Mem. Inst. Butantan, v. 53, supl. 1, p. 59-70, 1991

THE IMPACT OF RECOMBINANT DNA ON THE CONTROL OF ANIMAL HEALTH

Ingrid E. Bergmann, Pan-American Foot-and-Mouth Disease Center, Rio de Janeiro - RJ, Brasil

INTRODUCTION

Although there are at present a large number of different products for controlling diseases and improving productivity of livestock approximately one hundred billion dollars is lost annually to the world economy due to death losses, treatment costs or reduced productivity. The advent of recombinant DNA techniques has the potential to develop a variety of new diagnostic procedures as well as new vaccination strategies to help in the campaigns against economically important diseases of livestock.

Foot-and-mouth disease virus (FMDV) will be adopted to exemplify the impact of recombinant DNA of the new developments.

The causative agent of foot-and-mouth disease (FMD) 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 4 coat protein VP_1, VP_2, VP_3 and VP_4. VP_1 is the only structural polypeptide that, when purified and injected into cattle, is capable of inducing neutralizing antibodies [1,2,3]. 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 proteins.

This system provides an excellent model to explore many of the alternative possibilities of diagnosis and vaccines because: – The virus has a relatively simple structure compared to other agents; – Foot-and-mouth disease is obviously one of the major dis-

Correspondence to: Ingrid E. Bergmann, Pan American Foot-and-Mouth Disease Center, P.O. Box 589, 20.001 Rio de Janeiro - RJ, Brazil.

eases of domestic farm animals; – It is an acute disease characterized by vesicular lesions which lead to significant productivity losses; – After the acute phase of the disease, the virus established inaparent persistent infections which constitute an economic burden to cattle exporters because of the restrictions placed on the export of FMDV-positive animals or animal products to disease-free areas; – Therefore the disease causes one of the major economic impacts in livestock industry. It is estimated that in South American countries, at least 25% of the reduction in livestock productivity is due to this disease, causing losses and expenses of 500 million dollars annually; – Variability of the virus during replication leads to variants often not neutralized by the vaccines in use. Variability is critical during persistent infections[4,5]; – The potential of new biotechnological strategies should be evaluated in order to develop:

a) highly sensitive and specific methods for detection of carrier animals;
b) precise techniques for the characterization of FMDV to establish the similarities between field and vaccine strains;
c) more effective vaccines: Conventional vaccines have been available since 1938 and in addition, 800 million doses are used annually, so that efficacy of new alternatives will have a good point of reference.

DIAGNOSTIC PROBLEM IN SUSPECTED APHTHOVIRUS DISEASE

The identification of carrier animals infected with FMDV is usually made by the isolation of FMDV of material obtained from esophageal-pharyngeal (OP) fluids, in tissue culture [6,7,8,9]. Moreover, screening for antibodies against the FMDV-infection associated antigen (VIAA)[10-13], the FMDV RNA polymerase [14,15], in animal sera is also carried out routinely. These tests are internationally accepted for import/export testing and as an epidemiological tool to determine the spread of FMD in animal populations. In practice, these methods do not always yield conclusive results. No clear correlations could be established between these methods suggesting technical problems with the OP fluid sampling and/or the tests used to detect the anti-VIAA antibodies. Virus isolation procedures appear to be successful in the acute phase of the disease with extensive virus replication, however persistent virus from OP samples is only occasionally recovered during the whole carrier state.

The identification of serum antibodies against the VIA antigen by the immunodiffusion test in agarose gels (IDAG)[10-12] is not sensitive enough. Attempts to increase the sensitivity through an enzyme-linked immunosorbent assay (ELISA)[16] raised questions with regard to the development of VIAA antibodies in cattle vaccinated and revaccinated with vaccines produced in baby hamster kidney (BHK) cells, containing high concentrations of non-purified FMDV-antigens. Since the VIA antigen used for these tests is only partially purified from BHK-infected cells, traces either of FMDV capsid polypeptides or BHK antigens may be recognized by sera of animals immunized with BHK-produced vaccines, leading to false positive tests.

Another significant limitation of these methods is the requirement of a high security laboratory unit for handling FMDV, which constitutes a problem, especially in FMD-free areas.

