PART 1

Overview: the present state of veterinary vaccine development

The induction of immunity by veterinary immunoprophylactics

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Infectious disease continues to be one of the most important constraints on the efficient production of farm livestock in both developing and developed countries. While vaccination and the therapeutic or prophylactic use of drugs both play an important role in animal disease control, vaccination is increasingly being viewed as the more sustainable option. This view is influenced not only by the potential that vaccination offers for greater economic efficiency but also by the concerns that have been raised about the selection of drug-resistant pathogens and the potential harmful effects of drug residues in animal products and the environment. Vaccination has had a major impact on the control of epidemic viral diseases of livestock such as foot-and-mouth disease and rinderpest. However, there are many other important diseases for which efforts to develop effective methods of vaccination have been unsuccessful.

The advent of recombinant DNA technology in the early 1980s created exciting new opportunities to produce vaccines based on the use of expressed products of cloned genes. However, only a few such vaccines have been successfully developed. In retrospect, it can be seen that the immediate expectations of the new technology were unrealistically high, given the limited knowledge of the immunology of many of the target diseases and of how antigens are processed and recognized by the immune system. In the intervening decade there have been major advances in immunology which, coupled with further developments in the application of DNA technology, now provide a strong conceptual framework for the rational development of new vaccines.

This chapter will consider recent developments in immunology that are pertinent to understanding how the immune system controls infections and will discuss their implications for contemporary approaches to vaccine development.

APPROACHES TO VACCINE DEVELOPMENT

Most of the current veterinary vaccines are based on the use of either killed organisms or their products or live attenuated organisms. The development of these vaccines has not relied on knowledge of the immune responses that mediate immunity. Significant advances have been made primarily by the development of new culture techniques, improved attenuation procedures and better adjuvants. While there is some scope for further efforts to develop vaccines along these lines, there are many diseases for which the more empirical methods are unlikely to be successful.

Two main approaches to vaccine design can be considered using modern molecular technologies, namely the production of attenuated mutant organisms by deliberate molecular manipulation and the identification of antigenic components of pathogens that can be used to induce protective immune responses (these components are known as subunit vaccines). Unlike the traditional vaccine

strategies, the ability to exploit these new approaches to vaccine development is dependent on an intimate knowledge of the molecular structure of the target pathogens and an understanding of the mechanisms of immunity.

PROCESSING AND RECOGNITION OF ANTIGENS

Immune effector mechanisms

Studies in laboratory animal model systems have demonstrated that the immune system can respond in a number of different ways to control an infection. The type of response elicited by a pathogen depends largely on the nature of the organism and its site of replication within the host. In general, organisms that establish systemic infections and replicate extracellularly are controlled by antibody responses.

Secretory antibody responses also play an important role in the control of infections at mucosal surfaces. By contrast, cell-mediated immune responses are generally more important in controlling organisms which replicate intracellularly. The T lymphocytes that participate in cell-mediated immune responses may exert their effector function in a number of different ways. They may kill infected cells or release cytokines, which inhibit growth of intracellular organisms or which recruit and activate accessory cells such as macrophages, to perform these functions. Indeed, a number of different mechanisms may operate against the same organism. Stimulation of T lymphocyte responses is also essential to provide help, again in the form of cytokines, for the production of antibody by B lymphocytes. Hence, T lymphocytes have a pivotal role in the induction of virtually all specific immune responses. The way in which antigen is processed and recognized by the immune system determines the type of T cell response that is induced.

Recognition of antigen by B and T lymphocytes

The antigen recognition structures on B and T lymphocytes, namely immunoglobulin (Ig) and the T cell receptor (TCR) are both generated by a process of gene rearrangement whereby each of the two chains that make up the molecules is produced by bringing together two or three variable sequences, from a pre-existing library of variable genes, with a constant sequence to form a functional gene (Cooper and Burrows, 1989; Davis and Bjorkman, 1988). This mechanism, together with the further diversity created by a combination of different variable regions in Ig heavy and light chains and TCR alpha and beta chains facilitates the generation of a very large repertoire of B lymphocytes and T lymphocytes, each with a unique antigen recognition specificity.

Despite the similarity in structure of Ig and TCR molecules, B and T lymphocytes differ fundamentally in the way they recognize antigen. Immunoglobulins, whether on the surface of B lymphocytes or as secreted antibodies interact directly with foreign antigen, usually in the form in which it is initially encountered by the host, i.e. as an intact organism or as molecules released from the organism. Recognition of antigens by antibody is, therefore, often dependent on the conformational integrity of the molecules. By contrast, T lymphocytes only recognize antigens after they have been degraded and presented on the surface of other cells (Brodsky and Guagliardi, 1991). These antigen-bearing cells may be cells infected with foreign organisms or "professional" antigen-presenting cells, such as macrophages and dendritic cells, which have ingested antigen. The processed antigen, which is in the form of short peptides of less than 20 amino acids, is associated with major histocompatibility complex (MHC) molecules on the surface of the antigen-

presenting cell (Bodmer, 1984). The T cell receptor does not react with antigen alone but rather recognizes a combination of the antigenic peptide and the associated MHC molecule (Townsend and Bodmer, 1989).

The special role of MHC molecules

The MHC consists of a set of closely linked genes, many of which encode molecules involved in antigen processing and presentation (Trowsdale, 1993). There are two main types of MHC molecules, namely class I and class II. The most striking feature of these MHC molecules is the high degree of polymorphism they display among individuals of a species (Bodmer, 1984; Trowsdale, 1993). Class I molecules are heterodimers composed of a polymorphic heavy chain and a non-polymorphic light chain (β_2-microglobulin), the latter encoded outside the MHC. They are expressed on most cells of the body. Class II molecules are also heterodimers, both polypeptides being encoded within the MHC, but their expression in healthy animals is confined mainly to "professional" antigen-presenting cells, namely macrophages, dendritic cells and B lymphocytes. In most mammalian species examined, each class is encoded by two gene loci and, in each instance, both alleles are expressed.

