Contagious bovine pleuropneumonia vaccines: the current situation and the need for improvement


Summary: The control of contagious bovine pleuropneumonia (CBPP) has been clearly identified by the Organisation of African Unity/Inter-African Bureau of Animal Resources as a priority.

In the first part of this article, the authors introduce the past and present vaccines, based on the two classic strains, T1 and KH3. They describe the guidelines for vaccine production technology, and the quality control requirements for CBPP vaccines of the Office International des Epizooties.

The failure of the currently used T1-SR vaccine to provoke satisfactory immunity in cattle, particularly in the newly infected areas of Africa, is pointed out. Other shortcomings of the current CBPP vaccines are also highlighted.

Thus, there is a need to improve CBPP vaccines and the authors propose detailed emergency measures to address this problem.

In the second part of the article, a subunit approach using immunostimulating complex technology is outlined. The authors emphasise the importance of current research in cell-mediated immunity and immunopathology, which is aimed at improving the efficacy of CBPP vaccines.


INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an economically important disease of cattle caused by Mycoplasma mycoides subsp. mycoides (small colony, bovine biotype) (MmmSC). At some stage in history, CBPP has occurred in Europe, Asia and

* Centre de Coopération Internationale en Recherche Agronomique et pour le Développement-Département d'Elevage et de Médecine Vétérinaire (CIRAD-EMVT), B.P. 5035, 34032 Montpellier, France.

** Pan African Veterinary Vaccine Centre (PANVAC), Debre Zeit, P.O. Box 5536, Addis Ababa, Ethiopia.

*** Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Department of Microbiology, Virology Section, P.O. Box 585, BMC 751-23, Uppsala, Sweden.
the United States of America (USA) (41). Through a policy of restriction of animal movement, slaughter and compensation, CBPP has been eradicated from the USA and most of Europe. Australia also eradicated the disease, using a combination of vaccination, control of cattle movement and slaughter. CBPP remains endemic in Africa to this day. For a variety of reasons (mainly socio-cultural, geographic and economic), the measures that have been used successfully elsewhere may not be feasible in Africa at this time. Thus, it seems that vaccination is the only realistic method of choice for the control of CBPP in endemic zones in Africa. As a result of the current importance of CBPP, the directors of the Veterinary Services and of the veterinary vaccine production laboratories in Africa have designated CBPP vaccination programmes as the second priority after rinderpest. This is clearly indicated in the final recommendations of the two co-ordination meetings held by the Organisation for African Unity (OAU), Inter-African Bureau of Animal Resources (IBAR) and the Pan African Rinderpest Campaign (PARC), in Debre Zeit (Ethiopia) in March 1996 and in Dakar (Senegal) in June 1996.

Apart from the aborted JP-28 programme in West Africa, there has been no major systematic and well co-ordinated vaccination campaign against CBPP in Africa, as is the case for rinderpest.

Consequently, noting the resolution of the third African conference of Ministers responsible for livestock (1990), the OAU/IBAR has now identified the control of CBPP as a priority, to be included in the next phase of PARC. However, an important prerequisite of a well co-ordinated vaccination campaign is the availability of data on the production and quality of the vaccines for the target disease. Until the establishment of the Pan African Veterinary Centre (PANVAC) CBPP vaccine quality control testing programme, such data were not available for the continent. Secondly, except for the Office International des Epizooties (OIE) guidelines (31), there were no standard operating procedures (SOPs) for quality control of CBPP vaccine. With these shortcomings in mind, in 1990, PANVAC organised a workshop in Bamako, Mali, on CBPP vaccine production and quality control (35). That meeting, attended by experts in CBPP and specialists of the national CBPP vaccine-producing laboratories, recommended that:

a) all CBPP vaccines to be used in the PARC programme should be certified for quality by PANVAC

b) the T1SR vaccinal variant of M. mycoides subsp. mycoides be adopted as the only standard seed strain for CBPP vaccine production in Africa. This recommendation was based on the following points:
   - that T1SR had immunogenic potency similar to that of its parent, T1/44
   - that T1SR causes fewer post-vaccinal reactions than T1/44
   - that antigen yields are 10 to 1,000 times greater in media containing streptomycin than in media without added streptomycin
   - that the property of streptomycin resistance could be used as a marker for this vaccinal strain

c) PANVAC should solicit the assistance and collaboration of the Centre de Coopération Internationale en Recherche Agronomique et pour le Développement-Département d'Élevage et de Médecine Vétérinaire (CIRAD-EMVT) to establish an African depot of the Food and Agriculture Organisation (FAO) reference CBPP master seed representing the 49th passage of the T1SR variant, from which PANVAC would
prepare a fully tested seed lot to be distributed to all CBPP-vaccine producing laboratories in Africa. The tests on the seed lot should include both in vitro and in vivo examination.

d) all African CBPP vaccine manufacturers should operate a CBPP vaccine seed lot system and take steps to produce a freeze-dried product, in order to increase the shelf life of the vaccine.

e) PANVAC should prepare a common stock of immune serum against the seed lot and distribute it to manufacturers and national quality control laboratories to be used in the identity tests for CBPP vaccine.

CIRAD-EMVT and PANVAC have since produced a T1SR seed lot which has undergone the requisite in vitro testing, and awaits cattle safety and potency testing before being distributed to CBPP vaccine producer laboratories. PANVAC has also prepared a stock of CBPP hyperimmune serum against the PG1 reference strain, which can be supplied to laboratories for use in their in-house quality control tests.

PAST AND PRESENT VACCINES

Historical background

The history of vaccination against CBPP dates back to the era of Willems, when it was experimentally established that parenteral (subcutaneous) inoculation of CBPP-infective 'lymph' into areas of thick connective tissue in cattle protected those cattle against contact challenge (41). However, following the administration of such an inoculum, inflammatory reactions occurred at the injection site, which often led to cattle mortality. The need therefore arose to look for ways of making the inoculations safer. Further studies of CBPP showed that, whereas the disease was explosive at the onset (causing high morbidity and mortality), later stages of the course of the outbreak were marked by mild disease. It thus appeared that the agent of CBPP (for some as yet unclear reason) became less virulent with an increasing number of passages either in vivo or in vitro (3, 5).

