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PERSPECTIVES OF GENETIC ENGINEERING VACCINES AGAINST FOOT-AND-MOUTH DISEASE

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of wild and domestic cloven-hooved animals. It is an acute disease characterized by vesicular lesions which lead to significant productivity losses as well as to indirect economic losses due to embargoes on trading.

FMD virus (FMDV) is an aphthovirus belonging to the family **picornaviridae**. The virion is icosahedral, without envelope, of about 25 nm of diameter and consists of 60 copies each of four coat proteins VP1, VP2, VP3 and VP4, one or two copies of VP0, the uncleaved precursor of VP2 and VP4 and 3D (the viral RNA polymerase). The viral structural polypeptides VP1, VP2 and VP3 are arranged in 12 pentameric subunits each of which forms one of the 12 vertices of the icosahedron. The intact virion sediments at 145S. By lowering the pH to 6,5 or heating at 45°C the virion dissociates into 12S particles which consist of five copies each of VP1, VP2 and VP3 and an aggregate of VP4 molecules. The 12S particles have low immunogenicity, approximately 1% compared to that of the entire viral particle. VP1 is the only structural polypeptide that purified and injected into cattle is capable of inducing neutralizing antibodies (1, 2, 3) and is located at the apices of the virion. VP4 is internally located. The viral genome consists of a single-stranded RNA of approximately 8000 nucleotides.

The viral RNA is infectious and serves as mRNA. Upon translation a polyprotein is produced which is subsequently cleaved into a series of intermediate precursors which are further processed to give the mature non-capsid and capsid viral proteins (VP).

Methods used to control FMD vary with the individual country. In FMD - free countries (Australia, the United States, Japan, etc.) control occurs by slaughtering of infected or exposed animals as well as by having strict restrictions on importation of animals and animal products from countries where FMD is still a problem. In countries where the disease is endemic regular vaccination programs take place. Control of the disease is highly dependent upon strict quality control of the vaccines

and good campaigns which require a deep understanding of the epidemiological situation of the region.

Overall considerations of FMD vaccines

It is evident that effective vaccines are highly dependent upon the quality of the antigens, inactivant, adjuvants and adequate handling of the antigens during production and distribution. In addition, knowledge of biochemical, antigenic and immunogenic properties of the virus are essential aspects which should be taken into consideration for designing an appropriate vaccine. Several techniques for the analysis of nucleic acids and proteins are being increasingly used for studying biochemical properties. Such techniques include nucleic acid analysis through RNA fingerprinting and RNA sequencing as well as viral protein analysis through SDS-polyacrylamide gel electrophoresis (PAGE), isoelectrofocusing, two-dimensional gel electrophoresis, tryptic peptide analysis, etc. These biochemical methods together with recently developed serological techniques (RIA and ELISA) and the development of monoclonal antibodies provide ideal tools for the precise biochemical and antigenic characterization of active, evolving, vaccine and laboratory strains.

Crucial for an adequate vaccine design is also the understanding of viral biological properties such as variability, persistence and host range, lability of virions, etc., enabled by the application of the above mentioned methods together with recently developed DNA recombinant techniques.

Antigenic variation

Vaccination is complicated by the occurrence of the virus in 7 serotypes: the European types O. A and C, also present in South America, the South African Territorie types Sat 1, Sat 2 and Sat 3 and the Asiatic type Asia 1. In addition over 60 known subtypes resulted from variation within each serotype, with little cross-reactivity among them.

Immunization against one subtype may not protect against another one. Antigenic evolution while the viruses are replicating may frequently give rise to new subtypes and is one of the most important causes contributing to the spread of the disease in endemic regions. Therefore in order to assure efficient vaccination campaigns it is essential to monitor emerging strains and to assess the degree of similarity between the viral vaccine and the strains circulating. In Argentina during the last 3 years, 2 viral strains were replaced in the vaccine, the strains C Argentina 84 and C Argentina 85 replaced the strain C_3 Resende (4) and the strain A Argentina 87 replaced the strain A Argentina 81. We are intensively involved in the biochemical and serological characterization of field and vaccine strains. Figure 1 shows the significant differences of the selected vaccine strain A Argentina 87 when compared to the previously used vaccine strain A Argentina 81.