While it has been recognized since the early 1970s that class I and class II molecules are involved in presenting antigen to T cells, the precise molecular and structural basis of presentation was not elucidated until the late 1980s. A key event was the resolution of the structure of class I molecules by X-ray crystallography (Bjorkman *et al.*, 1987). This revealed a prominent cleft in the membrane-distal part of the molecule, which subsequent studies have shown to be the site of antigen binding. A similar structure has been described for class II MHC molecules (Brown *et al.*, 1993; Stern *et al.*, 1994) although the antigenic peptides that associate with class II are longer (13 to 17 amino acids) than those bound to class I (eight to ten amino acids). Much of the sequence polymorphism in MHC molecules occurs in and around the peptide-binding region and, although this variation does not affect the overall structure of the peptide-binding groove, it results in subtle differences that influence the nature of the peptides that each molecule will bind. Thus, each individual class I molecule tends to bind a different repertoire of peptides (Sette *et al.*, 1987; Rothbard and Gefter, 1991), so that T cells from animals expressing different MHC molecules will often recognize different epitopes from the same pathogen, and in some cases these epitopes may be on different proteins.

Although this variation might be expected to result in quantitative differences in the immune response to pathogens, and hence differences in susceptibility to disease, there are relatively few well-documented examples of strong associations of MHC with susceptibility to infectious disease in outbred species. This probably reflects the large number of potential T cell epitopes in most pathogens and the fact that most animals in an outbred population will be heterozygous and therefore will express several class I or class II molecules. Variations in the epitopes that are selected in individual animals may, however, affect the strain specificity of the T cell response if some of the epitopes vary between pathogen strains (Vitiello and Sherman, 1983).

As already indicated, the TCR recognizes a combination of self MHC molecule and bound peptide. Since the region of the MHC molecule recognized by the TCR, i.e. the peptide-binding groove, is polymorphic, each T cell will only recognize an antigenic peptide associated with a particular MHC molecule. This

phenomenon, known as MHC-restriction (Doherty, Blanden and Zinkernagel, 1976), has important practical implications for studies of T cell responses in outbred animals; T cells from one animal will recognize antigen presented on the animal's own antigen-presenting cells but will not recognize the same antigen on presenting cells from another animal of a different MHC phenotype.

Until recently, the capacity of a given MHC molecule to bind a large number of different antigenic peptides was difficult to explain. However, studies carried out over the last four years, involving the isolation and sequencing of peptides bound to class I molecules, have helped to resolve this issue. The heterogeneous mixture of peptides isolated from an individual class I molecule were found to be conserved at one or two residues, usually at positions 2 and 9, and these conserved amino acids were shown to be essential for binding the peptides to the respective MHC molecule (Matsumura *et al.*, 1992; Rammensee, Falk and Rotzschke, 1993). Thus, the amino acids at these positions represent an MHC binding motif, the antigenic specificity of the peptide being determined by amino acids at other positions. Information on the binding motif of a particular MHC molecule can be used to predict possible T cell epitopes within proteins of known amino acid sequences. There is some evidence that similar characteristics determine the binding of peptides to class II molecules (Rudensky *et al.*, 1992) although this has proved difficult to substantiate.

T cell subpopulations recognize antigen processed in different subcellular compartments

The identification of the cell surface molecules, CD4 and CD8, as markers for the two major subpopulations of T cells in mammals was followed by the demonstration that $CD4^+$ T cells recognize antigen presented by class II MHC molecules whereas $CD8^+$ T cells recognize antigen presented by class I MHC molecules. These interactions are accompanied by binding of the CD4 and CD8 molecules to conserved regions on the presenting class II and class I MHC molecules, respectively. $CD4^+$ T cells were shown to mediate help for B cell responses and delayed-type hypersensitivity reactions whereas $CD8^+$ T cells were responsible for cell-mediated cytotoxicity of virus-infected cells. However, for some time the factors that determined whether an antigen was presented by class I or class II MHC molecules were unclear. This was resolved by studies which showed that class I and class II molecules bind peptides generated within different subcellular compartments of antigen-presenting cells. Antigens derived from organisms that replicate in the cytoplasm of cells are degraded by proteases within the cytosol and the resultant antigenic peptides are transported into the endoplasmic reticulum where they associate with newly synthesized class I MHC molecules destined for the cell surface (van Bleek and Nathenson, 1992). By contrast, organisms or proteins taken into antigen-presenting cells by phagocytosis or endocytosis undergo enzymatic degradation within endosomes and associate within an endosomal compartment with newly synthesised class II MHC molecules transported from the Golgi apparatus before being expressed on the cell surface (Brodsky, 1992; Unanue, 1992).

These alternative routes of antigen processing are known as the endogenous and exogenous pathways, respectively. Clearly, processing of antigens by the endogenous pathway and presentation by class I will be confined to organisms, such as viruses and some bacteria and protozoa, that replicate intracellularly (in the

cytoplasm). The killing of such infected cells by $CD8^+$ T lymphocytes can occur early in the replication cycle and thus represents an effective means of limiting multiplication of the pathogens.