These observations were applied to the development of the present-day seed strains for CBPP vaccine. Thus, the current live attenuated vaccine strains are the products of the serial passage of M. mycoides subsp. mycoides in eggs and subsequently in broth culture media.

It is only on the African continent that routine vaccinations against CBPP are presently carried out. Over the years, the vaccines used were based on two strains, namely: T1 or KH3J, either as liquid or as lyophilised preparations (4, 16, 18). Each of these two strains has been adapted to grow in media containing streptomycin and the resulting variants have been designated T1SR and KH3J-SR, respectively (40). Inactivated vaccines against CBPP have been tested experimentally with varying results but such vaccines have not been tested in the field (12, 37). At least one publication reported protection of cattle with an inactivated oil-adjuvanted CBPP vaccine (12).

Strain T1 was isolated in Tanzania from a mild case of CBPP in 1951. It was then serially passaged in embryonated eggs up to the tenth passage and used extensively in East Africa in CBPP vaccination campaigns during the 1960s. The avianised vaccine, however, caused unacceptable reactions (thought to be due to allergic
reactions to egg proteins) and use was therefore discontinued. The strain was further passaged in chicken embryos up to the 44th level, from which broth culture CBPP vaccines are now produced and used either as liquid or freeze-dried preparations.

Strain T1/44 has been passaged three times by CIRAD-EMVT in media containing increasing concentrations of streptomycin (41). The resulting variant (T1SR) is now used in the production of combined CBPP-rinderpest vaccine, because this variant grows in the presence of streptomycin, which is contained in rinderpest vaccine culture medium. Strain T1/44 confers protection to vaccinated cattle which lasts for at least one year, but this strain still has some residual virulence to some taurine breeds of cattle.

T1SR causes relatively fewer post-vaccination reactions than T1/44. Reports in the literature indicate that T1SR also confers immunity similar to that of T1/44, but quantitative data on immunogenic comparisons of the two vaccine seed strains are lacking (39).

First isolated in the Juba area of southern Sudan, strain KH3J was passaged 88 times and then used for the manufacture of CBPP vaccines. As for T1/44, additional passages of KH3J in media containing streptomycin yielded a streptomycin-resistant variant named KH3J-SR, which has also been used in the manufacture of combined CBPP-rinderpest vaccine. KH3J-based CBPP vaccines were used extensively, particularly in west and central African countries. Because it is the most attenuated strain, KH3J does not cause any side effects and general reactions, but is poorly immunogenic, conferring protection to cattle which lasts for less than six months. For this reason, KH3J and its KH3J-SR progeny are no longer used to produce CBPP vaccines.

Vaccine production

The production of CBPP vaccine should be based on a seed lot system (31, 45) and governed by strict adherence to the principles of good manufacturing practice (GMP).

Various types of media are used but the common media are modified Newing’s tryptose broth (popularly known as Gourlay medium) and medium F66 (4, 35). Vaccine production protocols vary from one laboratory to another. Generally, sterilised media (pH 7.8-8.0) are heavily seeded with the vaccine mycoplasma inoculum at its exponential growth phase (roughly one part inoculum to 10 parts medium), and then incubated at 37°C for two to three days, with or without agitation. Experience at PANVAC has shown that agitation of the culture increases the rate of mycoplasma growth but does not significantly affect the final titre yield of the culture.

Vaccine cultures are harvested just before peak growth. Stabiliser is added, the culture is dispensed into freeze-drying vials and then lyophilised in shelf freeze dryers. At the end of the lyophilisation cycle, the vaccine vials are stoppered in a vacuum, or after backfilling with a sterile and dry inert gas, e.g. nitrogen.

OIE requirements stipulate: 'the minimum titre per vaccine dose must be at least $10^7$ viable mycoplasmas. It is recommended that production laboratories take into account the local transport conditions, and supply vaccines having titres of at least $10^8$ mycoplasmas per dose' (31).

The method prescribed for titration is the tube method. Comparative titrations at PANVAC have shown the microtitre technique to have comparable sensitivity and this has been selected as the method of choice (see PANVAC SOP for technique). The minimum requirements for titre per vaccine dose are considered to be the passmark.
Whatever culture procedures are used, it is important to ensure that:
- the ingredients for media formulation are of the highest possible quality
- a growth curve of the vaccine seed in the culture vessels being used in each production laboratory is established. This will allow determination of the optimum time to harvest the antigen
- the pH of the culture is not allowed to fall below 6.8 because mycoplasma titre falls rapidly with any further decrease in pH
- the medium destined for culture of streptomycin-resistant variants should contain streptomycin at a final concentration of 0.1 mg/ml
- the master seed lot must not be submitted to more than three passages to produce final vaccine because immunogenicity decreases as the number of passages increases
- lyophilisation should be commenced as soon as possible following vaccine culture harvest; always keeping the vaccine cool during the addition of stabiliser, dispensing and loading onto freeze drying shelves.

Only one producer laboratory in Africa currently manufactures CBPP vaccine in liquid form; the rest produce freeze-dried products. Liquid vaccine has a shelf life of only one month, whereas lyophilised vaccine can be stored for at least two years at -20°C with a minimum drop in titre. It is for this reason that the PANVAC workshop on CBPP vaccine production and quality control urged all producers to take steps to manufacture freeze-dried products (31).

**Vaccine quality control**

The OIE requirements for CBPP vaccine stipulate that seed lots and final vaccine batches should be tested for *in vitro* potency (titration), sterility, identity, innocuity (on laboratory animals), safety and potency in cattle (31).

Between November 1991 and June 1994, 180 batches of monovalent CBPP vaccine from six laboratories and 96 batches of combined CBPP-rinderpest vaccine from three laboratories were tested for quality at PANVAC. These represented a total of 21.8 and 26.308 million doses of monovalent CBPP and combined CBPP-rinderpest vaccines, respectively.

The quality assessment procedures used were described in the PANVAC SOPs for CBPP vaccine (21). It is demonstrated that the yearly pass rates for monovalent CBPP vaccine improved from 17% in 1991 to 85% in 1994. The corresponding values for combined CBPP-rinderpest vaccine for 1993 and 1994 were 31% and 53.3%, respectively.