Persistence

After infection or vaccination with live attenuated strains, FMDV is known to persist in oesophageal-pharyngeal regions of cattle and other ruminants for time periods of up to several years without signs of the disease. During this time, rapid evolution of viruses was found (5). We recently described a decreased reactivity of viruses persisting within the first 63 days post-infection to a set of neutralizing monoclonal antibodies (6). This demonstrates the high risk for the animal and for those susceptible hosts in the surroundings.

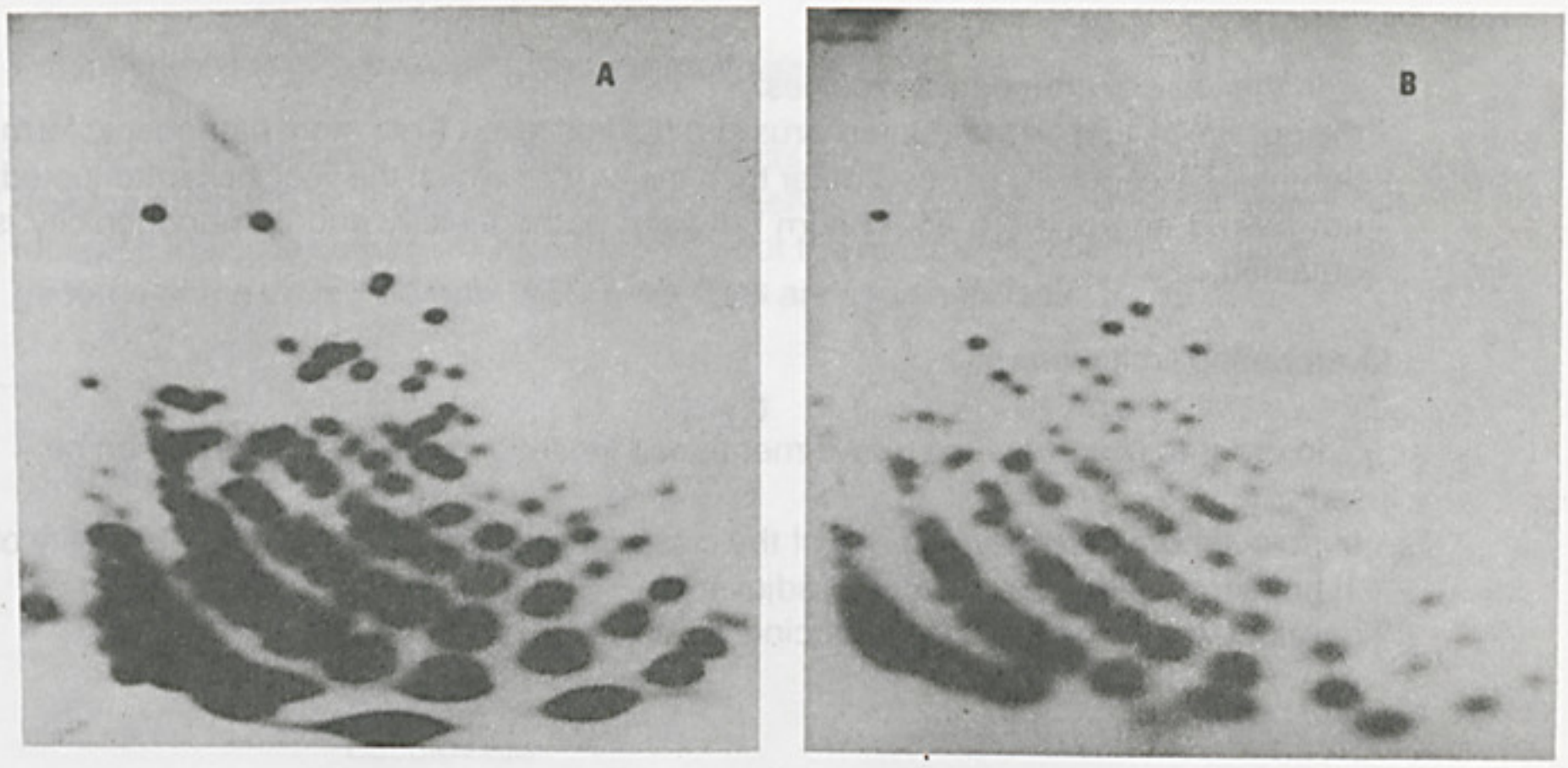

Fig. 1 - RNAse T_1 Two-D maps (Fingerprints) of (A) FMDV type A strain Argentina/81 and (B) FMDV type A strain Castellano-Argentina/87

Host range

The host-virus complexity of FMDV with more than 30 natural hosts allows the selection of viral populations with varying genetic and antigenic properties or with altered pathogenicity. This fact constitutes a risk when attenuated vaccines are used, since attenuation for one species (e.g., cattle) does not assure attenuation for others such as pigs.

Current FMD vaccine designs

At present, chemically inactivated whole-virus vaccines and to a much lesser extend live attenuated vaccines, obtained through serial passages of the virulent strains in non-susceptible hosts, are used for the control of FMD.

While the overall success of these vaccines in controlling the disease is widely recognized, there are a number of considerations which make it desirable to improve them.

In the case of killed vaccines:

- many viruses are difficult to grow in sufficient quantities to ensure enough antigenic mass per dose for effective immunization;
- handling large volumes of infected cultures constitutes a biological security risk;
- requirement for biological containment facilities;
- risk of incomplete inactivation (only a fraction of the production is tested for inocuity);
- risk of escape of viruses (in this regard, it has been demonstrated that in recent years some FMD outbreaks in Central Europe occurred near vaccine production units) (7);
- requirement of refrigeration during storage and distribution, especially in tropical countries;
- alterations of the antigenic structure due to inactivants;