INTRODUCTION OF NEW DETECTION STRATEGIES

So far we have chosen two approaches to potentially overcome the mentioned limita-

tions. The first approach was based on the use of new diagnostic approaches for the detection of FMDV in OP samples which included: a) molecular nucleic acid hybridization techniques (dot blot, northern blot or in situ hybridization), using cloned viral DNA as a diagnostic reagent for detection of the viral RNA sequences and b) amplification of specific viral genomic fragments using the polymerase chain reaction (PCR). The second approach was to develop highly sensitive and specific methods to detect in sera of suspected carrier animals the presence of antibodies against FMDV-nonstructural antigens, including others than the VIAA traditionally used. Immunochemical techniques (ELISA, immunodot and immunoblotting) using as serological probes highly purified bioengineered VIAA as well as other bioengineered nonstructural antigens were attempted.

One major prerequisite for the introduction of these strategies as diagnostic tools in suspected persistent aphthovirus infections was the molecular cloning of the aphthovirus RNA genome in order to get a complementary DNA (cDNA) as a probe for the FMDV genome, to be used in molecular hybridization tests. Besides, the cloned cDNA is further used to obtain the bioengineered serological probes, by expressing defined genomic regions, coding for the different nonstructural proteins, in *E. coli.*

Intact 35S viral RNA was prepared, copied into cDNA using oligo (dT) as a primer and reverse transcriptase. The cDNA was then fractionated on an alkaline sucrose gradient. The largest cDNA fractions were further inserted through standard techniques into a bacterial plasmid.

Clones covering the whole coding region of the genome were obtained.

CLONED APHTHOVIRUS cDNA AS A DIAGNOSTIC REAGENT

If one addresses nucleic acid hybridization as a diagnostic tool for the detection of aphthovirus carriers a major prerequisite is the demonstration that the radioactively labeled cloned cDNA detects specifically viral nucleic acids and does not hybridize to total cell RNA. When radioactively labeled cloned aphthovirus cDNA corresponding to the genomic region coding for the viral polymerase was hybridized to RNA immobilized on nitrocellulose paper, extracted from OP samples of control or experimentally persistently-infected cattle, specific hybridization was found only for FMDV-infected tissue RNA and not for total tissue RNA. The minimal detection level under high stringency conditions, that we observed was 1 pg of genome FMDV-RNA. Thus, the use of cloned cDNA provides a valuable diagnostic tool for detection of carrier cattle.

A main advantage of the nucleic acid hybridization approach in suspected FMDV infections is given by the fact, that detection of different aphthovirus serotypes is possible by using just one cloned cDNA as a probe. The molecular basis for this finding is given by the high degree of nucleic acid sequence homology among the different serotypes for example in the genomic region corresponding to the viral polymerase. The exact typing of the implicated strain could also be carried out by using serotype-specific DNA fragments corresponding to the viral genome encoding for the viral coat proteins.

Hybridization directly in fixed tissues is another interesting approach to be considered. It is a worthwhile tool especially for studies of viral pathogenicity, since it permits the direct identification of the virus-infected target cells and an estimate of the proportion of cells carrying the viral genome within a section.

Other probes for veterinary applications include: Pseudorabies, Bovine herpes virus I, African swine fever, Bluetongue, Marek's disease, Anaplasma marginale, Babesia

and Enterotoxigenic *E. coli.*

AMPLIFICATION OF SPECIFIC APHTHOVIRUS GENOMIC RNA SEGMENTS

A major breakthrough for diagnostic procedures is given by the polymerase chain reaction (PCR)[17]. It consists on the capacity to amplify specific segments of DNA through an *in vitro* enzymatic synthesis of a specific DNA fragment using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. This synthesis is achieved through a repetitive series of cycles involving denaturation, primer annealing and extension of annealed primers.

Since primer extension products can serve as templates in the next cycle, there is an exponential accumulation of specific fragments whose termini are defined by the 5' ends of the primers, so that 20 cycles can yield about one million fold amplification.

For the detection of viral RNA it is necessary to first obtain a cDNA copy by the reverse transcription, which is then used as target DNA.

