VII) the virus parameters were closed together. However the corresponding vaccines gave totally different results.

1) with the 18 hour culture, there was no protection: 3 cattle out of 3 got secondary lesions.

2) with 24 hour culture, there was a beginning of protection: one cattle was totally protected, the second showed only secondary lesions on one foot and the third one only on two feet.

3) with the 48 hour culture, there was complete protection on 3 cattle out of 3 vaccinated.

At the same period our attention was drawn by Planterose, Cartwright, Brown's (23). Scientific paper which stated that in Frenkel culture harvested at the 18th hour, there was up to 80% complement fixing activity a which was destroyed by heating at 56° during one hour. On the contrary our own experience had previously shown that the complement fixing activity with Frenkel culture O virus harvested at the 72nd hour, was clearly increased when treated in the same conditions. So we joined Planteroseet al's views established with foot-and-mouth disease virus, thanks to pig kidney cells. The heat labile component was present during one phase of virus growth. Its importance depended on the time at which

the virus was harvested. But from our point of view, we were more inclined to the last of Planterose's assumptions, i.e. a new capacity of the virus was appearing by increasing the duration of culture. At the first stage of growth the viral particles were imcomplete in one way or another. The complement fixing activity which remained stable after inactivation (56°c during one hour) testified of "virion" existence, Caspar et al. (24) that is to say of the ultime phase of virus development. This opinion appeared to take some consistance when after several trials we established the standard curves 1) of Asia 1 antigenic titer evolution throughout the duration of culture 2) of the same but after inactivation at 56° during one hour. These curves joined together (see Table VIII) at about the 36th hour. So the decisive experiment occurred. With 11 batches of vaccine prepared with a 45 hour duration virus culture, in which the antigenic titer of inactivated virus was equal if not higher than the normal one. We vaccinated 52 cattle. At the challenge 30 cattle out of 52 showed secondary lesions (see Table IX). In other words the average protection was only 45% Even if we compare the hoof lesions in ratio to the total number of hooves (84 out of 208) the percentage of 60 obtained in this manner is far from being a success.

Before going further a parenthesis must be opened. Inactivation by heating at 56°c during one hour produces turbidity in the solution, so complement fixing test reading becomes unfeasible. So we purified the solution by chloroform action Chloroform is added in equal quantity (only half if the virus has been previously filtered) and shaken at 4°c during one hour. We noticed that at 37°c both components are apparently destroyed since infectivity and antigenicity disappeared (see Table X). However this deleterious action may well be due to the chloroform impurities, since chloroforms of other origins do not give the same results :

— normal antigen : antigenic titer $\frac{1}{40} = 2$, infectious titer 10^{-5}

— antigen + chloroform: 1 hour at 37°c, A.T. $= \frac{1}{02} = 2$, I.T. $= 10^{-4}$

In any way this remark has some importance since now many laboratories use chloroform to purify the virus instead of filtration.

Let us come back to the prolongation of culture time for obtaining a more complete, more immunigenic virus. The problem had to be carefully studied but we should have liked a more accurate method for virus analysis. Since 1954 Randrup (25) but above all in 1959 Brown and Crick (26) showed that the 25 mg infectious component was also immunigenic whereas the 7 mg. complement fixing component was not. These two components could be undirectly detected

