'WINGED' HYPER-MUTATED EPITOPIC MIMETICS OF VP1 (135-146): A NOVEL MOLECULAR DESIGN FOR BROAD-SPECTRUM, MULTIVALENT SYNTHETIC VACCINE AGAINST THE FOOT-AND-MOUTH DISEASE VIRUS SEROTYPE A

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ABSTRACT

An assemblage of synthetic vaccines are engineered against continuously epitopic areas of the Foot-and-Mouth disease virus (FMDV) serotype A comprising residues flanking the RGD loop, belonging to a family of important structural motifs identified with integrin-binding proteins, of the immunodominant epitope VP1 ór antigen site A. In the paper, the importance of salient molecular endowments of the synthetic vaccines are emphasized: (a) a cocktail of sequence hyper-mutations for host recognition of known and possibly unknown intratypic ligand variabilities, (b) optimal utilization of previously mapped viral T and B cell epitopes for antigen presentation and (c) attachment of a potent non-viral T cell epitope as 'wing' to enhance cellular response. This molecular design for novel anti-viral immunogen is deemed to elicit strong neutralizing antibody repertoire and solid cell-mediated protection against FMDV challenge in economically important livestock.

INTRODUCTION

The Foot-and-Mouth disease represents an economic threat not only to the rural population but to the national economy as well, most especially, and for as long as, if agriculture is the country's major mode of production. The etiologic agent infects swiftly and unabatedly large areas, causing devasting effects on the productivity of several cloven-hoofed animals: cattle, buffaloes, sheep, goats and pigs. Aside from direct livestock losses, countries endemic of the disease suffer competitive market trade-offs amidst those countries which have been declared FMD-free.

The only geographic sectors considered free from FMD are North America, Japan, Australia, New Zealand and most European countries. Its control in many parts of world had been mainly through the 'stamp-out' policy requiring all animals in an infected herd to be slaughtered and later incinerated or buried. Such a policy, however, is most impractical and expensive in a Third World scenario. In the Philippines, the disease is highly endemic in at least seven provinces --- Metro Manila, Pangasinan, Bulacan, Rizal, Batangas, Masbate and South Cotabato (Cainglet, 1990). Studies and strategies have already been issued by inter-regional coordinated research programs, like those of the FAO Regional Animal Production and Health Commision for Asia (APHCA). In the Philippine situation, vaccination is deemed most practical and powerful in the eventual control and eradication of FMD in endemic areas.

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The Foot-and-Mouth Disease Virus

FMDVs constitute a genus in the picornavirus family. Classified into seven serologically distinct types: O, A, C, SAT1, SAT2, SAT3 and Asia 1. Each type displays extensive intratypic genetic polymorphisms as a result of mutations accrued during viral replication (Meyer *et al.*, 1994) during which, template-copying processes for RNA genomes have very limited capacity for proofreading, repair and avoidance of nucleotide misincorporation. This high mutation rate of an estimated 10^{-3} to 10^{-4} changes per nucleotide per viral cycle renders the FMDV this hallmark antigenic (inter- and intratypic) variation which is among the major reasons for difficulties encountered by veterinarians in controlling the disease through vaccination (Levy *et al.*, 1994).

The infectious particle contains a molecule of single-stranded RNA (approx. 2.6 MDa) and 60 cop-ies each of four viral proteins VP1 to 4. VP1 to VP3 have a molecular weight of approximately 24 kDa and VP4 with 10 kDa. An empty particle does not contain any RNA with VP2 and VP4 covalently linked as monomeric VP0.

Vaccine Prospectus

Imported live avirulent and (chemically)-inactivated whole vaccines occupy the current vaccination niche in FMD control in the Philippines which no doubt have diminished FMD incidence, morbidity and mortality. Medium-level biotechnology approach is employed in the generation of these attenuated strains through tissue culture (BHK cell lines and bovine tongue epithelial explants) or employment of animal hosts. However, some major drawbacks are encountered with these vaccines, e.g., limited shelflife, need for a cold chain, requirement for high-security facilities for mass production of the virus, assurance of complete inactivation of the virus; possibility of virulent reversions, and occasional occurrence of delayed-type hypersensitivity (Brown, 1987).