We have applied the PCR method to the amplification of defined segments from the genomic region coding for the structural polypeptides of partially purified FMDV-RNA of serotype O_1. Primers were chosen from highly conserved regions and purified. Successful amplification of fragments of 850 bp covering the whole VP_1 region and of fragments of up to 2000 bp covering the genomic region coding for the capsid polypeptides VP_3, VP_2 and VP_1, were obtained. In contrast, amplification of segments of more than about 2000 bp normally resulted in several low molecular weight bands and only rarely gave a specific reaction.

The specificity of the amplified material was confirmed by restriction enzyme analysis of amplification products of the correct molecular weight, and is being further confirmed by sequencing after subcloning into SP64.

Our future goal is to amplify FMDV-RNA segments from biological specimens such as cells persistently-infected with FMDV and biopsies from experimentally-infected animals. The method should be adequate for rapid diagnosis of FMD, even when minimal quantities of virus are present.

A great advantage of this extremely rapid and sensitive method for the detection of FMD is that nucleotide sequence information can be obtained directly from clinical samples without the prior need for virus amplification in cell culture, thus avoiding the possibility of *in vitro* induced selection and modification of virus populations.

DETECTION OF FMDV-ANTIBODIES IN SERA OF PERSISTENTLY-INFECTED ANIMALS

In order to overcome the limitations mentioned above, we have attempted the use of immunochemical techniques using bioengineered VIAA and other bioengineered aphthovirus nonstructural antigens.

Compared to the classical VIAA obtained from infected cell cultures, the use of bioengineered antigens offers several advantages: safety, does not require a high security laboratory for its production; yields, higher yields can be obtained in bacteria than in infected cells; and simplicity and costs, bacterial cultures require less space, time and effort than eukaryotic cell cultures and are significantly less expensive. In addition, sero-

logical probes can be prepared for nonstructural antigens which can not be purified from infected cells, so that the most suited nonstructural antigens of potential diagnostic significance can now be identified by studying the antibody response of the host to the different aphthovirus nonstructural proteins during different stages of persistence.

To obtain the most adequate bioengineered VIAA for use as a serological probe, we constructed a bacterial expression vector (carrying the inducible PL promoter of bacteriophage Lambda in front of a consensus Shine Dalgarno sequence) coding for the complete VIA antigen. The strategy was designed so that the polypeptide expressed would only have one additional amino acid the N-terminal methionine[18].

The recombinant polypeptide was expressed and the soluble fraction from the bacterial extracts was further purified by chromatography over a series of phosphocellulose column and poly(U)-Sepharose column. The identity of the purified protein was confirmed by immunoblotting with sera of convalescent animals. We further analyzed the potential of the bioengineered native VIAA to detect specifically anti-VIAA antibodies in sera of experimentally persistently-infected cattle.

Analysis by immunoblotting indicated that antibody binding to the bioengineered native VIA polypeptide was constant and it was detected in sera from infected cattle as early as at 7 dpi, giving a peak at 6 weeks postinoculation with the intensity of the bands decreasing gradually with time after infection, and being still positive at DPI 644, time by which all detections were negative by the classical IDAG test. In contrast to the results obtained previously[16], under the conditions used, no detectable reaction was obtained with sera from control cattle (obtained from FMD-free regions) and/or vaccinated cattle.

The induction of antibodies to other nonstructural proteins was analyzed by using a set of bioengineered antigens obtained by expressing defined regions of the genome in *E. coli*, as serological probes[19]. Western blot analysis with sera of experimentally infected cattle shows that although all antigens gave a positive reaction, antigens 3A and 3B gave the highest signal/noise positive detection when tested either with sera from convalescent or late persistently-infected cattle. Antibody induction to antigens 3A and 3B shows a peak 5 weeks later than that obtained for the VIA antigen, decreasing then gradually with the increasing weeks postinoculation, but to a lesser extent than for the VIA antigen. Again, none of the sera of FMD-free regions or from vaccinated cattle gave a positive reaction.

Our ultimate goal is to develop a rapid and simple assay for the detection of carrier animals. The use of additional nonstructural antigens together with immunoblotting, could provide a method for simultaneously screening a single serum for the presence of antibodies against multiple antigens which may fluctuate during the course of the disease.