Heterogeneity of $CD4^+$ T lymphocyte function

Mosmanm *et al.* (1986), working with mouse T cell clones, described two types of $CD4^+$ T cell, termed Th1 and Th2, that were distinguished by the cytokines they produced. This and subsequent studies established that activated Th1 cells secrete interleukin 2 (IL-2) and interferon-γ (IFN-γ) but not IL-4, IL-5, IL-10 and IL-13, whereas the converse applies to activated Th2 cells (Mosmann and Coffman, 1989). A similar dichotomy has been reported for human T cells (Weiranga *et al.*, 1990).

Studies of murine models of parasitic infections have proved invaluable in elucidating the biological significance of the differences in Th1 and Th2 cells. Infection of mice with *Leishmania major*, or immunization with *Leishmania* antigens, can induce either Th1 or Th2 T cell responses, depending on the strain of mouse and route of immunization. Th1 responses result in the control of infection and immunity, whereas Th2 responses lead to enhanced disease (Liew, 1990). This is just one of a growing number of examples in which the outcome of infection is strongly influenced by the cytokine profile of the responding T cells. The induction of Th1 responses in mice is associated with the activation of macrophages, the production of antibody of the IgG_{2a} isotype and the detection of delayed-type hypersensitivity reactions, while Th2 responses give rise to eosinophilia and production of antibody of the IgG_1 and IgE isotypes. The latter characteristics feature prominently in many helminth infections and there is evidence that Th2 responses are beneficial for the control of enteric nematode infections (Else and Grencis, 1991; Urban *et al.*, 1992).

Studies of the biological activities of the cytokines produced by Th1 and Th2 cells have demonstrated strong cross-regulation operating between the subsets: IFN-γ produced by Th1 cells inhibits the induction of Th2 responses and both IL-4 and IL-10 have inhibitory effects on the induction of Th1 responses (Mosmann *et al.*, 1991; Fitch *et al.*, 1993). Thus, the initial induction of a strong Th1 or Th2 response will tend to inhibit responses by the reciprocal subset. Nevertheless, in some infections, notably *Schistosoma mansoni* in mice, an initial Th1 response is followed by a switch to a Th2 response (Pearce *et al.*, 1991). The switch is believed to reflect the presence of antigens in the parasite eggs with a strong propensity for inducing Th2 responses. This may represent a deliberate strategy by the parasite to favour survival of the adult worms. The parameters that determine whether an antigen will stimulate a strong Th1 or Th2 response are poorly understood. However, there is evidence that the biochemical nature of the antigen and the type of antigen-presenting cell in which it is presented to T cells are important contributory factors.

IMPLICATIONS FOR VACCINE DEVELOPMENT

Induction of antibody responses

In the early 1980s, a series of studies demonstrated that antibodies raised against intact proteins recognized short peptide fragments of the proteins (Geyson, Meloen and Barteling, 1984). These observations encouraged the belief that it would be possible to use synthetic peptides for vaccination. A large number of studies aimed at stimulating immunity with peptides were undertaken, in which animals were immunized with synthetic peptides representing B cell epitopes, conjugated either to other peptides from the same pathogen or to unrelated proteins,

to provide the necessary T helper cell epitopes. With a few exceptions, these attempts at immunization were unsuccessful. In many instances, the synthetic peptides completely failed to induce antibodies against the parent protein or organism while, in other cases, antibody responses did occur but were at best only partially effective.

Subsequent studies of protein structure have highlighted the fact that so-called linear epitopes have a degree of conformation and that this conformation may differ subtly from that adopted by the respective synthetic peptides. The process of conjugating a peptide to a carrier molecule may also affect the conformation of the peptide, resulting in antibodies of low avidity for the pathogen in question.

One pathogen for which a degree of success has been achieved by immunization with a synthetic peptide is foot-and-mouth disease virus. Immunization with a peptide consisting of two linked peptide sequences representing residues 141-158 and 200-213 of the VP1 capsid protein resulted in a proportion of immunized cattle (Di Marchi *et al.*, 1986). Structural studies of the virus demonstrated that the 141-158 component of the peptide corresponds to a superficial loop on the surface of the virus particle (Acharya *et al.*, 1989) and suggested that the peptide successfully reproduces this loop structure. Nevertheless, it is still unclear whether the failure to achieve protection in all animals immunized with the peptide was due to subtle conformational differences in the antibody recognition site or to inadequacies in the helper T cell response.

Another factor that may limit the success of immunization with peptides is the use of an unrelated carrier protein to stimulate T cell help for antibody production. No anamnestic T cell response will occur following the challenge of immunized animals with the pathogen, and immunity will therefore rely largely on the pre-existing antibody induced by vaccination.

The use of intact, purified or recombinant proteins for immunization can overcome some of the problems associated with immunization with peptides. Such proteins are likely to contain several T cell epitopes and, if appropriately produced, should have the correct conformation for recognition by antibodies. However, the latter is not always true. For example, the integrity of antibody epitopes on individual viral capsid proteins may be dependent on the structural interaction with other protein components of the capsid. This is the case for several of the neutralizing epitopes on the surface of foot-and-mouth disease virus; of the three capsid proteins only one (VP1) retains any immunogenicity following purification, and it is much less immunogenic than killed intact virus (Bachrach *et al.*, 1975).

The methods by which recombinant proteins are produced can also affect the integrity of epitopes recognized by antibodies. Thus, if glycosylation is required for antibody recognition, production of the proteins in bacteria will be inappropriate and proteins produced in insect cells may be defective because of differences in the sugar side chains added by these cells compared with mammalian cells. Differences in the folding of proteins produced in bacteria may also result in the disruption of some B cell epitopes.