A more advanced approach involves the production of synthetic peptide vaccines which are considered to be at a technological pinnacle of current vaccine production. The advantages of using synthetic vaccines have been thoroughly reviewed (Brown, 1988). Since these vaccines are chemically defined they contain absolutely no infectious agents nor the possibility for virulent reversions. Large-scale vaccine factories and downstream processes are no longer required resulting in more economical, simplified and cost-effective production scheme. In addition, synthetic peptide vaccines have long shelf-lives and do not require refrigeration which is, of course, advantageous once adopted in Third World countries, particularly in the Philippines, where problems in the delivery of labile live vaccines have been a persistent obstacle.

In view of the promise shown by these new-generation vaccines in many advanced countries, the production of a novel molecular architecture of a candidate vaccine was designed to target a great diversity of variants within serotype A of the FMDV. Amidst the major problem of immunologic divergence in a rapidly mutating virus, the conceptual strategy is to exhaust and strategically localize all possible mutations against a linearly continuous epitope of a defined serotype. This study would be of significance in the development of novel approaches in controlling viral diseases in livestock, and in addition, bridge the fields of veterinary medicine and biotechnology.

METHODOLOGY

Choice and Analysis of Viral Sequences

RNA sequences coding for the capsid protein (VP1) of the Foot-and-Mouth disease virus serotype A were downloaded from a sequence databank. These were taken from the following subtypes: A5, A22, A24, A32, Aarg79 and Aven76; reversed transcribed and finally translated into protein sequences where the appropriate open reading frames were read using the MICROGENIE™ software. Epitopes along the protein were later localized using the software as discussed.

Biophysics of Epitope Prediction

Secondary Structure Prediction. Conformational algorithms by Garnier and Robson (1978) resulting from the application of information theory and other biasing parameters, e.g., circular dichroism measurements of globular proteins, were used to determine the secondary structures: (A)= a-helix, (B)=b-pleated sheet, (T)=b-turn and (r or blank)=random coil of the designed vaccines and the viral proteins, VP1. The algorithm is described by the equation below:

$$I(S_j;R_1,R_2,\ldots R_{last}) \cong \sum_{m=-8}^{m=+8} I(S_j;R_{j+m}),$$

where I (information content) represents the likelihood that residue j will assume conformation S. Eight residues flanking residue j contribute significantly to the value of I resulting to the observed conformational state. Caveat: the accuracy of this algorithm is only 49%, however, when read carefully, may be improved up to 85% (Sanders, personal communication).

Determination of Regions of High Solvent Accessibility. In a 7-residue window, assigned values for hydropathies (multiplied by a factor 10) were obtained using the method by Kyte and Doolittle (1982). The corresponding value for each amino acid residue was based on its transition thermodynamics from liquid (water) to vapor and the transfer free energy in terms of being buried in the tertiary folded structure. The latter principle was derived from structural analysis of several proteins (Kyte and Doolittle, 1982).

Localization of Residue Palindromicity and Charge Symmetries. Residuewise and charge palindromes were scanned with respect to a central axis along the length of the peptide using the method described by Nazarea and Nazarea (1987a and b). Electrical charges of either 0, -1 and +1 were assigned to neutral, negative and positive residues, respectively. Occurrences of palindromicity in a region of disordered secondary structure represent promisingly strong signals or markers for an epitope along a peptide (Nazarea and Nazarea, 1987a and b).

Assessment of Secondary Structure Retention. Because it is essential that the synthetic peptide vaccine under consideration be similar to the corresponding region of the native viral antigen, retention of the secondary structure in the individual peptides and the final vaccines *vis-à-vis* that of the entire VP1 region was checked by applying the method of Garnier and Robson (1978). The percentage of secondary structure retention was calculated by the equation,

$$\% \text{ structure retention} = \frac{\text{number of correct secondary structures in the peptide segment against the native protein}}{\text{number of residues in the peptide segment}}$$

Hyper-mutations of epitopes

From the epitope mapping of VP1 polyprotein sequence, using the above-mentioned computational algorithms, peptide 135 to 146 was chosen as foundation for the vaccine design. Mutational events were deduced from the RNA and peptide sequence alignment which led to the identification of regions of hyper-variable or mutational 'hotspots' within the epitope. The behavior of these regions served as basis for the epitope mutations. Amino acid substitutions and deletions were constrained to functional changes in order to limit the number of iterations that would constitute the peptide vaccine.