Examples of other animals diseases which have immunochemical diagnostic assays include: Bluetongue, Salmonella, Pseudorabies, Parvovirus, etc.

MOLECULAR CHARACTERIZATION OF APHTHOVIRUS STRAINS

Control of FMDV 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 type 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. Therefore, the constant monitoring of field strains is essential to ensure that vaccines in current use are effective, i.e., contain strains sufficiently similar to those circulating in the field.

Several techniques for the analysis of nucleic acids and proteins are being used. Such techniques include nucleic acid analysis through RNA fingerprinting, and sequencing as well as protein analysis through SDS-PAGE, isoelectrofocusing, two-dimensional gel electrophoresis, etc. These biochemical methods together with the development of monoclonal antibodies and their use in ELISA tests provide ideal tools for the precise biochemical and antigenic characterization of active, evolving, vaccine and laboratory strains.

We are intensively involved in the biochemical and serological characterization of field and vaccine strains.

Fingerprinting is used routinely for comparing closely related strains, and so: evaluate genetic stability of strains during vaccine production, establish possible vaccine origin of field outbreak and monitor origin behaviour and fate of new strains. Examples of outbreaks caused by viruses of serotype C_3 which showed minor serological variations from the prototype strain C_3 Resende as well as very similar fingerprinting patterns, occurred in Argentina between 1982-1984[20]. The similarity of the isolated strains with that of the prototype strain, was taken as an indication that these strains had not circulated for long in the field and that they had been freshly introduced through escape of a laboratory or an incomplete inactivated vaccine.

The significance of the evolution of viruses in the field can be exemplified by the characterization of strains isolated from an outbreak caused by strains serologically identified as a C_3 variant which took place in Argentina during 1984 and up to 1986. This outbreak could not be controlled by the vaccines in use containing the prototype strain C_3 Resende [20]. Representatives of this outbreak were isolated, studied and later included in the vaccines in order to finally control the outbreak. Fingerprinting showed them to be significantly different from the prototype strain C_3 Resende. Moreover, relevant changes in the structural polypeptides could also be shown. Although the epidemiological origin of these strains has not been traced, it is possible that in endemic regions viruses could be maintained under conditions which they are replicating such as occurs in persistently-infected cattle. We have demonstrated that during persistence, variability is very critical. During this time, rapid evolution of viruses occurs. We found a clear cut relationship between the degree of genomic variations and the number of days postinoculation, indicating a gradual and progressive evolution of the strains during the persistent stage. In addition, we described a decreased reactivity of FMDV persisting at 63 dpi to a set of neutralizing monoclonal antibodies. Sequencing data also showed the accumulation of mutations which represented $2x10^{-2}$ substitutions/nucleotide/year, 60% of the changes resulted in changes of amino acids. Some of the changes occurred in amino acid residues 40-50 and 135-153, considered to be important immunological domains [5]. This fact demonstrates the high risk for the animals and for those susceptible hosts in the surroundings.

PROSPECTIVES FOR THE DEVELOPMENT OF NEW VACCINES

Although vaccination to prevent virus infections has been practiced for over two centuries, the process has changed relatively little since the time of Jenner. Most of the immunogens of choice used today are still killed or attenuated viruses. Recent attention has focused upon the design and production of vaccines consisting of non-viable (non-replicating) and non-infectious portions of the pathogenic agent that are still capable of eliciting a protective immune response.

Potential vaccine designs include:

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

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 VP_1; VP_1 isolated and used as a vaccine elicits neutralizing antibody responses and protects cattle and swine from infections;
- 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.

The aphthovirus structural gene coding for VP_1 was expressed in *E. coli*. Although a significant level of expression was obtained, the protein produced, evoked no neutralizing antibody response up to 30 days postrevaccination.

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 [21]. It becomes always more evident that VP_1 in the virion adopts a conformation which is highly dependent from a substantial interaction with the other structural proteins [22].

In the case of other infectious agents, expressed in prokaryotic systems, the first outstanding example of a genetically engineered bacterial vaccine which became commercially available was against the somatic pili of enterotoxigenic *E. coli* strains, the cause of diarrheal diseases in young livestock [23].