by complement fixing test but without knowing what was due to one or another. This same year Mussgay (27) treated the foot and-mouth disease virus by arcton 63, the aqueous phase retained the 25 mg components, the organic phase carried away the 7 mg. component. Later on Brown and Cartwright (28) showed that Arcton 113 did not modify, even after several treatment, the infectious component whereas the same solvent eliminated the 7 mg component With another fluorocarbon freon 113, we unfortunately did not obtain the same results and we even used this solvent to purify the virus to be able to use it as antigen in the agar gel diffusion test. It enabled us to obtain the two precipitin lines and this freon (see Table XI) has no action on the complement fixing activity. Recently Brown and Newman (30) used the treatment with arcton 113 as an index for establishing the immunigenic value of a vaccine. Like them, we used arcton 113 (Imperial chemical Industries, Ltd) and studied the growth of the 25 mg component throughout the evolution of virus culture. Detection of this component was carried out, thanks to the half quantitative comlement fixing method as described by Joubert Girard, Mackowiak and Camand (31). Undoubtedly, this method is f r from being so accurate as Brooksby's 50% method (32) it is nevertheless sufficient Arcton treatment was carried out in different ways: addition of half the volume of arcton 113, shaking at 4°c with either the omnimixer during 4 minutes, or the shaking machine during one hour, or by hand during 90 minutes, centrifugation 2,000 rpm. during 5 minutes, aqueous phase extraction, repeat until there is no longer any precipitate at the interface between arcton and virus solution, generally this occurred after 6 or 7 treatments. All our results are described in diagrams (Table XII and XII bis). With Frenkel culture in Erlen, the 25 mg. component reaches its peak near the 36th hour. To avoid any interference with the seed virus, these cultures were carried out by seed virus adsorption at 37° during one hour, then washed twice with phosphate buffer and completed with normal medium. With Frenkel culture in tanks, it sometimes occurs that the 24 hour culture gets a good yield in the large component (see culture 9 and 12 TAs CP 14) but generally is not like that (culture 13, 14, 15, 17, TAs CP 14). However the most demonstrative example is given by culture 15 TAs CP 14. In this culture between the 24 and 33th hour we note that the total antigenic titer remains the same, the infectious titer declines whereas arcton treated virus is regularly growing up. Generally tank culture grows quicker than Erlen culture and in spite of the use of a lot of antibiotics, it is difficult in the end to avoid microbe development. Then, it is

nearer the 33rd hour that the cultures are harvested. Still one question not yet elucidated: are prolonged cultures apparently richer in immunigenic component, good for seeding new cultures ?

Starting from culture 15 TAs CP 14 harvested at the 33rd hour and with high grade in an arcton treated element we carried out a vaccine according to the rules given at the beginning of this article. Formalin percentage was 0.05. Ten cattle were vaccinated and at the challenge all were protected (see Table XIV. production 41 B) compared with the 60% general average given by a 24 hour culture, the action is noticeable. And this action is surely linked to the 25 mg. component since in some vaccine productions of 24 hour virus culture with high grade in arcton treated component 19 cattle out of 19 have been correctly protected.

Formal

Many years ago, Girard and Mackowiak (33) showed consecutively to Moosbrugger 34) that the foot-and-mouth disease vaccine, Vallee-Schmidt-Waldmann type, which was generally regarded as inactivated was only attenuated. And this for two reasons 1) foot-and-mouth disease virus to which is added 0.05% formalin and heated at 26°c during 48 hours was still infectious for cattle when injected intradermolingually, 2) lyophilised vaccine (virus + formalin + alumine hydroxyde) inoculated in the same conditions was also infectious. If the vaccine in itself was totally innocuous, that was due to its incorporation in alumine. Later Wesslen and Dinter (35) studying foot-and-mouth disease inactivation by formalin reached the same conclusions. Girard and Mackowiak's trials has also proved that foot-and-mouth disease virus mixed with 0.1% formalin and heated at 46°c during 48 hours was apparently totally inactivated. Fogedby (36) confirmed the results.

These trials were again repeated in Thailand with A type at the beginning One cattle intradermolingually injected with 5 ml. of $\frac{4}{14}$ A virus solution, treated with 0.1% fomarlin and heated at 26°c during 48 hours remained uninjured. On unweaned mice we obtained the same results (see Table XIII). It is worthwhile remarking that in all these experiments formalin must contain 40% of formic aldehyde. When this percentage is not reached, which is generally the case each formalin batch is titrated before use we add more formalin to obtain 40%

Then, trials were repeated with Asia 1 virus. The latter, heated at 26°c during 48 hours with various quantities of formalin was injected intradermolingually

to three cattle. In the first case with 0.1 ml. in 5 sites for each formalin percentage. The result was as follows:

0.1%	formalin	0 0 0 0 0
0.075%	formalin	0 0 0 0 0
0.050%	formalin	0 0 0 0 0
0.025%	formalin	+ + + 0 0

In the second case with 5 ml. for 0.1 and 0.05% formalin in respectiveiy at the bottom and at the top of the tongue. There was no reaction. In the third case with 5 ml. for 0.05 and 0.025% formalin in the same conditions. There was no reaction with 0.05% (reading at the 48th hour), with 0.025% the epithelium could be peeled off. From these results we could conclude Asia 1 virus needs less formalin to be inactivated 0.025% might be used for virus attenuation in the vaccine.