- requirement for several periodical vaccinations;
- eventual bacterial contamination could degrade the antigen through proteolytic enzymes.

In the case of attenuated vaccines:
- the possibility that an attenuated virus strain could revert to its more pathogenic form.
- potential susceptibility of hosts other than the one for which the vaccine is attenuated;
- not always an optimum equilibrium between pathogenicity and immunogenicity is obtained;

Overcoming limitations

In order to overcome the above mentioned limitations, two approaches can be used:
- try to overcome some limitations of the classical vaccines by improving the quality of 1) antigens, 2) inactivants and 3) adjuvants.
- developing new generation of vaccines.

Improving classical vaccines

Antigens

In the case of FMDV, the quality of the antigen is highly dependent upon the conditions used to grow the virus.

In the last years, methods of tissue culture have advanced considerably which improved the industrial production of antigens. Vaccines have been produced successfully and without the need to concentrate the antigen from pig kidney cells growing on microcarriers (8). Spier and Whiteside (9) showed that the production of FMDV type O_1 from BHK cells grown on microcarriers gave higher infectivity and complement-fixing activity than suspension cultures.

However in all these cases, large volumes still had to be manipulated. Alternative methods of high density cell cultures need to be studied, involving standard perfused microcarriers (glass beads, gelatine, biosilon, polyacrylamide, cytodex I, II or III) ceramic matrix, microencapsulation, hollow fibers, collagen spongue, etc. in which densities varying from 10^7-10^9 cells/ml could be reached.

Inactivants

An improvement in vaccine production came with the introduction of virus inactivation by means of aziridine derivatives.

Formaldehyde has been the traditional agent used for the inactivation of virus infectivity and is still widely used. However, formaldehyde inactivation does not follow first order kinetcs and failures have occurred. The introduction of aziridine derivatives, in particular BEI (10, 11) together with more effective safety tests, have given great confidence in the safe application of these vaccines.