A recently developed baculovirus expression system utilizes the highly expressed and regulated *Autographa californica* nuclear polyhedrosis virus polyhedrin promoter modified for the insertion of foreign genes [24]. Encouraging results were obtained expressing bluetongue virus neutralization antigens VP_2 and VP_3 [25], pseudorabies gp 50 [26], Rift Valley fever virus antigens G-1 and G-2 [27] and simian rotavirus SA11 capsid antigen VP_6 [28].

SYNTHETIC PEPTIDES

It is fortunate that FMDV has been one of the most studied with regard to immuniza-

tion with peptide antigen and the results suggest that there could be a practical outcome to the work.

Several approaches predicted amino acid residues 144-159 and to a lesser degree 200-213 of VP_1 as good candidates for eliciting a neutralizing response [29-30].

According to these predictions we synthesized a series of peptides, covering various regions of the polypeptides, 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. Synthetic peptides representing each of the two potential immunogenic regions of VP_1 induced high levels of antibodies which recognized intact virus, but only residues 140-160 were protective. 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[31]. The potential importance of sequences 200-213 in enhancing the response of sequences 141-160 was recently suggested [32]. Moreover, a very encouraging result was obtained using tandem peptide sequences fused to bacterial proteins. Already protection of cattle and swine has been achieved with one injection of as little as 40μg of peptide. However, only limited animals were used in the trial [33].

A key issue in the field of peptide vaccines is the potency of the adjuvant. Several new adjuvants have been tried, including the use of what has been termed immunostimulating complex (ISCOM), in which the virus proteins are incorporated into cagelike structures by complexing them with saponin, a plant glycoside.

In the case of other animal infectious agents, encouraging results were obtained with vaccines against rabies and rotavirus [34-35].

LIVE ATTENUATED VACCINES

Live viral vaccines offer significant advantages over other types of viral vaccines, namely, induction of more effective local immunity, longer duration of immunity and spread of the vaccine strain among susceptible hosts within the population. Such vaccines, however, are at present only of limited use due to a) the possibility that an attenuated virus strain could revert to its more pathogenic form; b) potential susceptibility of hosts other than the one for which the vaccine is attenuated and c) an optimum equilibrium between pathogenicity and immunogenicity is not always obtained. Such limitations could not be overcome in the late fifties when these attenuated vaccines were developed [36].

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. Infectious DNA can be specifically modified to obtain attenuated 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 identification 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.

A remarkable feature was a common increased electrophoretic mobility of polypeptide 3A in the attenuated strains analyzed. Sequencing data indicates a genomic deletion of approximately 60 nucleotides, depending on the attenuated strain [39]. The potential relevance of this genomic region for determining the attenuated phenotype, is being further studied by introducing an equivalent deletion in an infectious cDNA clone, recently constructed by the laboratory of Dr. Ewald Beck [40].

With regard to other infectious agents, a modified virus strain of pseudorabies virus was obtained by engineering a mutation into the thymidine kinase (TK) gene so that the activity of TK is destroyed and thus the virus cannot multiply in the central nervous system of pigs [41]. Similarly the TK gene has been deleted from infectious bovine rhinotracheitis vaccine strains which would allow a similar development of a cattle vaccine [42]. Further attenuation of pseudorabies was obtained through deletions in the internal and terminal repeat region of the pseudorabies vaccine strains [43].

One of the major disadvantages of live attenuated vaccines is that the immune response which is elicited cannot be easily distinguished from the one provoked by natural infection so that many times disease control measures are complicated. One approach to overcome the diagnostic problem is through the introduction of specific gene deletions. Such an approach was effectively used for pseudorabies. The gene coding for the glycoprotein was removed from the live vaccine, which prevents antibodies being evoked to this glycoprotein and so allows vaccinated pigs to be identified from pigs naturally infected [44].

Another way to overcome this limitation is by combining the advantages of subunit and live attenuated vaccines through the use of vaccinia vectors.