The need for multiple T cell epitopes

Since T lymphocytes recognize small processed fragments of antigens, the conformational structure of antigens is generally not a constraint for the induction of T cell responses. However, because T cells from animals of different MHC types tend to recognize different peptide sequences within an antigen, immunization with short polypeptides containing only one or two potential T cell epitopes is

likely to induce a response in only a proportion of animals. This will be true both for T helper cell responses for antibody production and for effector T cell responses. This further strengthens the argument for using one or more intact proteins in subunit vaccines so that there will be sufficient numbers of potential T cell epitopes to ensure that the majority of individuals within an outbred population will respond to the antigens. In some circumstances, there may be a case for excluding particular T cell epitopes from a vaccine construct. For example, if an antigen contains a particularly dominant T cell epitope that is variable between strains of a pathogen, exclusion of such an epitope might result in a response that is less strain-specific.

Constraints on stimulation of $CD8^+$ T cell responses

Because of the need for antigen to be processed by the endogenous pathway for recognition by $CD8^+$ T cells, the immunization of animals with killed organisms or their component proteins generally fails to induce $CD8^+$ T cell responses. Therefore, alternative antigen delivery systems must be considered when developing subunit vaccines required to stimulate $CD8^+$ T cell responses. These could include the use of virus vectors or vaccination with "naked" DNA, both of which result in the expression of proteins within the cell cytosol. Recent studies have also provided evidence that the active component of the adjuvant saponin, when used to prepare antigen-complexed structures known as immunostimulating complexes (ISCOMs), facilitates the transfer of antigen across cell membranes and the induction of $CD8^+$ T cell responses (Takahashi *et al.*, 1990).

Influencing the cytokine response

The recognition that the cytokines produced by $CD4^+$ T cells to a large extent determine their function and that antigenic stimulation may result in the activation of T cells producing different patterns of cytokines is of major importance when considering immunization strategies. It is, therefore, desirable to know whether the protective responses against the target pathogens involve Th1 or Th2 $CD4^+$ T cell responses. However, since the early events in antigen processing that result in a bias to Th1 or Th2 responses are still incompletely understood, strategies for preferential induction of one or other response are not yet well established. The use of adjuvants that give a bias in the response, for example the induction of Th1 responses by mycobacteria, is one approach that can be pursued. Experiments in mice involving administration of recombinant cytokines or cytokine-specific antibodies at the time of immunization have implicated IL-4 and IL-12 as promoters of Th2 or Th1 cell responses, respectively (Swain *et al.*, 1991; Locksley, 1993). These findings indicate that, by administering cytokines with antigen or by including cytokine genes in molecular vaccine constructs, it may be possible to influence the cytokine profile of T cell responses.

More than one mechanism of immunity

It is becoming increasingly apparent that immunity against a given pathogen may be achieved by alternative immune mechanisms. This is clearly the case with complex protozoan parasites which undergo differentiation through several developmental stages that differ antigenically and replicate in different cell types. Thus, with malaria parasites, antibody against the infective sporozoite stage can block infection, class I MHC-restricted cytotoxic T cell responses are generated against the hepatic intracellular stages and other, as yet poorly understood, cell-mediated mechanisms operate against the intra-erythrocytic stages.

Similarly, different mechanisms may operate against migratory helminth parasites. However, there is also evidence that more than one immune mechanism may be effective in the control of virus infections. For example, it is well established that maternally derived antibody protects offspring from infection with the morbilliviruses (measles, rinderpest) and also interferes with vaccination (Albrecht *et al.*, 1977). Yet a proportion of cattle successfully immunized against rinderpest virus with a recombinant vaccinia virus expressing the F glycoprotein were found to produce little or no rinderpest-specific antibody (Yilma *et al.*, 1988; Belsham *et al.*, 1989), indicating that immunity must have been mediated by T cell responses. Similar results have been obtained with infectious bursal disease virus in chickens, using a recombinant avipox virus expressing the VP2 protein, although the immunity achieved with the recombinant was incomplete (Bayliss *et al.*, 1991).

Clearly, if sufficient antibody of appropriate specificity and biological activity is present in an immunized animal to prevent infection with the respective virus, cell-mediated effector mechanisms will not be required for protection. However, if some of the challenge virus escapes initial neutralization by antibody, it is likely that cell-mediated immune responses will be beneficial, if not essential, for clearance of the infection. This will be particularly true for viruses that spread by cell to cell contact. There may also be some flexibility in the type of T cell response that is employed. In mice immunized against influenza A virus, the transfer of either $CD4^+$ or $CD8^+$ T cells into native recipients resulted in the clearance of challenge virus, the important common feature being the cytokines produced by the two cell types (Lukacher *et al.*, 1986). With viruses such as that which causes foot-and-mouth disease, and for which antibody responses are undoubtedly of major importance in mediating immunity, T cell-mediated responses may provide an additional mechanism for clearing the virus. Yet the role of T cell effector mechanisms in foot-and-mouth disease has been largely ignored.

These observations indicate that the induction of highly effective immunity with subunit vaccines may necessitate the inclusion of more than one antigenic component in a vaccine and the use of an antigen delivery system that is effective at inducing both humoral and cell-mediated immune responses. The poor efficacy of some of the currently used killed vaccines may be due in part to their limited ability to stimulate T cell-mediated components of the protective immune responses.

Pathogens that subvert host immune responses

A common objective in the development of a vaccine is to mimic the immune responses that occur during recovery from natural infection. While this is an appropriate approach for many pathogens, some organisms have evolved stratagems for modifying host immune responses in order to establish persistent infections. Helminth and protozoan parasites have been particularly adept at developing a variety of escape mechanisms. One of the strategies employed by parasites such as *Leishmania* sp. and *Schistosoma* sp. is to direct the T cell response to produce cytokines that are inappropriate for parasite clearance (Sher *et al.*, 1992). In the case of *Schistosoma mansoni*, this results in the development of characteristic egg granulomas that are responsible for clinical disease. Nevertheless, schistosome-infected (SCID) mice, which are unable to mount a granulomatous response, develop severe hepatitis that is believed to be due to release the of parasite proteases (Amira *et al.*, 1993). Thus, the "deviated" immune

response not only favours parasite persistence but may also have a role in protecting and ensuring the survival of the host, albeit with some pathology. Any approach to designing a vaccine for such parasites must be based on an understanding of which components of the host's immune response are responsible for protection as well as on ensuring the avoidance of immune responses that potentiate disease.