Another important alternative is the use of an enzymatic approach consisting on the activation of a FMD virion-associated ribonuclease at alkaline pH in the presence of monovalent ions such as K+, Cs+ or NH_4+. A first order kinetic of inactivation was obtained with maximal preservation of the antigen (12)

Adjuvants

Great interest is now focused on the optimal conditions to present the antigen to the immune systems. This is important not only for improvement of classical vaccines but also for the successful development of new vaccines. In the case of FMD, we showed that replacement of the classical hydroxide-saponin adjuvant by oil adjuvant gave longer lasting and enhanced immunity in young and adult cattle and swine (13, 14).

Prospectives for the development of new vaccines

During this decade, one of the prime objects in research on FMD has been to develop methods for the production of the essential immunizing components without recourse to tissue culture methods and without the need to handle the infective genome of the virus. Potential vaccine designs are shown in Table 1.

Table 1

I. **Subunit vaccines**
  a) Expression of VP1 in:
    Bacteria
    Eukaryotic systems:
    Yeast
    Poxvirus
    Baculovirus
  b) Synthetic peptides
II. **Attenuation** by direct gene manipulation
III. **Anti-idiotypes**
IV. **Antisense:** Virus resistant cells
V. **Complementation** of a virus defficient strain in cells constitutivelly producing the defficient protein
VI. **Anti-cell receptor:** antiviral

We have pursued approaches I and II and the rest of this work will be separated into two parts: one describing the potential of subunit vaccines and the other part describing the potential of attenuated vaccines.

I. Subunit vaccines

This approach became possible when some structural features necessary for eliciting a good immune response were identified:

- The neutralizing activity is largely confined to VP1; VP1 isolated and used as a vaccine elicits neutralizing antibody responses and protects cattle and swine from infections (1, 2).
- The neutralizing activity was generated by fragments obtained by cyanogen bromide or enzymatically spanning the regions corresponding to amino acid residues 145-154 and 201-213 (15).

a) Expression of VP1 in bacteria

The structural gene coding for VP1 of the prototype strain C_3 Resende was isolated and expressed in two different expression systems:

- the pPLc24 system which carries the pL promoter of phage lambda, inducible by temperature in cells containing a temperature sensitive mutant repressor protein (16);
- the pUR expression system consisting of a set of 3 vectors carrying the lac promoter induced by IPTG (17).

A significant level of expression was obtained after induction of both of these systems. Figure 2^A shows the expression of the fusion protein at different times post-induction in the pUR system. The induction of a 145 Kd VP1- β galactosidase fusion protein can be clearly observed and distinguished from the 116 Kd β galactosidase protein. The β galactosidase - VP1 nature of the induced protein was further confirmed by its reactivity with antibody against virus on a Western blot, figure 2^B. Part C of the same figure corresponds to the preparative gel used to establish whether the fusion protein is able to elicit an antibody response. Cattle were

immunized with partially purified preparations of the fusion protein. The vaccine was prepared by pulverizing a slice of the polyacrylamide gel containing the protein and emulsifying in complete Freund's adjuvant. The vaccine was given subcutaneously to 18 bovines on days 1 and 30 up to 500 ug were inoculated. Serum samples were taken every 7 days. No significant neutralizing antibody response was detected up to 30 days post revaccination.