Vaccinia virus behaves as a live-virus vaccine. Therefore it stimulates cell-mediated immunity, but has the advantage that certain selected gene sequences of a heterologous agent can be inserted and expressed. Moreover, due to its large DNA capacity, it is possible to construct multivalent vaccines for different serotypes of the same virus or even against entirely different pathogenic agents. The products obtained are properly glycosilated and transported to membranes and therefore mimic the native state [45].

The large number of animal hosts for vaccinia enables its use for immunization in veterinary medicine. Other advantages of this system include:

– it is cheap to mass produce;
– it is possible to use poxviruses specific for each animal species;
– no animal reservoir is known;
– it is stable in lyophilized form at room temperature;
– it can be administered by the oral route.

Significant results were obtained with live vaccine of recombinant vaccinia virus which expresses the glycoprotein of rabies virus. Racoons fed with vaccina/rabies recombinant virus developed rabies neutralizing antibodies and were resistant to rabies challenge up to 200 days after feeding [46].

After inoculation of live recombinant vaccinia-G protein of vesicular stomatitis virus (VSV), mice were protected against lethal encephalitis and cattle protection correlated with the level of neutralizing antibody produced following vaccination [47].

More recently veterinary researches have employed vaccinia virus recombinants as experimental vaccine against Rinderpest Sindbis, Marek's disease, fowl pox and feline leukemia.

An interesting strategy was attempted for FMDV, combining the synthetic peptide

approach with recombinant vaccinia virus. However no neutralizing antibody response was elicited after inoculation into rabbits [48].

The potential hazards of human and animal virus vector vaccines should be carefully investigated before they are released for field use. The occurrence of generalized vaccinia after administration of samllpox vaccine to asymptomatic carriers of human immunodeficiency viruses (HIVs) has been reported [49].

Other interesting alternatives include:

Infectious resistant cells

Consists on blocking the production of viral proteins by inserting antisense genes (DNA fragments complementary to the viral genome) into cells. So far, attempts to use this approach have been reported for AIDS treatment and for FMD.

Anti-idiotypes

Since the observations that anti-idiotypes can mimic foreign antigens, their potential utilization in vaccines has been pursued [50]. In fact, many groups reported induction of protective immunity in experimental animals upon administration of anti-idiotypes for parasites (*Schistosoma mansonii, Trypanosoma rhodesiensi*), viruses (polio type I and II, hepatites B) and bacteria (*Streptococcus pneumoniae* and *E. coli*). Their main potential is against infectious agents which evade neutralizing by antigenic variation and for carbohydrate antigenic determinants that cannot be genetically engineered.

CONCLUDING REMARKS

At present, several new approaches to the development of animal vaccines were undertaken. Although their potential is unquestionable, an overall success will depend on the identification and expression of protective epitopes, and a deeper understanding of the molecular definitions of virulence, immunological mechanisms and molecular biology of infectious agents. Further studies are required to explain why vaccines prepared with antigens obtained by genetic engineering or peptide synthesis have been of relatively poor immunogenicity in cattle and swine when compared with antigens obtained through classical inactivated vaccines. Very encouraging is the use of new diagnostic test, which are of great importance for veterinary medicine diagnostic and particularly with regard to detection of carrier animals.

ACKNOWLEDGMENTS

I would like to thank Ewald Beck, Paulo Augé de Mello, Ivo Gomes, Erika Neitzert, Beatriz H. Tiraboschi, Viviana Malirat, Maria Aparecida Affonso Boller, Pedro Jeovah Vieira Pereira and Ronaldo de Albuquerque for their scientific contributions and Carla Prete for typing this manuscript.

REFERENCES

1. LAPORTE, J.; GROSCLAUDE, J.; WANTYGHEM, J.; BERNARD, S.; ROUZE, P. *Compte Rendu Hebdomaire des Seances de L'Academie des Sci. Serie, D276:* 3399-3401, 1973.