The most common reason for vaccine batch failure is failing to establish or respect the *MmnsSC* growth curve in laboratories manufacturing CBPP vaccines. Bacterial and fungal contamination are also a chief reason for vaccine failure.

In the case of combined CBPP-rinderpest vaccines, a significant number of vaccine batches were rejected due to *Mycoplasma* and/or *Acholeplasma* contamination. The mollicute contaminants have been identified as *A. laidlawii*, *M. arginini*, *M. orale* and *M. hyorhinis*. The sources of some of these contaminants have been traced to use of contaminated rinderpest virus seed, contaminated animal serum used during vaccine preparation and also mycoplasma-infected cell cultures used for vaccine production.

*M. orale* is of human origin and the presence of this contaminant suggests that personnel involved in vaccine manufacture can constitute a major source of
mycoplasma contamination, unless there is strict adherence to the codes of good laboratory practice and good manufacturing practices (GLP/GMP).

It has previously been reported that most CBPP vaccine production laboratories do not readily attain the OIE recommendation of delivering vaccine at $10^8$ viable mycoplasmas per cattle dose, and that the titres of passed vaccine batches are only marginally above the minimum OIE requirement of $10^7$ mycoplasmas per cattle dose (31).

Contagious bovine pleuropneumonia vaccine strain: present situation

In December 1995, the Animal Health Service of the FAO indicated through a circular that, in newly infected areas in eastern and southern Africa, the currently used T$_1$-SR CBPP vaccine may not be as effective as would be expected of a T$_1$/44 strain when applied at the regular field dosage. Some doubts are evident as to the efficacy and identity of the T$_1$-SR strain.

Consequently, the FAO advises that: ‘Until experimental evidence (including efficacy tests in cattle) is obtained for the current variant of the T$_1$-SR strain, T$_1$/44 should continue to be the vaccine strain of choice’.

Immediate measures

Immediately after this announcement, a group of scientists from OAU/IBAR, CIRAD-EMVT, the FAO and PANVAC proposed a comprehensive programme for immediate improvement in this situation, including the following:

\( a \) preparation of a T$_1$/44 master seed bank to dispatch, upon request, to the African laboratories manufacturing CBPP vaccines

\( b \) preparation of batches of two experimental T$_1$/44 and T$_1$-SR vaccines, for use in a cattle safety and potency test protocol to be designed

\( c \) a vaccination-challenge protocol to be implemented in different African countries to report definitively on the comparative efficacy of the T$_1$-SR and T$_1$/44 strains.

This programme was introduced and discussed during a workshop organised by the European Union (EU) (DG VIII), 24-25 May 1996, during the annual OIE General Session in Paris, to report on the strategies and guidelines of choice for the control and eradication of CBPP.

The need for urgent implementation of the three different steps of this programme is clearly indicated in the final recommendations of the two above-mentioned OAU/IBAR-PARC co-ordination meetings held in Debre Zeit and in Dakar.

In June 1996, a PANVAC-EMVT CBPP reference seed strain, denominated T$_1$/44/2 (batch PAN-002), was prepared at PANVAC, Debre Zeit, from T$_1$M44 (16/11/70, Kevevapi, Muguga, Kenya) with two passages in liquid medium. The product has passed all PANVAC and EMVT in vitro quality control tests and was immediately supplied by PANVAC to vaccine-producing laboratories in Africa. According to information from W. Masiga (Director of OAU/IBAR), T$_1$/44 had, in the past, satisfactorily undergone safety and efficacy tests in cattle in Muguga.
In July 1996, two experimental T₁/44 and T₁-SR vaccines for use in a vaccination-challenge protocol were prepared at EMVT. They have passed all PANVAC and EMVT in vitro quality control tests.

In collaboration with PANVAC, CIRAD-EMVT has proposed a comprehensive vaccination-challenge protocol to compare the efficacy of the T₁-SR and T₁/44 strains in experimental conditions to the OAU/IBAR, to the EU and to a bilateral co-operation funding agency for special funding. These experiments should be conducted in different African countries, with challenge from different virulent strains, to report on possible modifications of virulence factors in regard to west Central African and east southern African strains, since an increase in the virulence of the latter could provide an explanation of these new outbreaks in East and southern Africa. On the other hand, taking into account the possible difficulties of experimental transmission of the disease, it is prudent to conduct these protocols on multiple different geographical sites and regions.

These experiments will be implemented immediately, as soon as financing is available.

THE NEED FOR IMPROVEMENT IN CONTAGIOUS BOVINE PLEUROPNEUMONIA VACCINES

General considerations

Despite its long usage, CBPP vaccine still has certain constraints, particularly those relating to production, quality control, immunogenicity and use at the field level. Some of these unresolved issues, and possible suggestions of means of addressing such shortcomings, may be outlined as follows.

Vaccine seed strain identity testing

The OIE norms for CBPP vaccine prescribe that CBPP vaccine seed lots and final vaccine batches should be tested for identity. The test of choice is the mycoplasma growth inhibition test on agar medium. The advantage of this test is its high specificity and simplicity. However, the test is not highly sensitive, and cannot distinguish between wild and vaccinal strains of *M. mycoides* subsp. *mycoides* (SC). The agar gel immunodiffusion, biochemical and colony characterisation tests also suffer from the same shortcomings.

At EMVT, various other tests are usually performed, such as the immunoperoxidase test with specific monoclonal antibodies (MAbs) and polymerase chain reaction (PCR) with specific primers to identify *MmmSC*.

The test for streptomycin resistance also seems to be imprecise. Some T₁/44 vaccines tested at PANVAC were found to be partially resistant to streptomycin.