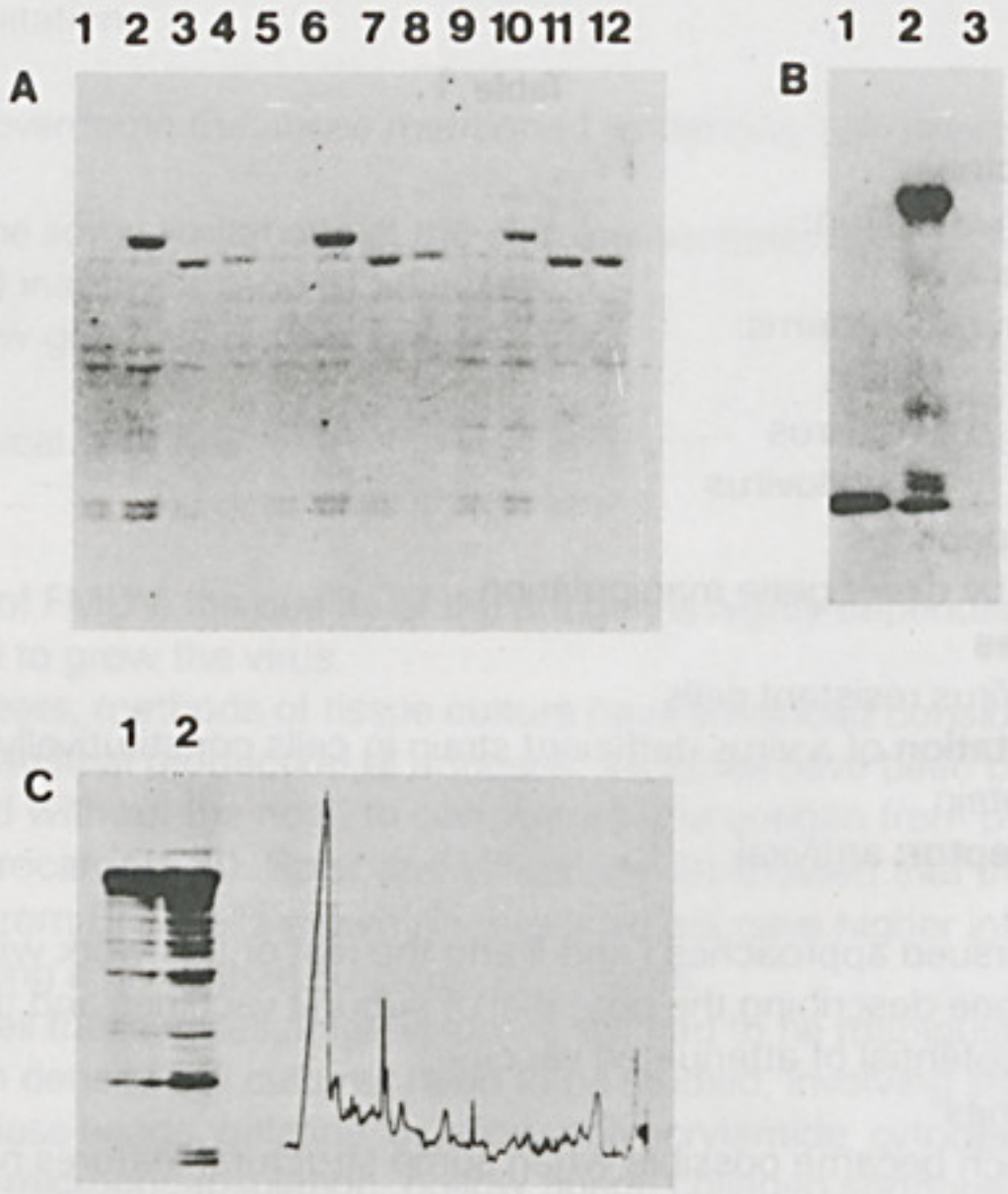

Fig. 2. Expression of cloned FMDV-VP1 gene in the pUR system.
SDS-polyacrylamide gel analysis of total cellular proteins stained with Coomassie brilliant blue.
a) Different times post-induction, lanes 1, 5 and 9, no induction; lanes 2, 3 and 4, 4 hours post-induction; lanes 6, 7 and 8,2 hours post-induction; lanes 10, 11 and 12, 1 hour post-induction, in the 3 different expression vectors lanes 1, 2, 5, 6, 9 and 10 correspond to the pUR 289 vector: lanes 3, 7 and 11 correspond to the pUR 288 vector and lanes 4, 8 and 12 correspond to pUR 278 vector.
b) Autoradiograms of proteins transferred to nitrocellulose and reacted with I^{125}-labelled VP1-specific antibodies, lane 1, isolated VP1, lane 2 expressed after 3 hours induction in the pUR 289 vector; lane 3, no induction.
c) Preparative polyacrilamide gel electrophoresis and corresponding scanning.

These results are not surprising considering that the isolated protein is weakly immunogenic possessing less than 0,1% of the activity of the virus particle (18). Meloen in 1982 demonstrated that neutralizing monoclonal antibodies raised against intact virus do not recognize isolated VP1 (19). It becomes always more evident that VP1 in the virion adopts a conformation which is highly dependent from a substantial interaction with the other structural proteins.