A recent study by Graves (37) brings a correction. Residual infectivity seemed bigger than we previously supposed. Virus treated by heat (30°c during 68 hours) and 0.1% formalin was injected by the same route on 6 cattle at 0.1 ml. dosis in twenty sites per tongue. After 72 hours 2 vesicules were noted out of 120 sites. This fact is interesting to know, but besides it is possible to find special susceptibility according to the breed or to the individual, this does not modify the difference in formalin action for Asia 1 virus and other types particularly O, A and C.

On ten batches of vaccine prepared from 24 hour Asia 1 culture, attenuated with 0.025% formalin, two out of 40 cattle got secondary lesions. That is to say 95% were protected. Formalin quanttiy is of course only one factor. The second one and most important is the virus value. And with two vaccine productions (No. 10 and 14-1964) treated also with 0.025% formalin there were 6 cattle out of 10 with secondary lesions, and we can read the same phenomenon with 45 hour culture (see table IX). But when 0.025% formalin has been added to virus (see Table XIV 4IA) of which the quantity of 25 mg. components was known, out of 9 cattle 5 even had no local tongue lesions and 4 just got this local lesion. That means a very high protection.

In another hand Brown and Crick (38) have shown that agar gel diffusion test reveals precipitin lines for each elementary component. With the help of Asia 1 vaccine as antigen we researched these precipitin lines. Vaccine was concentrated 25, 50, 100 times by dialysis against polyethylene glycol (Carbowax 20 M, Union Carbide Co.) according to Kohn's method (39). Serum was hyperimmunized guinea

pige serum, diluted at $\frac{1}{2}$ $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$. The test was working. During a short time we thought of using it as a quantitative method to detect the immunigenic value of the vaccine. As a matter of fact, precipitin lines appeared with a 25 times concentrated vaccine, We might be able to couclude to a better immunigenic value than for the vaccine requiring to be concentrated 100 times. Trial was repeated several times. Simultaneously we opposed results from the in vivo control. There was no correlation. However this trial was repeated with Asia 1 vaccine formalinized at 0.025%. This time and on the contrary to what occurred preceedingly with Asia 1 vaccine in activated with 0.05 % formalin, the precipitin test showed two precipitin lines corresponding to the two components. The infectious line is very thin and closer to the antigen cup as usual. In this way we had fresh proof that 0.05% formalin was too important for Asia 1 virus and would disintegrate the immunigenic component too much. We also had a new hope of finding a new comparative method for vaccine titration. A similar method, but more accurate has been used by Beale and Mason (40) for poliomyelitis vaccine evaluating and might be studied with foot-and-mouth disease vaccine. As far as our results (see Table XIV) were concerned, the only conclusion was that the immunigenic component preciptin line involves the protective value of tested vaccine. However it is a necessary but not sufficient condition. And yet the necessary condition is only relative, since one vaccine (41 B Table XIV) with just one precipitin antigen line, but rich in arcton treated particules, produced a good vaccine. As for the sufficient condition, if it was true for cattle, it was not for pigs (see other communication on pig vaccination). Whatsoever, the two precipitin lines obtained with a concentrated vaccine remain a good marker for the protective value of this vaccine.

Conclusion

To be really effective, the Asia 1 formalinized, saponized foot-and-mouth disease vaccine must contain 25 mg. of saponin per dosis and be attenuated with 0.025 % formalin. Generally the best immunigenic virus is obtained after 30–36 hour cultures. The determination of its potential value is carried out, thanks to the research of antibodies fixing the complement but with arcton treated virus as antigen. This new test should take the place of the research of infectious titer and normal antigenic titer which has never involved the immunigenic value of a vaccine.

(For tables and references see the French Text)