These observations call for a detailed molecular analysis of the CBPP vaccine strains, e.g. by use of MAbs and/or sequencing. The results obtained would then be evaluated with the aim of designing specific and sensitive methods of determining identity, which would thus be useful not only for vaccine quality control testing but also for epidemiological studies of CBPP, as well as in monitoring field vaccination campaigns (39).
**Immunogenicity and virulence**

Unlike many other live vaccines, CBPP vaccine confers protection to cattle of relatively short duration (one year for strain T₁ and six months for KH₃J). This implies that the least attenuated strains are better immunogens than those which have been subjected to many passages. Unfortunately, the least attenuated strains still retain some residual virulence, which can cause adverse post-vaccination reactions. It is established that the serial passage of CBPP vaccinal strains decreases both virulence and immunogenicity. Hence, some residual virulence is necessary for the development of strong immunity in vaccinated cattle. It is for this reason that the OIE norms require that the number of passages between a tested master seed lot and the final vaccine should not exceed three, in order to maintain the seemingly delicate balance between the virulence, immunogenicity and safety of the CBPP vaccine. With such a narrow production margin, the operation of a proper seed lot system becomes difficult.

A search for alternative CBPP vaccines with an efficacy equal to or better than the current vaccines still remains an option. For instance, killed and adjuvanted vaccines have been reported to give a satisfactory level of protection to vaccinated cattle (11, 12), but this has not been confirmed by extensive field studies.

**Mycoplasma antigen yields**

At present, most CBPP vaccine producers are unable consistently to attain 10⁸ mycoplasmas per dose in the final freeze-dried CBPP vaccine. This is due either to low mycoplasma titre of the vaccine culture or high losses during downstream processing. This would seem to suggest that the optimised culture conditions which ensure high mycoplasma titre yields should be defined. The suitability of fermenters for this purpose may be worthy of investigation, but possible antigenic drift must be taken into consideration. Post-harvest losses of titre can be minimised by shortening the handling period prior to lyophilisation and selection, which should also be optimised, and by employing good freeze-drying preservatives. Skim milk is the best, both in terms of efficacy of preservation and cost and availability.

**Thermostability of contagious bovine pleuropneumonia vaccine**

Present CBPP vaccines, whether liquid or lyophilised, should be kept cool during storage, transportation, reconstitution and use in the field. This is necessary because *M. mycoides* is readily inactivated by high temperatures. In order to ensure that the minimum number of mycoplasmas in each dose of the vaccine is preserved, CBPP vaccines must always be kept cool, hence the absolute requirement of a cold chain (9, 49). Maintenance of a cold chain is an expensive undertaking. It is already established that a regulated and prolonged lyophilisation regime of rinderpest vaccine, using certain chemical stabilisers, engenders some measure of thermostability in the freeze-dried vaccine (25). This type of rinderpest vaccine has been used in places with high ambient temperatures, and the users reported good seroconversion rates.

If this is the case, perhaps various lyophilisation cycles can be investigated with CBPP vaccine, and the resulting vaccine subjected to thermal degradation tests, in order to determine whether such a vaccine is able to resist high temperatures.
Potency assessment of contagious bovine pleuropneumonia vaccine

The virulence and immunological mechanisms involved during *M. mycoides* subsp. *mycoides* SC infection, or following vaccination against CBPP, are not fully elucidated. Consequently, there is no *in vitro* test at present which can be used to assess accurately the immune status of bovines with respect to CBPP.

As a result, the OIE norms require that potency assessment of CBPP vaccines should be achieved by virulent contact challenge of vaccinated animals. The results obtained are then evaluated using the clinical, pathological and serological scoring system of Hudson and Turner (15, 16).

This procedure is expensive, time-consuming and does not always give reproducible or consistent results.

These facts would seem to suggest strongly that cheaper methods of vaccine efficacy testing must be sought, e.g. laboratory animal models. In this respect, it will be noted that Smith (46) has demonstrated the production of mycoplasmaemia and passive immunity in mice following inoculation with *M. mycoides* var. *mycoides*. These observations merit further investigation.

A detailed understanding of the immunology of CBPP might permit the design of *in vitro* immunological tests with a strong *in vivo* correlation, which can be used as cheaper and quicker methods for vaccine potency testing.

Research on CBPP vaccine development is currently performed in the context of a new network on CBPP, sponsored by the EU (DG VIII). This network is divided into four components, two of which are devoted to the development of new vaccines and to immunopathology and the immunological response (cell-mediated immunity) in CBPP.

A subunit approach towards an improved contagious bovine pleuropneumonia vaccine

**Introduction**

The immune responses induced by the present CBPP vaccines are relatively short-lived and regular vaccinations are required. Vaccinated animals exposed to natural infections maintain virulent mycoplasma in the lymph nodes for extended periods of time and this situation causes recurrent severe disease. In addition, these animals are sources for the spread of the pathogen. Thus, new vaccine strategies should be considered and special attention should be paid to the use of new and better adjuvanted formulations.

**Mechanisms of pathogens to evade immune mechanisms**

One major problem is the property and capacity of CBPP to cause chronic infections persisting through life, which is due to the ability of the pathogen to evade the immune system. Generally, vaccines are lacking against infectious agents causing persistent or chronic infections. The mechanisms which the pathogens use to persist in the body under the pressure of an immune response are different from agent to agent, but these mechanisms are built into the structure of the micro-organism. It has been shown, for example, that a herpesvirus, i.e. Epstein Barr virus (EBV), has a nucleotide sequence analogous with that of IL-10 (24), a cytokine which down-regulates the T-helper 1
The TH1 type of immune response considered to be a basis for protective immunity to this virus. In general, a vaccine concept based on subunits facilitates the possibility of evading components which have a negative regulatory effect on the ensuing immune response. Secondly, when protective antigens are identified, it might be possible to clone these antigens and possibly to design an efficient antigen presentation system. Thirdly, subunits can be combined with an efficient antigen delivery system to modulate the immune response properly, thereby facilitating the development of a protective vaccine.

**The immunostimulating complex**

The immunostimulating complex (ISCOM) is formulated as a particle which combines a multimeric presentation of antigen with a built-in adjuvant. Consequently, it can be placed within the category of particulate adjuvant formulations. The ISCOM particle, first described by Morein et al. (27), is a cage-like structure of about 40 nanometres (nm) (1, 34) (Fig. 1), composed of the saponin adjuvant Quil A, cholesterol, antigen and phospholipids (23). The ISCOM, with its incorporated antigens, is a very stable construct which remains intact after lyophilisation, limited freeze-thawing and prolonged storage at +4°C. Storage limitations are generally determined by the stability of the antigen. In pharmaceutical terms, the ISCOM can be considered as a carrier for a combination of antigen and adjuvant, targeted at antigen-presenting cells (APCs) and the lymphatic system.