2. BACHRACH, H.L.; MOORE, D.M.; MCKERCHER, P.D.; POLATNICK, *J. Immunol., 115:* 1636-1641, 1975.
3. CAVANAGH, D.; SANGAR, D.V.; ROWLANDS, D.J.; BROWN, F. *J.Gen.Virol., 35:* 149-158, 1977.
4. COSTA GIOMI, M.P.; GOMES, I.; TIRABOSCHI, B.; AUGÉ DE MELLO, P.; BERGMANN, I.E.; SCODELLER, E.A.; LA TORRE, J.L. *Virology, 162:* 58-64, 1988.
5. GEBAUER, F.; DE LA TORRE, J.L.; GOMES, I.; MATEU, M.G.; BARAHONA, H.; TIRABOSCHI, B.; BERGMANN, I.E.; AUGÉ DE MELLO, P.; DOMINGO, E. J. *Virol., 62:* 2041-2049, 1988.
6. BURROWS, R. *J. Hyg. ,64:* 81-90, 1966.
7. SUTMÖLLER, P. & COTTRAL, G.E. *Arch. ges. Virusforsch., 21:* 170-177, 1967.
8. LECUW, P.W.; VAN BEKKUM, J.G.; TIESSINK, J.W.A. *J. Hyg., 81:* 415, 1978.
9. STRAVER, P.J.; BOOL, P.H.; CLAESSENS, A.M.; VAN BEKKUM, J.G. *Arch. Ges. Virusforsch., 29:* 113-126, 1970.
10. HEDGER, R.S. *J. Hyg., 66:* 27-36, 1968.
11. COWAN, K.M. & GRAVES, J.H. *Virology, 30:* 528-540, 1966.
12. MCVICAR, J.W. & SUTMÖLLER, P. *Am J. Epidemiol., 92:* 273-278, 1970.
13. ALONSO, A.; AUGÉ DE MELLO, P.; GOMES, I.; ROSENBERG, F. *Bol. Centr. Panam. Fiebre Aftosa, 17/18:* 17-22, 1975.
14. POLATNICK, J. & WOOL, S.H. *J. Virol., 40:* 881-889, 1981.
15. POLATNICK, J. *Arch. Virol., 84:* 269-275, 1985.
16. ALONSO, A.; GOMES, M.P.D.; MARTINS, M.A.; SÖNDAHL, M.S. *Prev. Vet. Med.,* 9: 233-240, 1990.
17. MULLIS, K.; FALDONA, F.; SCHARF, S.; SAIKI, R.; HORN, G.; ERLICH, H. *Cold Spring Harbor Symposio on Quantitative Biology,* v. 51, n. 1, 267-273, 1986.
18. NEITZERT, E.; BECK, E.; AUGÉ DE MELLO, P.; GOMES, I.; BERGMANN, I.E. *Virology*, In Press, 1991.
19. STREBEL, K.; BECK, E.; STROHMAIER, K.; SCHALLER, H. *Journal of Virology, 57:* 983-991, 1986.
20. BERGMANN, I.E.; TIRABOSCHI, B.; MAZUCA, G.; FERNANDEZ, E.; MICHAILOFF, C.A.; SCODELLER, E.A.; LA TORRE, J.L. *Vaccine,* 7, 1988.
21. BACHRACH, H.L.; MOORE, D.M.; MCKERCHER, P.D.; POLATNICK, J. *In Proceedings, International Symposium of Foot-and-Mouth Disease* (II), (C.Mackowiak R.H. Regamey, Eds). *Symposia Series in Developments in Biological Standardization,* Karger, Basel. *35*: 150-160, 1976.
22. MELOEN, R.H.; BRIARE, J.; WOORTMEYER, R.J.; VAN ZAANE, D. J. *Gen. Virol., 64:* 1193-1198, 1983.
23. HILDEBRAND, D. *Animal Nutrition Health* , 20-22, 1983.
24. VERNE, A.L. & SUMMERS, M.D. *Biotechnology, 6:* 47-55, 1988.
25. INAMURA, S.; GHIASI, H.; ROY, P. *J. Gen. Virol., 68:* 1627-1635, 1987.
26. KURODA, K.; HAUSER, C.; RUDOLF, R.; KLENK, H.D.; DOERFLER, W. *Embo J. , 5:* 1359-1365, 1986.
27. TAKEHARA, K.; MIN, M.K.; BATTLES, J.K.; SUGLYEMA, K.; EMERY, V.C. *Virology, 169:* 452-457, 1989.
28. ESTES, M.K.; CRAWFORD, S.E., PERRARANDA, M.E.; BURNS, J.; CHAN, W.; ERICKSON, B.; SMITH, G.E.; SUMMERS, M.D.*J. Virol., 61:* 1488-1494, 1987.
29. STROHMAIER, K.; FRANZE, R.; ADAM, K.H. *J. Gen. Virol., 59:* 295-306, 1982.
30. PFAFF, E.; MUSSGAY, M.; BOEHM, H.O.; SCHULZ, G.E.; SCHALLER, H. *Embo J., 1:* 869-874, 1982.
31. BERGMANN, I.E.; AUGÉ DE MELLO, P.; SCODELLER, E.; LA TORRE, J.L. *Mem. Inst. Butantan, 50, (Supl.),* 1988.
32. DIMARCH, R.; BROOKE, G.; GALE, C.; CRACKNELL, V.; DOELT, T.; MOWAT, N. *Science, 232:* 639-641, 1986.