Other laboratories reported that vaccines produced with several A strains through VP1 specific fusion proteins were protective in cattle. However, significant amounts of polypeptide and revaccination regimes were required (20).

Alternative hosts as well as alternative genomic fragments should be pursued so that the surface epitopes reach the appropriate conformations required for protection *in vivo*. Moreover, more information is required on the importance of the immunological response to internal viral antigens in relation to T-cell immunity.

b. Synthetic peptides

The fact that the entire VP1 is not necessary for eliciting protective antibodies in animals, encouraged us to the use of this approach.

The prediction of the location of immunogenic sites within VP1 capable of eliciting a neutralizing antibody response was based on several approaches which include: immune response in mice of proteolytic and chemical fragments of VP1 (15); comparison of predicted amino acid sequences in different serotypes; hydrophilicity plots superimposed on amino acid sequence variation plots and identification of helical regions displaying hydrophilic and hydrophobic zones on opposite sides of the helix (21). These methods predicted amino acid residues 144-159 and to a lesser degree 200-213 of VP1 as good candidates for eliciting a neutralizing response. According to these predictions we synthesized a series of peptides, covering various regions of the polypeptide, corresponding to the sequence of serotype O_1 Kaufbeuren by using the solid phase Merriefield process. The peptides were linked to keyhole·limpet haemocyanin and tested for immunogenicity in guinea pigs. 200 ug of peptides were injected subcutaneously with complete Freund's adjuvant and revaccination was at 29 days with incomplete Freund's adjuvant.

As can be seen in Table 2 synthetic peptides representing each of the two potential antigenic regions of VP1 induced high levels of antibodies which recognized intact virus, but only residues 140-160 were protective. No cross neutralization activities were observed with other serotypes. Despite the optimal results obtained in guinea pigs with the peptide corresponding to residue 140-160, when this peptide was inoculated in cattle, very low levels of neutralizing antibodies were obtained even after revaccination.

Table 2

Protection of guinea pigs vaccinated with synthetic peptides corresponding to the VP1 region of FMD virus O_1 Kaufbeuren against challenge with the homologous virulent virus

Sample N.º	Challenge of guinea pigs with virus O_1 Kaufbeuren	Neutralization of viruses by anti-peptide serum[b] microneutralization test performed in tissue culture				
		O_1 Kaufbeuren	O_1 Campos[c]	A_{24} Cruzeiro	A Venceslau	C_3 Indaial
89-34 - 1	0/5[a]	<1.2	ND[d]	ND	ND	ND
89-34 - 2[e]	6/6	3.9	2.4	≤1.2	≤1.2	≤1.2
89-34 - 3	1/8	≤1.2	ND	ND	ND	ND
89-34 - 5	0/2	≤1.2	ND	ND	ND	ND
89-34 - 6[f]	0/6	2.4	2.25	≤1.2	≤1.2	≤1.35
89-34 - 8	0/5	≤1.2	ND	ND	ND	ND
89-34 - 9[f]	1/6	3.3	3.3	≤1.2	≤1.2	1.50
89-34 - 10[e]	6/6	4.35	3.3	≤1.2	≤1.2	1.35

[a] Protected/challenged (challenged with $10^4 ID_{50}$ of guinea pigs adapted O_1 K FMDV)
[b] Values are expressed as $-\log_{10}$ of serum dilution that protects 50% against 100 $IDTC_{50}$
[c] By fingerprinting O_1 K and O_1 Campos show a high degree of homology (better than 95%).
[d] Not done.
[e] Oligopeptide corresponding to amino acid residues 140-160 of VP_1
[f] Oligopeptide corresponding to amino acid residues 200-213 of VP_1.

It is evident that additional work is required which should establish:
- the importance of additional peptides of another genomic regions;
- optimal carrier proteins;
- alternative methods of coupling;
- use of immunological potentiators;
- use of mixtures of peptides as multivalent vaccines;
- studies of immunological memory;
- significance of a priming effect.