Exclusional Scanning for Non-Viable Epitope Mutations

Non-viable epitope mutations are defined as the sequence iterations whose epitopic attributes, i.e. secondary structure, hydrophilicity and palindromicity, significantly depart from those of the native viral protein, namely (in this case), the VP1 polyprotein. Exclusional scanning assay was performed by screening epitomorphs, which were strung together with glycine bridges, for the major structural motif exhibited by the 6 subtypes studied. Secondary structure retentions were calculated from the feasible candidates and those epitopes belonging to the 100 percentile cluster was designated as 'high-stringency epitomorphs', while those having lower retentions belonged to the 'low-stringency epitomorphs'.

Final Vaccine Design

Viable epitomorphs were intercatenated using glycine tripeptides. The epitopic mutants that exhibited the highest secondary structure retention were chosen as the constituents of the final vaccine. Computation of secondary structure retention followed suit for final confirmation of immunogenic value of the vaccine candidate.

RESULTS AND DISCUSSION

Immunity against viral infections is mediated by the synergistic action of both humoral and cellular immune mechanisms. Constituting the humoral response is the generation of high neutralizing antibody titers which saturate binding to viral envelope or capsid proteins causing the prevention of viral attachment and entry into the host cells. This involves the major histocompatibility proteins (MHC) class II-restricted presentation of viral antigens to the helper T cells for B cell activation and antibody production. It should be, however, emphasized that the protective value of humoral immunity is limited only during the early phases of viral infection, prior to the sequestration of the viral particles by the host cells. Essential in its pathologic control is the induction of non-specific immunity and/or cell-mediated immunity which reactivates cytotoxic T lymphocyte (CTL) response. The best defined virus-specific CTLs are the $CD8^+$ cells that recognize viral antigens associated with class I MHC molecules (Abbas *et al.*, 1989).

Available knowledge on the amino acid motifs in MHC-bound peptides priming the immune cell lineage has aided in the development of experimental approaches for inducing cell-mediated and humoral responses to FMDV challenge which are normally achieved upon immunization of the host by a killed whole-virus vaccine (Beker *et al.*, 1994). The precise location of B and T cell epitopes have been established within a peptide containing the major immunogenic site VP1 spanning residues 135 to 160 of FMDV O1 Campos (Zamorano *et al.*, 1994). Similarly, in serotype A22 T-cell epitopes were directed within peptides 135-159 and 170-189 from *in vitro* viral recognition experiments using peptide-activated mouse lymphocytes (Volpina *et al.*, 1994).

Infectious viral particles digested by trypsin resulted in a 99% decline in neutralizing titers. Having found VP1 as the major protein altered during trypsinization, workers deduced that this may be a major target by immune surveillance (Wild *et al.*, 1969). Alignment of protein sequences from several serotypes and subtypes furnished information on its possible sites of function. This was confirmed by overlapping peptide studies (Strohmair *et al.*, 1982), and later on, by x-ray diffraction structural elucidations (Verdaguer *et al.*, 1994) which identified potential structure-function relationships.

VP1 harbors a region known as the G-H loop or antigen site A (residues 132 to 159), the most prominent topographic feature of the virion. This contains a central highly conserved Arg-Gly-Asp sequence (RGD: 141 to 143 in serotype C) sharing conformational similarities with important integrin motifs (Lea *et al.*, 1995) and has implicated the possible role of integrin receptors as loci of attachment during viral infection. Immunogenic value of these areas has been undermined. In fact, it was discovered that 57% of the total virus-binding activity in immunized swine corresponded to neutralizing antibodies against the G-H loop (Mateu *et al.*, 1995).

Baseline Observations on VP1 Epitope Mutations

The importance of the VP1 polyprotein as a major immunogenic determinant has disadvantageously affected vaccination efforts due to the generation of antigenic variants.