Bloodsucking ectoparasites have developed a number of mechanisms to avoid the clotting of imbibed blood and to minimize adverse effects of inflammatory mediators released at the site of feeding. In the case of ticks, which feed continuously for several days, significant hypersensitivity reactions are induced by salivary proteins. While in previously exposed animals this results in a reduction in the number of ticks that engorge, such animals still carry significant tick burdens (Willadsen, 1980). Over the last ten years, researchers in Australia have developed an alternative strategy for vaccination against the one-host tick *Boophilus microplus* based on immunization with proteins from the tick gut, to which the host is not normally exposed (Willadsen, McKenna and Riding, 1988; Willadsen *et al.*, 1989). Antibody induced by these "concealed" antigens, when ingested by the tick, causes damage to the gut wall and results not only in tick mortality but also in a markedly reduced fecundity of surviving ticks. A similar approach is also being pursued for vaccination against the bloodsucking nematode *Haemonchus contortus*.

Several of the large DNA viruses have evolved molecular mechanisms that could potentially modify antiviral immune responses. The vaccinia virus genome contains a number of genes with homology to host receptors for the cytokines IL-1, IL-6 and IFN-γ. Some of these genes are expressed as soluble proteins (virokines) that are able to bind the respective cytokines (Smith, 1993). There is also evidence that serine protease inhibitors expressed by vaccinia may inhibit intracytosolic processing of antigens destined for association with class I MHC (Townsend *et al.*, 1988). Several of the herpesviruses and adenoviruses have been shown to express genes that inhibit the assembly of class I MHC molecules (Lippé *et al.*, 1991; Hill *et al.*, 1994). Identification of the precise role of these genes in determining the virulence of the viruses and their immunogenicity is relevant not only for understanding the pathogenesis of disease caused by the viruses but also in considering the use of animal poxviruses and herpesviruses as vaccine vectors.

NEW APPROACHES TO VACCINE DESIGN

Molecularly defined attenuation

The simplest way of producing a vaccine that mimics the immune responses induced by natural infection is to select an attenuated mutant. The use of live organisms also has the advantage of providing longer-lasting immunity than can be attained with killed antigen. Many of the traditional vaccines are based on the use of attenuated organisms that were either identified by chance or selected by the prolonged culture of the organisms. Advances in knowledge of the molecular structure of viruses and of the function of individual genes in replication and assembly now provide the opportunity to produce targeted mutations that result in altered virulence. The deletion of whole genes negates the possibility of reversion to virulence as a consequence of point mutations. Moreover, viruses with mutations in several genes can be produced. A strain of pseudorabies virus (Aujeszky's disease virus), in which the genes encoding the GI glycoprotein and thymidine kinase have been disrupted, has been shown to be avirulent and to

stimulate immunity against challenge with native virus (Moormann *et al.*, 1990). This virus is being marketed as an attenuated vaccine.

A similar approach is being used with bacteria by targeting genes involved in bacterial metabolism. The most thoroughly investigated system is *Salmonella* spp., in which mutation of a number of genes has been shown to result in attenuated organisms that retain immunogenicity (Dougan, Hormaeche and Maskell, 1987). Again, the incorporation of double or triple mutations minimizes the risk of reversion to virulence. The best characterized of these are the *aro* mutants (Dougan *et al.*, 1988), in which the mutations interrupt the pathway for biosynthesis of aromatic metabolites resulting in organisms that are dependent on nutritional elements not available in mammalian tissues. Such *aro* mutants have been used successfully to vaccinate calves against *Salmonella typhimurium* (Jones *et al.*, 1991).

Immunization with recombinant proteins

The use of purified recombinant proteins for vaccination can be considered where specific antigens have been identified as the targets for protective antibody and/or $CD4^+$ T cell responses. However, as already indicated, the expression system used to produce the protein should not alter important antibody recognition sites on the protein. Generally, recombinant proteins need to be administered in a potent adjuvant.

The human hepatitis B vaccine, which consists of recombinant viral core protein, is the most notable success in this area (McAleer *et al.*, 1984); immunity has been achieved with antigen produced in *Escherichia coli* or in yeast. Recently, a vaccine for *Boophilus microplus* tick infestation in cattle, based on the production in *E. coli* of an antigen that is normally expressed in the tick gut, has been put into commercial production in Australia (Willadsen *et al.*, 1989; Rand *et al.*, 1989). A recombinant protein from the tapeworm *Taenia ovis* has been shown to be effective at immunizing sheep against tapeworm infestation (Johnson *et al.*, 1989), and immunization of cattle with a recombinant sporozoite surface antigen from *Theileria parva* has been found to protect a proportion of animals against experimental challenge (Musoke *et al.*, 1992).

Production of virus capsids

The capacity of the hepatitis B core protein to assemble spontaneously into virus-like particles (McAleer *et al.*, 1984) may partly account for the success of this vaccine, since assembly should allow the protein to adopt a conformation similar to that of the native virus particle.