Moreover additional information on the 3-D structure of the virion through X-ray crystallography is essential. The poor performance in cattle compared to the promising results in guinea pigs would be indicative that attempts with peptide containing domains which react with helper T-cells should be pursued. Recently, the potential importance of sequences 200-213 in enhancing the response of sequences 141-160 was suggested by the serological evidence that a peptide containing both sequences was more reactive with neutralizing monoclonal antibodies against the intact virus than either sequence alone (22). Moreover a vaccine prepared from two peptides 141-158 and 200-213, linked by a diproline spacer, in order to promote adequate folding, and having cysteine residues at each end of the linked peptide, for the purpose of polymerization of the molecule as a means of eliminating the need of a carrier, elicited protection in cattle even when no revaccination was applied. However, it required significant amounts of peptide and complete Freund's adjuvant (23). In addition, it was shown that tandem peptide sequences fused to bacterial proteins elicited high levels of neutralizing antibodies and protected pigs against challenge. However, only limited animals were used on the trial (24).

II. *Live attenuated vaccines*

Live viral vaccines offer several significant advantages over inactivated or subunit viral vaccines, namely induction of more effective local immunity and greater duration of immunity. Such vaccines, however, are at present only of limited use due to the considerations mentioned above, which could not be overcome in the late fifties when these attenuated vaccines were developed (25).

The observation for several picornaviruses that viable virus can be rescued from cloned cDNA was an essential breakthrough to study the genetic determinants of attenuation and to construct safe attenuated vaccines (26, 27) Infectious DNA can be specifically mutagenize to obtain modified strains which can replicate and retain antigenic identity without propensity for virulence. Moreover, once a stable attenuated strain is identified or generated through adequate alterations of an infectious clone, and provided that the genetic determinants of attenuation are not located in the immunogenic regions, one can extend the attenuated phenotype to other serotypes, by introducing through recombination via cDNA *in vitro* the genes for immunogenicity from a new strain into the genome of an ideal avirulent strain. To make such an approach feasible indentification of viral genes that specify virulence is of critical importance. Therefore, the biological and biochemical characterization of several attenuated strains of different serotypes was undertaken.

As expected due to the high variability of RNA viruses and to the multiple passages used to generate these attenuated strains, molecular weight and charge differences between the polypeptides of the attenuated strains and their corresponding wild type strains were observed randomly throughout the entire genome (28, 29). A remarkable feature, however, was a common increased electrophoretic mobility of the precursor polypeptide P3 in all attenuated strains when

compared to their wild type strains. Preliminary data identifies a genomic deletion in the region of polypeptide 3A of the attenuated strains (Figure 3).