Immunization against one subtype may not protect an animal from infection against a different subtype. Immunogenically divergent viruses are not just artifactually found in the field, but they also pose real problems during vaccine production. In fact, when growth conditions of the virions were altered *in vitro* several differences in antigenic reactivities had been discovered (Piatti *et al.*, 1995). To obviate these difficulties, the strategy is to create a vaccine composed of an assemblage of epitopes that mimics known field isolates and, in addition, potential FMDV quasi-species within a particular serotype. Diversification of serotype A was performed with the iteration of epitomorphs, or epitopic mutants, through deliberate placement of amino acid substitutions and deletions within the chosen basal epitope KYAVGGSGRRGD (residues 135-146 in A24 and A32). Technically, when all residues of the 12-mer peptide were mutated a total of 20^{12} epitopes would result which, from an economic standpont, is quite unrealistic. In order to circumvent the unnecessary need to produce these epitomorphs, analysis of the mutational events within available subtypes at protein and RNA levels should be determined to situate the putative mutational 'hotspots' and to serve as guide in the placement of mutations within the epitopes.

As shown in Table 1, alignment of the peptide sequences belonging to the 6 subtypes revealed the apparent mutational 'hotspots' characterized by the presence of amino acid substitutions at residues 137-139 and 141-142, and a deletion at residue 141. The changes were noted to be polar to hydrophobic (res. 137, 138 and 141), acidic to hydrophobic (res. 139) and silent hydrophobic (res. 142) transitions. Curiously, despite the multiple amino changes within subtypes, there were only three distinctive structural groups which were: Aarg79, Aven76, A24 and A32 [brT hook]; A22 [rbr fold]; and A5 [TrT clamp]. Our observations are consistent with the evidences presented indicating that these viruses are confined within antigenic clusters which modulate the diversification observed in the field (Mateu *et al.*, 1992). Evolutionary routes of European, South American and Asian isolates revealed that although silent mutations were linearly associated in time, there was no net accumulation of amino acid substitutions (Martinez *et al.*, 1992). Amino acid divergence had been found characterized by limited sequence variation at key residues thus resulting in a myriad of escape mutants (Mateu *et al.*, 1994) that may have occurred with or without immunologic selection (Borrego *et al.*, 1993).

From these data alone, with the given observed maximum mismatches of 4, it could be drawn that by taking four mutational events that occupied the 5 mutational 'hotspots' at a time, a total number of 800,000 combinations of epitope mutants would need to be iterated. Moreover, if mutations were constrained to only the

functional shifts, the final vaccine may still consist of 155,850 epitopes which was likewise impractical and contraindicative.

A closer examination of the RNA sequences displayed a 3-codon deletion, minimized accumulation of point mutations and the absence of frameshifts. The conserved nucleotide sequences, with homologies ranging from 55.6-96.3%, presented a paradoxical situation in the context of field antigenic diversity. Apparently, this phenomenon of constrained or limited mutational events may point to the requirement for critical mutations at RNA level as implied in Table 2.

Utilizing this information on the RNA mutational 'hotspots', we were able to limit our iterations to 612 epitomorphs representing the most probable functional changes in critical amino acids based on the RNA sequence. Such intra-typic mutational comparisons served as decision-factors for conformational restrictions to allow the epitopes of the final vaccine to constitute just the critical amino acid replacements which would likely mimic escape mutants from antibody selection in the natural host.

Iteration and Screening of Epitomorphs

Since VP1 contains multiple continuous epitopes, it would be safe to assume in the present case that any iterated mutational event on the p135-146 peptide would have a direct biological significance. This may not necessarily hold true in the case of discontinuous and pointwise epitopes.

In *in vivo* situations, selection by the host's antibodies and fixation of mutational events play major roles in the rapid evolution of FMDV (Rojas *et al.*, 1992) which had been explained within the context of episodic positive Darwinian selection from immunogenic pressure (Elena *et al.*, 1992). The simulated epitope viability assay was based on our assumption that amino acid changes within the VP1 moiety must alter the epitopes to selectively delude neutralizing antibodies, yet at the same time, avoid abrogating its virus-host interaction function. From a point of view of viral fitness, it could be inferred that although a great number of mutations can be ascribed to a module of essential function , there would only be a handful of changes beneficial to the virus.