Generally, membrane proteins are incorporated into ISCOMs, and the first ISCOMs were prepared with membrane proteins derived from the virus. However, membrane proteins from bacteria should likewise be incorporated.

**FIG. 1**

Electron micrograph of immunostimulating complexes

An electron micrograph of negative stained immunostimulating complexes containing proteins of *Mycoplasma mycoides* (magnification × 120,000)
The initial phase of immunisation

For classic adjuvants, such as aluminium hydroxide or oil adjuvants, the depot effect at the site of injection is considered to be an important immunopotentiating factor. In contrast, antigens in ISCOMs are rapidly transported from the site of injection to the draining lymphatic organ. Local inflammatory reactions following the injection of ISCOMs are, therefore, negligible compared to those of aluminium hydroxide (47) or oil adjuvants, and granuloma are not observed following injection of ISCOMs. A temporary redness may be seen locally after the subcutaneous injection of high doses of ISCOMs. After the intraperitoneal immunisation of mice with radio-labelled antigens via ISCOMs, a comparatively high proportion of the antigens become cell-associated and are transported to the spleen, where these antigens remain for a longer period of time than the same antigens in the micelle form (53).

The unique property of ISCOMs of inducing an immune response over both major histocompatibility complex (MHC) classes, I and II, trigger speculation about the internalisation of the ISCOM-borne antigens and their intracellular transport. The general belief in regard to antigen presentation is that, after injection of non-replicating (exogenous) antigens, these antigens are taken up by the APCs. In these cells, the antigens enter the endosomatic-lysosomal pathway, processed by proteolytic enzymes at an acid pH in the lysosomes to peptides of various length, ranging from 13 to 20 amino acids, before association with the MHC class II antigen (44). Subsequently, the processed antigen is transported to the cell surface for interaction with T-helper cells. Endogenous antigens are produced in the antigen-presenting cell either because of infection, e.g. with a virus, or as a cancer antigen. These antigens are processed by cytosolic proteolytic enzymes to nonapeptides and then transported by aid of the transportation molecules TAP1 and TAP2 to the endoplasmatic reticulum. Here, they join the MHC class I molecules for further transport to the cell surface, where they are available for interaction with CD8+ cytotoxic T-cells (20, 26, 36, 38).

Using electron microscopy, ISCOM particles carrying influenza virus antigens could be directly located in endosomes (53). In macrophages, a significant proportion of the antigen was detected inside the cells, not only bound to the plasma membrane or to other membrane structures (Fig. 2), but also outside vesicles in the cytosol (51).

Macrophages internalise ISCOM-borne antigen more efficiently than monocytes or naive B-cells. Splenic dendritic cells were also less active than macrophages but more efficient than monocytes or naive B-cells in taking up ISCOM-borne antigen. Both naive B-cells and dendritic cells proved to be more efficient than peritoneal macrophages and monocytes in their capacity to present the antigen internalised by aid of ISCOMs and to stimulate T-memory cells.

Cytokine studies showed that ISCOMs and the matrix of the ISCOMs induce macrophages to produce IL-1 (52) and IL-6. In vitro, the production of membrane-bound IL-1 reaches a peak after 16 h of stimulation (51, 52).

ISCOM-borne antigens induce a strong cell-mediated immune response, measured by cytokine production. The T-cell products IL-2, INF-γ, IL-4, IL-5 and IL-20 are important factors in determining the class and isotype distribution in the antibody response, as well as for determining regulatory and effector T-cell functions, including the expansion of cytotoxic lymphocyte clones. Cell culture fluids from splenocytes originating from mice immunised with influenza virus ISCOMs, cultured with the
same ISCOMs, contained high levels of IL-2 and INF-γ. In comparison with non-adjuvanted micelles containing the same antigens, IL-4 production was allowed and IL-10 production was down-regulated (28).

*Induction of protective immunity*

Although vaccines have existed for almost 200 years, comparatively little has happened as regards new developments in the presentation of antigen in the vaccine. Even today, most licensed vaccines consist of whole micro-organisms: live, attenuated or killed. In spite of this, vaccines have been of the utmost medical importance to both humans and animals in, for example, the eradication of smallpox or as a prophylaxis against diseases such as polio or rabies in humans. Vaccines can also be of great economic importance, as is the case with foot and mouth disease in cattle and swine. A long list of effective vaccines can be produced.

However, new techniques are required for presenting antigen in vaccines aimed at preventing persisting infection. Such techniques should be based on the crucial components, i.e. the protective antigens. Reconstituted in an immunogenic form, these
antigens, together with an immunomodulator, e.g. adjuvant, may create an immunogen which can evade the strategy of the micro-organism to evoke an immune response, allowing the micro-organism to persist in the host. In fact, experimental ISCOM vaccines have been prepared which induced protective immunity where conventional techniques failed. For example, an ISCOM vaccine containing the envelope glycoprotein of feline leukaemia virus (FeLV) proved to induce protective immunity and neutralising antibody to FeLV in cats (1, 32, 33). EBV causes persistent infection in humans. In parts of China, EBV causes a nasopharyngeal carcinoma, and this virus is the most common cause of cancer. The cottontop tamarin monkey is used in an experimental model for EBV-induced tumours.

Recently, a mycoplasma ISCOM vaccine was also constructed. The vaccine contained membrane protein from *Mycoplasma gallisepticum*, which induced protection as measured by gross air sac lesions (48). In contrast to the commercial vaccines tested, no side effects encompassing growth retardation have been observed with the ISCOM vaccine.