In the case of non-enveloped viruses in which the capsid is composed of several proteins, the surface conformation adopted by each protein in the virus particle is dependent on interactions with the other proteins. Thus, the isolated proteins are often poorly immunogenic. This problem may be overcome if non-infectious viral capsids can be produced by expressing all of the capsid proteins in a single construct. This has been achieved with bluetongue virus (Roy, 1992). Bluetongue virus, the prototype of the orbiviruses, is made up of seven proteins, three of which are within an inner core surrounded by two concentric protein layers formed by VP3 and VP7. The remaining two proteins, VP2 and VP5, are attached to the outer VP7 layer. Empty virus-like particles have been produced by expressing the four main structural proteins (VP2, VP3, VP5 and VP7) in a single recombinant baculovirus. These particles closely resemble native virus particles in structure. Immunization experiments in sheep have shown that they protect against challenge with bluetongue

virus of the homologous serotype and that they are much more immunogenic than purified VP2 or VP5 proteins (Roy, French and Erasmus, 1992).

Similar studies are under way with foot-and-mouth disease virus.

Live vectors

The potential use of viruses or bacteria as live vectors for vaccination has been a major focus of experimental investigation over the last ten years. Vectors may be naturally occurring apathogenic organisms or attenuated mutants produced by genetic manipulation. Such vectors offer many of the advantages of live vaccines and can potentially be used to express antigens from more than one pathogen. The virus vectors in particular provide a means of inducing $CD8^+$ T cell responses, which are not readily generated by killed vaccines.

Initial studies of virus vectors were focused on vaccinia, which had already been used with great success in smallpox vaccination programmes and was known to have a wide host range (Mackett and Smith, 1986). Being a poxvirus, it also had a large genome in which non-essential regions could be identified for the insertion of foreign genes. Recombinant vaccinia viruses expressing viral genes have been used successfully in experimental studies to immunize cattle against rinderpest and vesicular stomatitis and dogs and foxes against rabies (Yilma *et al.*, 1988; Belsham *et al.*, 1989; Blancou *et al.*, 1986). Field vaccination of foxes against rabies, using baits containing a vaccinia recombinant, has also been successful. Despite these successes and the demonstrated safety of the vaccines employed, concern has been expressed about the widespread release of vaccinia now that smallpox vaccination has stopped. Consequently, attention has turned to animal poxviruses.

Capripox virus, which infects cattle, sheep and goats, is found throughout large areas of Asia and Africa where it causes sporadic outbreaks of disease. An attenuated strain of the virus that had been used locally in Africa to vaccinate against the disease (Kitching, Hammond and Taylor, 1987) has been developed as a vector, and recombinant viruses expressing rinderpest virus glycoproteins have been shown to protect cattle against challenge with rinderpest virus (Romero *et al.*, 1993). A disadvantage of the capripox virus is that it cannot be used outside the areas where infection is endemic owing to its notifiable status. Nevertheless, there are many potential applications of this vector in Africa and Asia.

Avipox and canarypox viruses have been developed as potential vaccine vectors and the former has been used successfully to vaccinate chickens against Newcastle disease and infectious bursal disease (Boursnell *et al.*, 1990a and 1990b; Bayliss *et al.*, 1991). These viruses can also be considered as vectors for mammals, since they produce abortive infections in mammalian cells, i.e. the synthesis of virus proteins occurs but no infectious virus is produced; there is therefore no risk of the virus spreading to other animals.

The herpesviruses have large genomes which, like that of the poxviruses, can accommodate large inserts of foreign DNA. The potential of a number of animal herpesviruses to serve as vaccine vectors is currently being explored. These include pseudorabies virus, bovine herpesvirus 1, equine herpesvirus 1 and turkey herpesvirus. Adenoviruses are also being investigated, particularly in the context of delivering antigens to mucosal surfaces.

The coexpression of cytokine genes and foreign antigens in virus vectors offers a potential means of enhancing or modulating immune responses to the expressed antigens. Experiments with vaccinia virus in mice have yielded promising results with a number of

cytokines (Ramshaw *et al.*, 1992). This approach may be of particular value in providing a bias towards Th1 or Th2 T cell responses.

Attenuated mutant strains of several different *Salmonella* species have been studied extensively for their potential as vaccine vectors (Dougan, Hormaeche and Maskell, 1987; Dougan *et al.*, 1988). Foreign antigens can be expressed from plasmids or following the integration of the genes into the bacterial genome. Much of this work has been carried out in mice and has focused on the induction of immune responses to other bacterial antigens and on the capacity to stimulate immunity at gut mucosal surfaces. Mice immunized with a *Salmonella* recombinant expressing a malaria circumsporozoite antigen were found to be protected against malaria in the absence of antibodies (Sadoff *et al.*, 1988). Since this immunity is mediated by $CD8^+$ T cells, this finding suggests that *Salmonella* spp. may be able to introduce antigens into the endogenous processing pathway.

A variety of other bacteria are being investigated as potential vaccine vectors. Because of its potent capacity to stimulate Th1 T cell responses, the mycobacterium bacillus Calmette-Guérin (BCG) may have particular utility as a vector. A recombinant BCG expressing a surface antigen from the protozoan parasite *Leishmania major* has been shown to protect mice against cutaneous leishmaniasis (Connell *et al.*, 1993), a disease that is known to require Th1 responses for immunity.