Effects of amino acid changes as iterated, individually or in multiples, showed that out of the 612 total epitomorphs screened, 72 were grouped as 'high-stringency epitomorphs', 164 as 'low-stringency epitomorphs' and 376 as non-viable mutants. In spite of the strategic placement of mutations, the 'high-stringency cluster', representing the epitomorphs which were able to conserve secondary structures, comprised barely 8% of the mutant population. Subtle amino acid changes at the G-H loop was sufficient to compel large modifications in antigenic specificity accompanied by significant alterations in some internal regions involving protomer-protomer contacts (Lea *et al.*, 1994).

Intuitively, omission of this exclusional scanning procedure could result in the inclusion of garbage sequences or non-viable epitopes within the vaccine. In such an event, a 'clouding-effect' may occur such that neutralizing antibodies produced by the viable epitopes might be overshadowed by those elicited by non-viable peptides. Another unfavorable possibility could be the emergence of epitopic suppression.

Vaccine Construction

Three types of potentially high-efficiency synthetic vaccines were designed: a pentavalent vaccine comprised of 5 distinct epitopes that would be capable of raising antibodies against the 6 subtypes mentioned; and the winged 'high-stringency' and 'low-stringency' hyper-mutated vaccines.

Pentavalent vaccine. In the last decade, a gamut of synthetic vaccines had emerged focusing mainly on the production of monovalent peptidomimetics comprising of chemically synthesized peptides that mimic the areas p140-160 of the VP1 capsid protein. By far, only monovalent vaccines have been

extensively used in field trials which often gave discouraging results attibuted to the low titers of neutralizing antibodies. In some cases, although this region of the VP1 was able to elicit an antibody response in cattle, protection conferred were often poor due to a recessive T cell response (van Lierop *et al.*, 1995). Another approach involves the employment of molecular principles in epitope prediction which had been performed by Natividad (1991), a pioneering work on the methodological development of multivalent vaccines against FMDV, with the construction of four novel trivalent peptides bearing inter-species potency against O1BFS, C3Indaial and A24Cruzeiro strains. Along this line, we have molecularly designed an intra-species pentavalent vaccine that targets six subtypes of FMDV serotype A (Figure 1). This vaccine was obtained by permuting the epitope from the five subtypes, taking three at a time, to come up with a total of 60 possible epitope arrangements and combinations. Products of the permutations were ranked according to their ability to mimic structurally the native viral protein, and the final two combinations had been strung together with glycine bridges to obtain the pentavalent vaccine. Secondary structure comparison of the pentavalent vaccine with native viral VP1 epitope showed a portentous 100% structural retention implying the vaccine's second-order capacity for molecular mimicry in stimulating immune response against FMDV.

'Winged' Hyper-Mutated Vaccines. Two molecular constructs of novel vaccines are designed to epitopically cover the 'gyrations' of a wildly mutating RNA virus. They are essentially composed of epitomorphs which, to a great extent, have the capability to mimic all the possible functional amino acid changes by field, untyped and theoretical FMDV mutant isolates. Accordingly, these peptidomimetics should have distinctive activities. The 110 kD 'high-stringency vaccine' with an isoelectric pH of 6.6 is a diversified peptide vaccine strung together with epitomorphs of strictly a brT hook-character and is shown to have 100% secondary structure retention value when matched with a stretch of the native epitope counterpart in the VP1 polyprotein (Figure 2). After intensive exclusional scanning of all iterated epitopes, those which were able to maintain pliable brT hook-motifs were clustered and strung to fabricate a 239 kDa 'low-stringency vaccine' having an isoelectric pH of 6.7 (Figure 3). At this point, we still do not know to what extent this 'low-stringency vaccine' would evoke FMDV-specific humoral response. Nevertheless, comparison between the 'high-' and 'low-stringency' vaccines would be of interest as fine-structured tools for answering important virological questions regarding the structure-function relationships of these families of protein motifs and topographies, their antigenic role and biochemistry.

The GGG-beaded epitomorphs, representing the final vaccine core, was post modified by intercatenating at the amino-terminus with tandem-repeated tripeptides of the highly conserved RGD motif previously shown to evoke neutralizing antibodies in rabbits and guinea pigs (Novela *et al.*, 1993). Also, we covalently attached tri-homotypic peptide strings from a known nontoxic variant of diptheria toxin to the carboxy terminus of the large peptide vaccines. This string of tetradecapeptides in an earlier study were able to enhance a non-specific T cell response using a glutathione S-transferase fusion protein model (Pilliai *et al.*, 1995). The vaccine polypeptide wings as we had earlier denoted, in essence, is a built-in molecular adjuvant and a chaperone for MHC recognition to improve and optimize the immunologic response to viral infections.