_Proparation of an experimental immunostimulating complex contagious bovine pleuropneumonia vaccine_

*Mycoplasma mycoides* contains a great number of membrane proteins which have been processed to be incorporated into an ISCOM (Fig. 3). A number of carbohydrate-containing molecules were also detected in the ISCOM prepared from this mycoplasma. So far, it is not possible to say whether these carbohydrate molecules are part of the glycolipids or glycoproteins or both. As shown in Figure 1, ISCOM

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**FIG. 3**

_Sodium dodecyl sulfate polyacrylamide gel electrophoresis on Mycoplasma mycoides proteins_

Protein pattern in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie Brilliant blue. Lane A: whole mycoplasma; Lane B: immunostimulating complexes prepared after solubilisation with non-ionic detergent; Lane C: molecular weight markers.
structures are readily formed, and through analytical ultracentrifugation, their incorporation into ISCOMs is verified. The immunogenicity of these ISCOMs is very high in mice with serum titres measured by enzyme-linked immunosorbent assay (ELISA), after one immunisation, ranging from 100-150,000, and after one boost, titres of 600,000 are obtained (B. Morein and I. Abusugra, unpublished findings). The specific antibodies were distributed among all immunoglobulin G (IgG) subclasses, encompassing a high IgG2a titre, indicating that a TH1-type of response is involved. These experiments continue and the cell-mediated immune response is now being evaluated. Preliminary experiments indicate that strong proliferative responses are induced in mice.

The first experiments performed in cattle show that a dose of 50 μg of mycoplasma antigens in ISCOMs induces high serum antibody responses after one immunisation, and that strong booster responses are obtained. The antibodies induced after one immunisation revealed a growth inhibitory effect in an in vitro assay.

In various reports, it is stated that ISCOMs induce a mucosal immune response following intranasal or oral modes of administration. The mycoplasma ISCOMs were also found to induce potent serum antibody, as well as secretory IgA responses in lung extract following intranasal immunisations of mice (B. Morein and I. Abusugra, unpublished findings) (Fig. 4). Obviously, it would be of interest to test whether mucosal administration of mycoplasma ISCOMs would improve the mucosal immune response in cattle, as well as deliver improved immune protection. In practical terms, it might be difficult to design an intranasal vaccine for cattle.

**Fig. 4**

**Intranasal immunisations of mice with Mycoplasma mycoides subsp. mycoides immunostimulating complexes**

Immunoglobulin response in lung extract measured by enzyme-linked immunosorbent assay after two intranasal immunisations of mice, six-weeks apart, with different doses of immunostimulating complexes containing *Mycoplasma mycoides* subsp. *mycoides* antigens.
Prospects for future contagious bovine pleuropneumonia vaccines

An experimental CBPP vaccine based on ISCOM technology is sufficiently well developed to be evaluated in cattle against experimental as well as natural challenge infections. The outcome of such experiments will reveal whether protection is obtained or not. In the case of a positive response, future work will focus on obtaining an efficient vaccine production system, in tandem with studies aimed at characterising a protective immune response. In the case of a negative response, an increased effort should be made to characterise protective immunity, in order to enable the construction of a vaccine delivery system inducing the right kind of immune response with selected protective antigens (29). In such a way, Jan et al. (17) have identified and purified two lipoproteins located in the plasma membrane of MmmSC and considered as important surface antigens in mycoplasmas. ISCOM technology offers this possibility by incorporating alternative immunomodulators with the selected protective antigens into a particle for the induction of a defined and desired immune response.

Immunopathology and cell-mediated immunity in contagious bovine pleuropneumonia

The actual live vaccines used against CBPP present some limitations, due to the short duration of protection (less than one year) and the risks associated with the stability of the vaccinal strain after numerous passages. Therefore, there is a need to develop new vaccines which overcome these limitations by identifying the relevant antigens involved in protection against CBPP.

Vaccine efficacy should rely on the type of immune response induced in the animal host by the introduction of an MmmSC strain. Therefore, one must know which aspects of the immune response are responsible for protection and the onset and duration of protection. An immunological memory is defined by the acquired property of the immune system to respond more rapidly and more intensively to a second antigen stimulation. The development of this immunological memory is the prerequisite for good protection since, generally, resting memory cells have a long lifespan. Therefore, the improvement of vaccines for CBPP implies the need for studies to understand the immunopathological mechanisms leading to a state of disease or immunity during this infection.

Until now, very few studies have been performed concerning the immunopathology of CBPP. Furthermore, most of these studies have focused on humoral responses rather than on cell-mediated immunity. However, while humoral immune responses play a major role in protection against systemic infections, they only reflect an early cell-mediated reaction, which occurs on mucosal surfaces and the immediate surrounding lymph nodes.

CBPP is characterised by a primary broncho-pneumonic invasion due to inhalation of infective droplets, an early secondary lymphatic involvement and tertiary vascular lesions, which lead to the characteristic necrosis and encapsulation (50). Therefore, to understand the immunopathological mechanisms involved in CBPP, and according to the kinetic of the disease, specific and non-specific immunity must be checked at the lung level.

Theoretically, only rarely do bacteria enter the lung alveoli. Most are rapidly removed from the upper respiratory tract (i.e. the trachea and bronchi). Among the first barriers against infection are the mucus, which blocks the adherence of bacteria to
epithelial cells, and the ciliary movement of these cells, which removes the trapped foreign particles. However, a pathogenic effect has been described for *M. mycoides* subsp. *capri* (belonging to the same cluster as *MmmSC*) when this mycoplasma interacts with the epithelial membrane. Owing to its ability to release hydrogen peroxide (another pathogen factor), *M. mycoides capri* inactivates the ciliary activity of tracheal epithelial cells (6). The next non-specific defence mechanism in the host is the phagocytosis of bacteria occurring in the alveoli. The predominant phagocytic cells of normal, non-inflamed lung tissue are alveolar macrophages. Following infection, other cells, such as polymorphonuclear cells, migrate into this infected area. The outcome of the initial interaction with phagocytic cells seems to play an important role in determining the progression of infection. It was observed for both arginine and glucose-using mycoplasma species that they have the capacity to attach to phagocytic cells and to survive on the cell surface without being engulfed, unless opsonizing antibodies are present (8). Indeed, it has also been reported that *M. bovis* and *M. dispar* are killed by bovine alveolar macrophages in the presence of bovine antisera (14). However, the presence of a capsule, as in the case of *MmmSC* and *M. dispar*, may render the mycoplasma resistant to phagocytosis (43). Owing to their capacity for attachment, *M. bovis* and *M. dispar* are also able to inhibit the phagocytosis of other organisms. Indeed, these mycoplasmas inhibited the killing of *Escherichia coli* by bovine neutrophils (14). However, studies still have to be performed to determine the proper interaction between *MmmSC* and bovine alveolar macrophages. Bacteria which survive some of the non-specific defence reactions (e.g. mucociliary movement and phagocytosis) colonise deeper tissue sites.