33. BROEKHUDSEN, M.P.; BLOM, T.; VAN RIJN, J.; POUWELS, P.H.; KLASEN, E.A.; FASBENDER, M.J.; ENGER-VALK, B.E. *Gene, 49:* 189-197, 1986b.
34. ARNON, R. *TIBS, 11:* 521-524, 1986.
35. BARROS-CUESTA, F.; PETIT, A.; PERY, P.; FEDON, J.; GARNIER, J.; COHEN, J. *Ann. Inst. Pasteur/Virol., 138:* 437-450, 1987.
36. BERNAL, C.L.; CUNHA, R.G.; HONIGMAN, M.N.; GOMES, I. *Proc. 5th Pan-Amer. Cong. Vet. Med. Zoot., 1:* 42-58, 1966.
37. ALMOND, J.W.; STANWAY, G.; CANN, A.J.; WESTROP, G.D.; EVANS, D.M.A.; FERGUSON, M.; MINOR, P.D.; SPITZ, M.; SCHILD, C.C. *Vaccine, 2:* 177-184, 1984.
38. RACANIELLO, V.R. & BALTIMORE, D. *Proc. Natl. Acad. Sci., 78:* 4887, 1981.
39. GIRAUDO, A.T.; BECK, E.; STREBEL, K.; AUGÉ DE MELLO, P.; LA TORRE, J.L.; SCODELLER, E.A.; BERGMANN, I.E. *Virology, 177:* 780-783, 1990.
40. ZIBERT, A.; MAASS, G.; STREBEL, K.; FALK, M.M.; BECK, E. *Journal of Virology, 64:* 2467-2473, 1990.
41. KIT, S.; KIT, M.; LAWHORN, B. et al. High *Technology Route to Virus Vaccines,* 82-99, 1985.
42. KIT, S.; SHEPPARD, M.; ICHIMURA, H. et al. *Am. J. Vet. Res., 48:* 780-793, 1987.
43. KIT, S.; KIT, M.; MCCORNELL, S. *Vaccine , 14:* 55-61, 1986.
44. KIT, S.; GAVI, H.; GAIRES, J.D. et al. *Archives of Virology, 86:* 63-83, 1985.
45. DESMETRE, P. *Biofutur,* 27-31, 1986.
46. RUPPRECHT, C.E.; WIKTOR, T.J.; JOHNSTON, D.H. et al. *Proc. Natl. Acad. Sci. , 83:* 7947-7950, 1986.
47. MACKETT, M.; YILMA, T.; ROSE, J.K.; MOSS, B. *Science, 227:* 433-435, 1985.
48. NEWTON, S.E.; FRANCIS, M.; BROWN, F.; APPLEYARD, G.; MACKETT, M. *Vaccines, 86:* 303-309, 1986. *Cold Spring Harbor Laboratories.*
49. REDFIELD, R.P.; WRIGHT, D.C.; JAMES, W.D.; JONES, T.S.; BROWN, C.; BURKE, D.S. *N. Engl. J. Med., 316:* 673, 1987.
50. EICHMANN, K.; EMMRICH, F.; KAUFMANN, S.H.E. *CRO Crit. Rev. Immunol.,* 1987.