Any new vaccine introduced would have to meet stringent requirements, that is, at present cattle or buffaloes should be protected against intradermal challenge (*via* the tongue) with 100,000 ID_{50} of virus after administration of a single vaccine bolus. The use of an adjuvant to enhance the immune response is recommended. Currently, aluminum hydroxide is popularly used, however, for cattle. It was found that addition of saponin can enhance responsiveness to both primary and secondary immunizations (Cheung *et al.*, 1984).

It is hoped that these novel potential products arising from our basic collaborative research would attract an outpouring of resources and support from both private and government sectors as long-term

investments in S&T. Clearly, the molecular biology work that had been undertaken should in the near future be paralleled by sound and critical inquiry into the economics of synthetic peptide-vaccine production coupled to genetic engineering strategies, efficiency of immmune challenge in field trials, licensing, intellectual property rights ownership and its eventual commercialization.

Declaration of prior claim: *The authors hereby declare that the present disclosure of the results of this research does not in any way diminish the authors' claim to any product or products resulting from information disclosed herein.*

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Table 1. Intratypic peptide sequence comparison between the epitopic residue within the VP1 polyprotein

Subtype	Residue	Sequence
Aarg79	135–146	K Y **T V G** G **S G** R R G D
Aven76	135–146	K Y **T V D** G **S G** R R G D
A5	140–150	K Y **S T G** G **P** R R G D
A22	135–146	K Y **S A G** G **M G** R R G D
A24	132–143	K Y **A V G** G **S G** R R G D
A32	91–102	K Y **A V G** G **S G** R R G D

*boxed residues represent the mutational 'hotspots'

Table 2. Comparative analysis of RNA sequences of the different subtypes based on the epitope of VP1

Subtype	Amino Acid Residue	Sequence
Aarg79	135–146	AAA UAC ACC GUG GAU GGU UCA GGC AGG
Aven76	135–146	AAA UAC ACC GUG GAU GGU UCA GGC AGG
A5	140–150	AAG UAC UCC ACG GGC GGU _ CCG AGA
A22	135–146	AAG UAC UCC GCA GGU GGU AUG GGC AGA
A24	132–143	AAG UAU GCU GUG GGU GGU UCA GGC AGA
A32	91–102	AAG UAU GCU GUG GGC GGU UCA GGC AGA

*boxed residues represent the mutational 'hotspots'

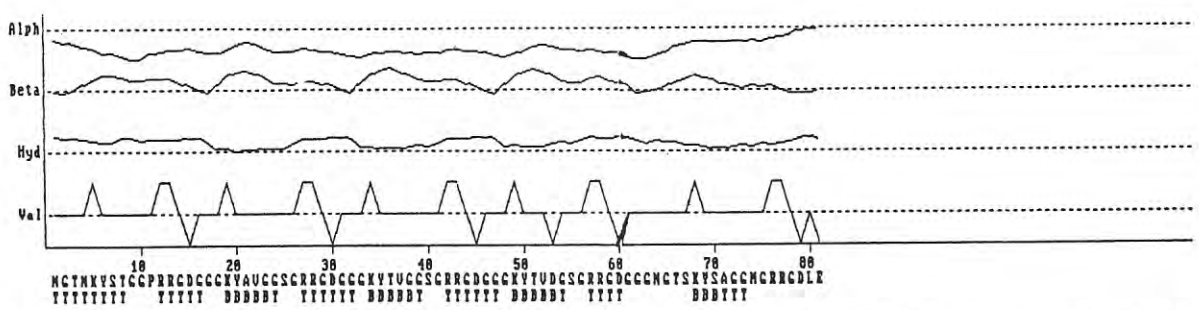

Figure 1. Graphical representation of epitopic parameters of the 7.8 kDa pentavalent FMDV serotype A vaccine