Immunization with nucleic acids

The recent discovery that the injection of DNA into animals can result in immune responses to proteins encoded by the DNA has opened up a completely new approach to the development of subunit vaccines. Vaccination of mice with plasmids containing the influenza haemagglutinin gene has been shown to result in protection against virus challenge (Ulmer *et al.*, 1993; Fynan *et al.*, 1993). The injected genes appear to be expressed within host cells without integration of plasmid into chromosomal DNA. While studies of the transfection efficiency of injected DNA have shown that muscle is 100 to 1 000 times more permissive than other tissues, protection has also been achieved following inoculation by the intravenous, intranasal and intratracheal routes and by delivering the DNA intradermally using a "gene gun". The immunization of mice with DNA induces antibody responses as well as $CD4^+$ and $CD8^+$ T cell responses. The induction of immune responses to bovine herpesvirus 1 in cattle has been investigated in one study (Cox, Zamb and Babiuk, 1993); a plasmid containing the gII viral gene stimulated neutralizing antibodies and resulted in a marked reduction in nasal shedding of the virus following challenge.

Targeting of antigen at mucosal surfaces

The stimulation of protective immune responses at mucosal surfaces presents a particularly challenging problem because of the requirement for such responses to be induced locally. Non-viable antigen administered orally is susceptible to proteolytic degradation and is absorbed in only small amounts through the gut wall. Moreover, such "dietary" antigens tend to induce tolerance rather than an active immune response. One way of overcoming this problem is to use bacterial or viral vectors, such as *Salmonella* spp. or adenovirus, which replicate in the alimentary tract.

Other strategies that facilitate the uptake of protein in the gut and the induction of an active response are currently being investigated. One of these involves the use of cholera toxin, which is a multimeric protein composed of a single A subunit and a pentameric B subunit; binding to

epithelial cells occurs by interaction of the B subunit with GM_1-gangliosides while toxicity is mediated by ADP-ribosyl-transferase activity of the A subunit (Spangler, 1992). Oral administration of either subpathogenic doses of intact toxin or purified B subunits results in induction of a specific immune response. Moreover, protein antigens administered along with, or covalently linked to, the toxin or its B subunit elicit strong antibody and $CD4^+$ T cell responses (Dertzbaugh and Elson, 1993). Whether or not the B subunit retains the full adjuvant activity of the intact toxin has not been fully resolved.

Another approach to delivering antigens to the gut is to encapsulate antigen in microspheres with an outer biodegradable polymer coat that protects the antigen against rapid enzymatic degradation. Microspheres with a diameter of 5 to 10 µm have been shown to be taken up efficiently by the Peyer's patches and to induce IgG and IgA antibody responses in mice.

CONCLUDING REMARKS

In the last decade, a clear picture has emerged of how antigens are processed and recognized by the immune system and significant progress has been made in understanding the immune mechanisms that operate against different types of pathogens. This new knowledge, together with further advances in technologies for genetic manipulation, has led to a variety of new approaches to the attenuation of pathogenic organisms and to the design of antigen delivery systems appropriate for inducing particular immune responses. There has also been progress in other areas of research, not discussed here, such as the targeting of antigens at particular cell types in the immune system and studies to develop new adjuvants and understand their mode of action. These developments offer exciting potential for the production of a new generation of vaccines.

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Genetically engineered vaccines

B.W.J. Mahy

Genetic engineering is a term used to describe experimental or industrial approaches to modifying the genome of a living cell without normal sexual or asexual transmission of genetic material. In a broader sense, it is used to describe genetic manipulation of the genome of a virus and is often used synonymously with the term recombinant DNA technology. The ability to manipulate the genome of a living cell or virus was made possible by a series of discoveries made 20 to 30 years ago.

First was the recognition of bacterial restriction enzymes by Arber and Dussoix (1962). These are endonucleases that recognize specific DNA sequences and cleave the DNA only at those sites (Meselson and Yuan, 1968). Coupled with other enzymes involved in DNA synthesis such as ligases (DNA-joining enzymes), it became feasible to introduce deliberately specific foreign DNA sequences into DNA molecules which are then described as recombinant DNA. When the recombinant DNA replicates, the foreign DNA is also replicated. If the foreign DNA is inserted into a bacterial plasmid or phage DNA which undergoes rapid multiplication together with the host bacteria, it is possible to generate large quantities of the foreign DNA. This process is called molecular cloning (Sambrook, Fritsch and Maniantis, 1989).

Another crucial discovery was the existence of reverse transcriptase, an enzyme present in oncogenic RNA viruses and which converts RNA into DNA (Baltimore, 1970). This allowed the conversion of cellular or viral messenger RNAs into DNA so that they could then be molecularly cloned and expressed to produce the polypeptide or protein specified by the original messenger RNA. Bacterial plasmids can serve both as cloning and expression vectors when introduced into appropriate host cells and, in the last few years, a range of expression vectors have become available that will replicate in bacteria, yeast, insect or animal cells to produce large quantities of a desired polypeptide or protein.

Such expressed proteins can be purified to serve as a vaccine antigen, as in the highly successful vaccine against hepatitis B prepared in yeast (*Saccharomyces cerevisiae*), which was the first genetically engineered vaccine licensed for use in humans. Alternatively, if the expression vector will replicate in animal or human cells, the expressed antigen may immunize the recipient host without the need for purification. This is the basis for the development of a wide range of candidate vaccines for both human and veterinary use which depend on recombinant gene expression in an appropriate viral or bacterial vector. Such live recombinant vector vaccines offer great promise but, to date, have not been licensed for use in human subjects.

Recently, the discovery that plasmid DNA sequences can be expressed directly after injection into mouse muscle (Wolff *et al.*, 1990) without the need for a viral or bacterial expression vector has opened up an exciting range of possibilities for the design of vaccines for human or veterinary use through polynucleotide vaccination (Cohen, 1993; Ulmer *et al.*, 1993; Ulmer, Donnelly and Liu, 1994). In addition, since

the encoded antigens are expressed intracellularly in the recipient host, they are introduced into class I MHC molecules and this results in the priming of cytotoxic T cells (Townsend and Bodmer, 1989; Barber and Parham, 1994) so that both cellular and humoral immune responses are stimulated (Ulmer *et al.*, 1993).