Macrophages, being the main population in the lung, are not only important in non-specific immunity (phagocytosis). Macrophages also have a major role in specific immune responses as APCs. A study of the cellular composition of broncho-alveolar lavages, in *Bos taurus* and *Bos indicus*, established the presence of 80% to 85% macrophages, 10% lymphocytes (Tα/ß, Tγ/δ and B) and 5% to 10% of other cells (L. Dedieu and A. Bensaid, unpublished findings). Antigen processing and presentation by APCs is needed for the induction of cell-mediated immunity and then for the T-cells to respond to the presence of infectious agents. Class II MHC molecules, which are expressed by macrophages, are specialised for presenting exogenous proteins, which they encounter after endocytosis. Class II molecules interact with T-cell receptors on CD4 T-helper cells. The CD4 T-cells have a central role in the immune response, as helper cells for T-dependent antibody production through the production of cytokines.

As previously mentioned, there are few data concerning *MmmSC* and cell-mediated immunity. At the level of peripheral blood, lymphocyte transformation (LT) responses to *MmmSC* antigens have been studied. No LT response was observed when samples were taken from cattle infected with attenuated *M. mycoides* (14). The same results were observed with vaccinated animals. Two out of six animals developed an LT response in the presence of mycoplasma membrane antigens (T1 strain); none of these animals presented a positive intradermal reaction. However, both the LT test and production of an intradermal reaction gave positive results in cattle experimentally infected with a virulent strain. A contradictory report showed an *MmmSC*-induced immunosuppression as Roberts *et al.* observed the partial or nearly complete inability of lymphocytes from *MmmSC*-infected cattle to respond to a T-cell mitogen (42). However, more recent reports have shown that neither intradermal reaction nor lymphocyte proliferation correlates with protective immunity (14), and this applies to
other lung diseases (19). Concerning the type of T-cells involved in the immune response against CBPP, no published data are yet available. However, in a set of experimental infections recently conducted with the Afade strain, the lymph node and peripheral blood mononuclear cells from cattle recovering from CBPP responded to an in vitro stimulation with killed mycoplasma. In this case, the T½44 and Afade strains were used as antigens. B- and T-cells of the CD4 subtype responded, whereas, when using live mycoplasma, no stimulation could be observed. Lymphocytes incubated with live micro-organisms in general did not respond to mitogens (L. Dedieu and A. Bensaid, unpublished findings), except for those of one animal, which recovered from infection where the inhibition was only partial.

The presence of γ/δ T-cells in lungs and their rapid infiltration into inflammatory lesions could indicate that these cells have a role in the first line of defence against infectious pathogens. A study on the cell composition of broncho-alveolar lavages (BAL) (L. Dedieu and A. Bensaid, unpublished findings) and of peripheral blood mononuclear cells (7), performed with adult cattle, has shown the presence of γ/δ T-cells from 1% to 10% in the BAL and from 1% to 5% in blood. Until now, the nature of the specificity and immunological functions of these T-cells has remained unclear. Recently, the role of the T-cells in the immune response to mycobacterial infections has been described, as these γ/δ T-cells have been found to be highly responsive to mycobacterial antigens (2, 13). It was reported that, in contrast to α/β T-cells, the γ/δ T-cells appear to recognise only constituents associated with the whole organism and are not restricted by class I or II molecules. However, it is generally agreed that APCs are required for γ/δ T-cell activation. Indeed, mononuclear phagocytes appear to be effective APCs for M. tuberculosis-responsive γ/δ T-cells and it was demonstrated that these γ/δ T-cells have the potential to express cytotoxicity for those APCs and are capable of secreting a variety of cytokines (2).

Therefore, basic research on the immune mechanisms of protection and pathogeny should focus at present on the type of host response. This should be defined by studying the macrophages/monocytes, polymorphonuclear cells and lymphocyte subpopulations recruited in lungs, lymph nodes and blood.

A preliminary study has been conducted to search for a possible MmmSC-superantigen, since different mycoplasmas have been recognised as being polyclonal mitogens for both B- and T-cells (30). Studies conducted with MmmSC demonstrated that no lymphocyte proliferative response due to this mycoplasma was observed on any lymphoid tissues used (L. Dedieu and A. Bensaid, unpublished findings). Therefore, the presence of superantigen-like molecules in MmmSC is excluded.

Among the cytokines produced in the lungs, of particular interest are TNFα, TGFβ and IFNγ. TNFα is produced by activated macrophages, and also by other cells. It is considered as a major inflammatory mediator and might therefore contribute to the inflammatory process encountered in CBPP. Indeed, the presence of TNFα has been shown to increase in pulmonary alveolar macrophages from animals infected with Corynebacterium pseudotuberculosis, Pasteurella haemolytica or lentiviruses (10). At a local inflammatory site, vascular endothelial cells can react to TNFα in a thrombogenic pattern by producing procoagulant activity and necrosis of tissues. Indeed, thrombosis was described as an early event in the pathogenesis of CBPP (with numerous changes in haematological data) and necrosis occurs when arteries are affected (22). TGFβ stimulates the proliferation and activities of connective tissue cells, whereas this cytokine has anti-proliferative effects on most other cells, including
epithelial, endothelial and lymphoid cells. TGFβ is a potent immunosuppressive cytokine, which suppresses cell-mediated as well as humoral immunity. TGFβ can also function as an IgA switch factor, causing activated B-cells to secrete this isotype. The involvement of TGFβ in the progression of the pathogenesis of CBPP cannot be disregarded in respect of the formation of the sequestra and immunosuppression. IFNγ is produced by activated T-cells, such as CD4 T-cells, and can regulate antibody production by B-cells. IFNγ is a potent activator of macrophages and greatly augments their microbicidal activities. IFNγ also has an important immunoregulatory function, due to an ability to modulate the expression of class I and II MHC molecules on a variety of cell types, including B lymphocytes, macrophages and epithelial cells, allowing these cells to become active in antigen presentation and the induction of specific T-cell immunity.