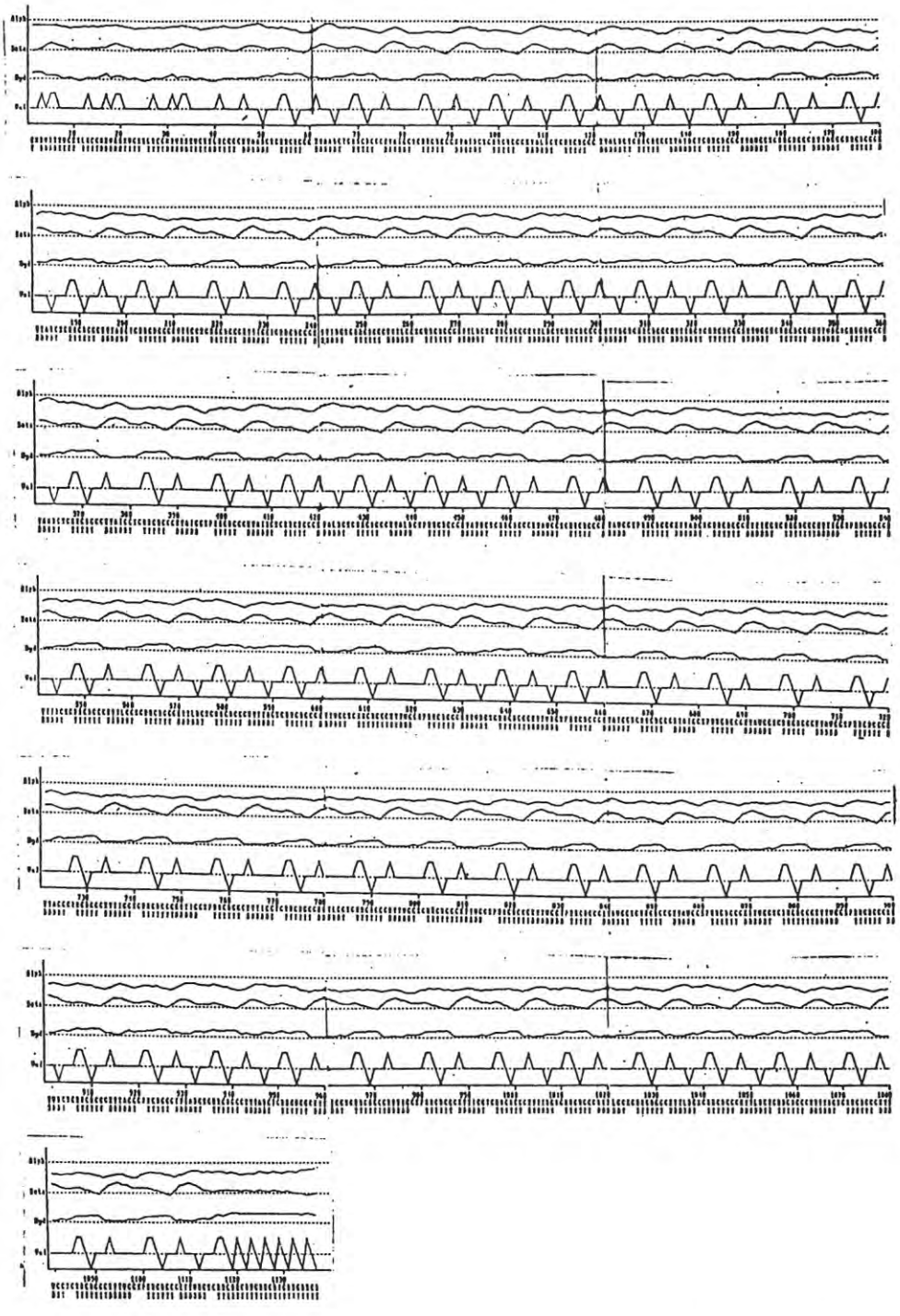

Figure 2. **Graphical representation of epitopic parameters of the 110 kDa 'winged' hyper-mutated high stringency vaccine against FMDV serotype A vaccine. The bi-assymmetric wings are shown as: (1) a C-terminal triple repeating element of a diptheria toxin tetradecapeptide (res. 1-42) and (2) an N-terminal tandem repeating RGD motif (res.1120-1137).**