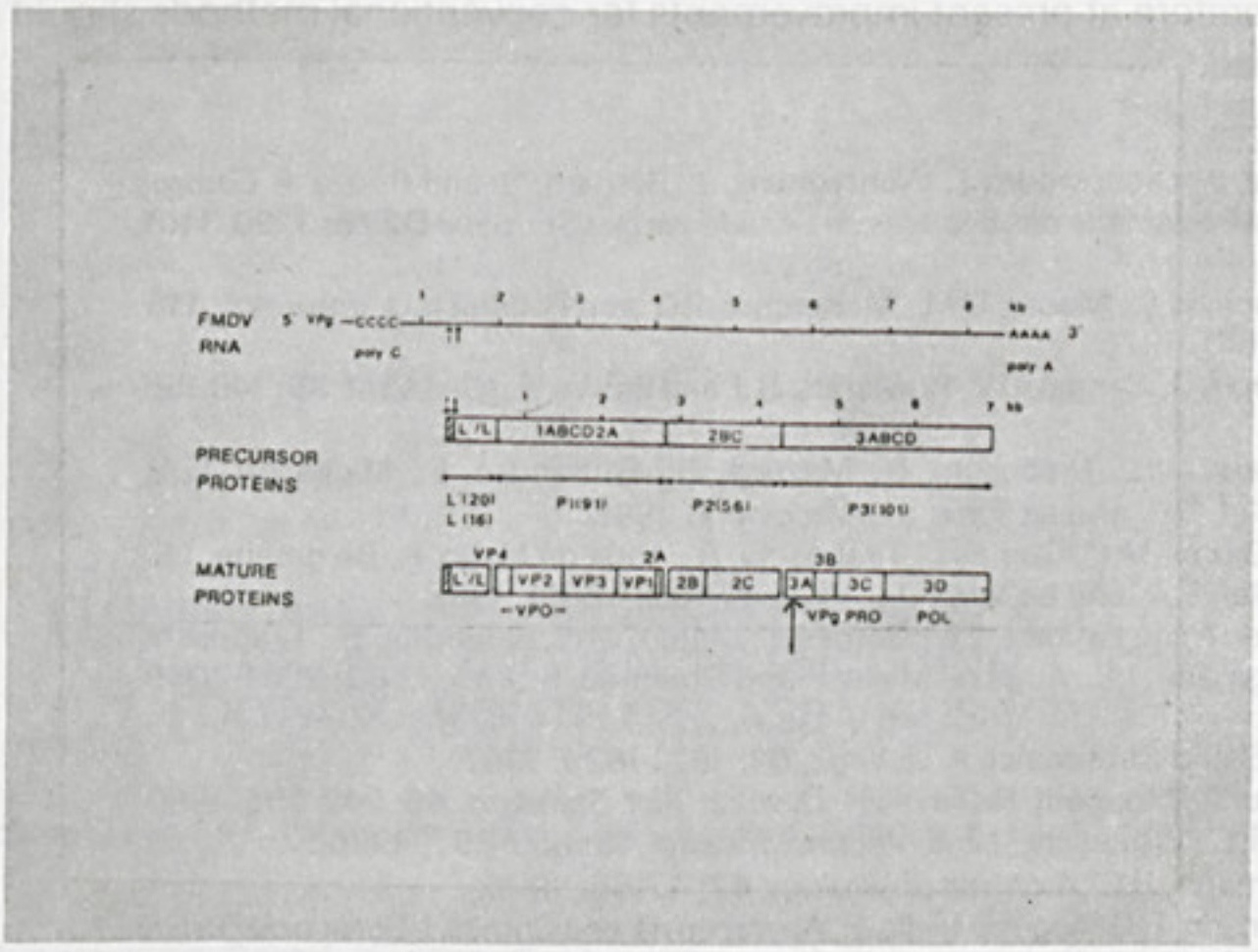

Fig. 3 - Biochemical map of FMDV

The potential relevance of this genomic region for the attenuated phenotype was indicated by the following facts:

- The biochemical and biological properties of intermediate strains isolated during the process of attenuation indicated a direct relationship between the appearance of the attenuated phenotype and the alteration in P3.
- A genomic deletion is in agreement with the fact that no revertants could be isolated for the strains studied.
- The biological properties of recombinant viruses between wild type and attenuated strains located the major genetic determinants of attenuation in the 3' half of the genome (30).

The fact that viable recombinant viruses were obtained constituted a crucial prerequisite for the development of attenuated strains for new emerging serotypes based on other stable attenuated strains.

As more becomes known on the functional and non-functional regions of the FMD genome, it may be possible to make other specific deletions to ensure attenuation for example for other hosts such as swine.

All these approaches are feasible provided that an infectious DNA clone is available. We are working in this direction but so far, we and no other laboratory could obtain the cloning of the entire FMDV genome as a contiguous cDNA sequence. It seems that the poly (C) tract or sequences 5' to the poly (C) tract are extremely difficult to clone since they were not observed in any clone reported until now by any laboratory. Uncoventional methods will need to be pursued in order to clarify whether the development of new safe live vaccines can be taken into consideration for FMD.

Concluding remarks

At present, the potential of biotechnological methods for improving FMDV vaccines is unquestionable. It should be noted, however, that the developments are

only beginning and much more needs to be known about immunological mechanisms and the molecular biology of FMDV before an overall success can be seen. Therefore at present improvements for conventional methods should not be disregarded.

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