Since antibodies exert protective effects at various levels (e.g. inhibition of attachment, inhibition of growth or metabolism, opsonization, promotion of complement killing, etc.), the humoral response must also be evaluated at a local and systemic level. The serum antibody response of MmmSC-infected cattle follows the usual course of IgM, IgG and IgA isotype-switch progression. Passive transfer of convalescent serum to naive cattle could confer protection against subcutaneous challenge with MmmSC (14). Host defences, such as phagocytosis and complement-dependent lysis, are mediated by antibodies produced at the local level. Indeed, for M. bovis-infected calves, the local humoral response consists of a predominance of IgA and IgG in the respiratory secretions. Immunity to M. bovis after vaccination with killed organisms was related to the antibodies in lung washings rather than to serum, but in this case IgG was the predominant antibody. Also observed was an accumulation of lymphocytic cells in the lungs of these infected calves. These cells in the infected tissues and the lymph nodes draining them are the major source of local antibody production (14).

In conclusion, understanding the complexity of the interactions between the multiple cells of the immune system and the roles of the regulatory factors produced by these cells will help to explain the immunopathology of CBPP. A comparison of lymphocyte recruitment, proliferation and cytokine release among infected, recovered and vaccinated animals will define both the protective responses and the pathogenic responses. Since the development of an immunological memory is the prerequisite for effective protection, memory T-cells should be particularly studied. Lymph nodes, which provide cells for the first line of defence, are the main organs in which immune responses take place and, more particularly, where memory cells are recruited. These immune memory T-cells can be used to screen for mycoplasma antigens obtained by protein fractionation. The identification of proteins involved in protection will then allow the development of new vaccines.

CBPP vaccines could be improved through various approaches. The first method consists of modifying the existing vaccines to eliminate elements acting as virulence factors, which can drive the host reactions towards the pathogenic outcome, while maintaining the same level of immunogenicity. The other approaches require the identification of immunodominant T-cell stimulatory peptide epitopes from selected proteins. These antigenic epitopes could then be delivered in new attenuated vaccines or in an optimised vehicle, e.g. as an adjuvant-based subunit vaccine, a live recombinant vaccine or as nucleic acid. Consideration should also be given to vaccine delivery, which will be influenced by the choice of vaccine and the desired immune
response. Future vaccines should also allow discrimination between infected and vaccinated animals. Therefore, safe marker systems must be incorporated into new vaccines.

**CONCLUSION**

CBPP is one of the main constraints for livestock in Africa.

Vaccination remains the method of choice for the eradication of this disease.

The present vaccines are not completely satisfactory. There is, especially in the newly infected areas of eastern and southern Africa, a problem of failure of immunity with the currently used T1-SR vaccine strain.

Emergency measures will help to ameliorate the situation but, in any case, with the present vaccines, duration of immunity is incurably short, and the border between attenuation and loss of immunogenicity of the vaccine strains is not clearly defined.

Thus, improvements to the CBPP vaccines are essential.

The strategy for an effective vaccine includes the selection of protective antigens in the form of a subunit vaccine, for instance, and ISCOMs should be an interesting way to construct a new generation of vaccines. It is expected that ISCOMs will aid in improving the immune response, reducing considerably the dose of antigens and diminishing the risk of toxicity or allergy.

On the other hand, immunological investigations for CBPP over the years have been confined to the development of serological diagnostic techniques, without necessarily understanding the relevant immunological mechanisms involved in protection. However, it is quite obvious that the humoral response is not the sole component of immunity for CBPP.

First, a better understanding of the immunopathology of the disease is necessary. Moreover, research on cell-mediated immunity should help in the screening of candidate antigens, to improve the efficacy of the CBPP vaccines.

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Résumé : La lutte contre la péripneumonie contagieuse bovine a été clairement définie comme une priorité par l’Organisation de l’unité africaine/Bureau interafricain des ressources animales.

Dans la première partie de cet article, les auteurs présentent les vaccins anciens ou encore utilisés produits à partir des deux souches classiques T1 et KH3J. Ils rappellent les directives relatives à la technologie de production des vaccins contre la péripneumonie contagieuse bovine ainsi que les conditions de contrôle de leur qualité fixées par l’Office international des épizooties.

Le vaccin actuellement en usage, produit avec la souche T1-SR, ne confère pas aux bovins une immunité satisfaisante, notamment dans les zones récemment infectées d’Afrique. Les insuffisances des autres vaccins antipéripneumoniques sont également examinées.

Ces vaccins doivent donc être améliorés ; les auteurs proposent des mesures d’urgence spécifiques.


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Resumen: El control de la perineumonía contagiosa bovina (PCB) ha sido identificado como una clara prioridad por la Organización para la Unidad Africana/Oficina Interafricana para los Recursos Animales.

En la primera parte de este artículo, los autores presentan las vacunas empleadas en el pasado y en la actualidad, basadas en dos cepas clásicas, T1 y KH3J. Exponen asimismo las líneas directrices que rigen la tecnología de fabricación de las vacunas, y los requisitos en materia de control de calidad establecidos por la Oficina Internacional de Epizootias para las vacunas contra la PCB.

Subrayan luego la incapacidad de la vacuna T1-SR actualmente empleada para conferir una inmunidad adecuada al ganado, en especial en las zonas de reciente infección de África. Los autores ponen de relieve asimismo otras deficiencias de las vacunas actuales contra la PCB.
Es evidente pues la necesidad de introducir mejoras en las vacunas contra la PCB. A tal efecto, los autores proponen un detallado conjunto de medidas de urgencia.

En la segunda parte del artículo se exponen las grandes líneas de un posible enfoque basado en la utilización de subunidades mediante la tecnología de complejos inmunoestimulantes. Los autores hacen hincapié en la importancia de la investigación actual en el campo de la inmunidad mediada por células y de la inmunopatología, orientada a la consecución de vacunas más eficaces contra la PCB.


REFERENCES