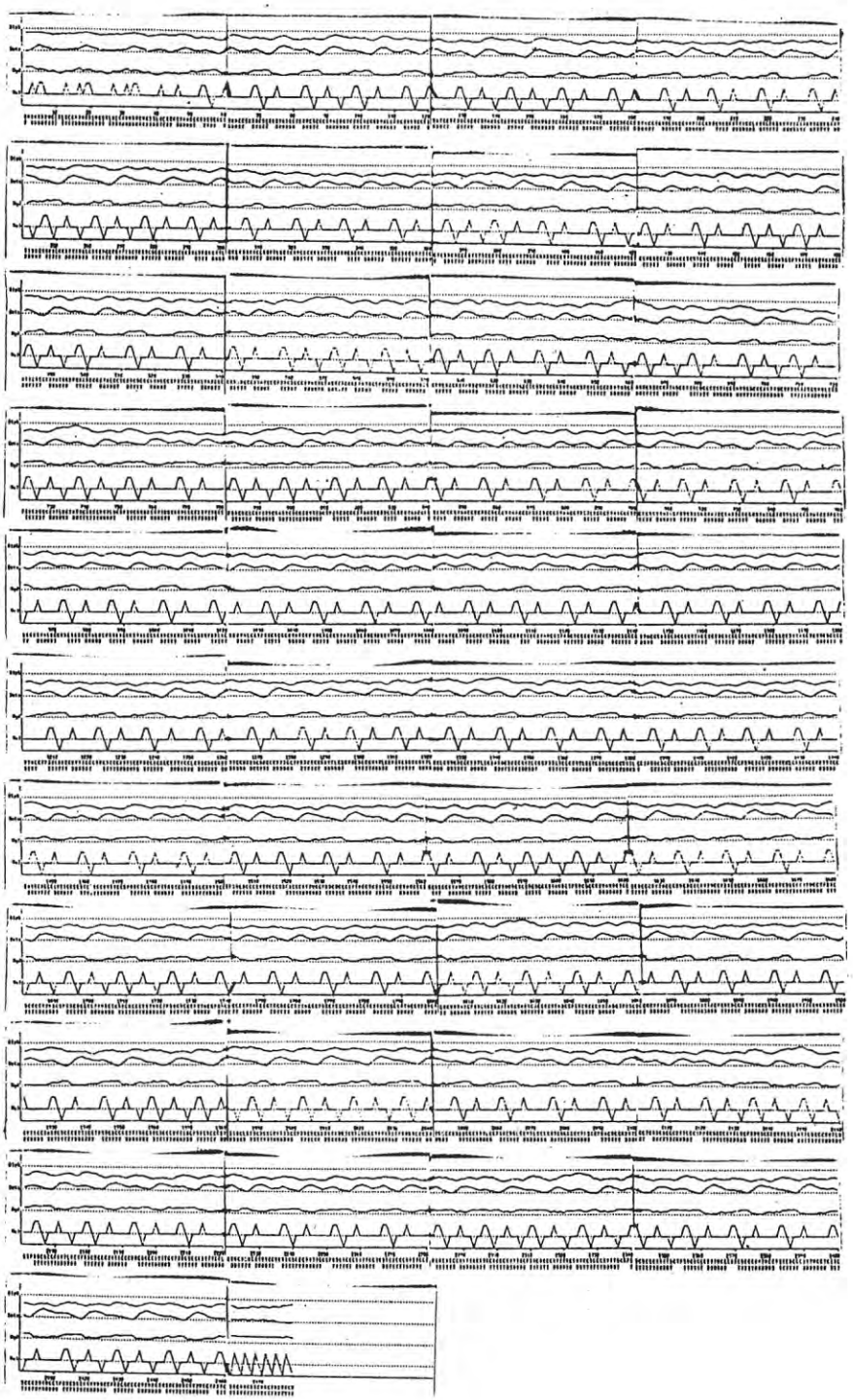

Figure 3. Graphical representation of epitopic parameters of the 239 kDa 'winged' hyper-mutated low stringency vaccine against FMDV serotype A vaccine. The bi-assymmetric wings are shown as: (1) a C-terminal triple repeating element of a diptheria toxin tetradecapeptide (res. 1-42) and (2) an N-terminal tandem repeating RGD motif (res.1120-1137).