Finally, the ability to determine the nucleotide sequence of the genomes of DNA or RNA animal viruses has led to a considerable body of research on the structure of the antigens they encode. In addition to expressing the genes in various vectors, attempts have been made to synthesize *in vitro* various polypeptides which correspond to these antigenic determinants or epitopes and to use the peptides themselves as candidate immunogens. Unfortunately, this approach has had only limited practical success, but some important early work was carried out with foot-and-mouth disease (Bittle *et al.*, 1982; Di Marchi *et al.*, 1986).

In this brief overview, the development and future prospects for genetically engineered veterinary vaccines will be described. The various types of vaccine will be considered separately according to the listing given in Table 1.

TABLE 1
Prospective genetically engineered vaccine types

Type	Examples
Attenuated live	Pseudorabies (Aujeszky's disease)
Defective live	ALVAC-FL (feline leukaemia)
Chimeric live	Capripox, rinderpest, lumpy skin disease
Subunit	Bluetongue
DNA polynucleotide	Avian influenza
Polypeptide	Foot-and-mouth disease

GENETICALLY ENGINEERED ATTENUATION

Many conventional vaccines currently employed in human or veterinary medicine contain live micro-organisms or virus that has been attenuated, for example by multiple passages in cell culture, to the point that it will multiply in the recipient host but no longer induce disease. Live vaccines offer several potential advantages compared with killed vaccines in that they usually induce a rapid immune response of long duration and also induce local immunity which may be critical in providing protection against subsequent infection by virulent micro-organisms. However, conventional methods of attenuation rely on spontaneous, random mutations occurring during multiple passages, and the basis for the attenuation is usually not known. An alternative approach is to use genetic engineering to define specific genes or regions of the genome which are responsible for virulence, then delete these in order to obtain a replicating, non-pathogenic virus for use as the immunogen.

This approach has been particularly fruitful with bacteria and DNA viruses with large genomes, such as herpesviruses and poxviruses. Since this type of attenuation involves the removal of parts of the genome, such bacteria and viruses may then be used to vector genes encoding a variety of foreign antigens, creating chimeric vaccines.

The experimental protocol for gene deletion and insertion usually requires some means of identification of the progeny which contain the desired genotype. In the original experiments to develop modified poxviruses for foreign gene insertion, the thymidine kinase (TK) gene was deleted and a positive selection of progeny which lack the gene was made by growing the virus in a TK cell line (lacking a cellular thymidine kinase) using

a medium containing 5-bromodeoxyuridine (BudR). Any progeny virus which contains the TK gene will convert BudR into a phosphorylated form which is toxic to the host cell. Consequently, only recombinant viruses with the TK deletion will survive (Mackett and Smith, 1986). A similar system can be used to select herpesviruses from which the TK gene has been deleted, for example bovine herpesvirus 1 (Bello, Whitbeck and Lawrence, 1992; Kit, Kit and McConnell, 1986). A deletion mutant of pseudorabies virus (suid herpesvirus 1) has been used successfully to vaccinate pigs against Aujeszky's disease. This genetically engineered virus, strain 783, contains three deletions: one that prevents expression of pseudorabies virus glycoprotein 1 (2 055 base pairs), one that inactivates the viral thymidine kinase (19 base pairs) and one of 100 base pairs that lies in the promoter-enhancer region of the immediate early gene (Glazenburg *et al.*, 1994).

A more general method for gene deletion and the detection of progeny virus with the required genetically engineered deletions is to use a heterologous reporter gene contained in a plasmid transfer vector. The reporter gene is inserted into a plasmid between flanking sequences of the gene to be deleted. When the virus genome DNA is cotransferred into cells along with the plasmid containing the reporter gene, recombination occurs between the homologous (flanking) sequences of the genome DNA and those in the transfer vector. This yields some progeny containing substitution of the virus gene by the reporter gene, and these can be detected in a variety of ways depending on which reporter gene is used. The most convenient plasmid transfer vectors contain a chromogenic reporter such as β-galactosidase which allows an easy visual selection of plaques containing recombinant virus (Chakrabarti, Brechling and Moss, 1985).

In the early development of poxviruses as gene vectors, it was estimated that infectious virus could be produced containing at least 25 000 base pairs of foreign DNA (Smith and Moss, 1983). More recently, a highly attenuated candidate vaccinia virus vector vaccine (NYVAC) has been constructed by the deletion of 18 genes which are involved in pathogenesis and host range (Tartaglia *et al.*, 1992). The resultant virus replicates well in Vero cells or chick embryo fibroblasts but poorly in cells of human, equine, murine or swine origin, and provides a safer alternative to vaccinia virus as a potential gene vector vaccine (Tartaglia *et al.*, 1994).

The potential of NYVAC as a vector vaccine against pseudorabies virus has been demonstrated. NYVAC recombinants expressing pseudorabies virus glycoprotein gp50 protected pigs against challenge with live virulent virus (Brockmeier *et al.*, 1993).

GENETICALLY ENGINEERED DEFECTIVE VACCINES

Recently, the potential of non-replicating (defective) poxvirus as vectors for foreign genes has been explored. Avipoxviruses do not replicate when introduced into mammalian hosts, but they may still act as gene expression vectors in mammals as well as birds, since replication is blocked at a stage after early gene expression, but before DNA replication. The basis for the avian poxvirus vector was an attenuated licensed vaccine for canaries (ALVAC) (Taylor *et al.*, 1991 and 1992; Cadoz *et al.*, 1992). When the glycoprotein gene of rabies virus was introduced into ALVAC by recombination, a highly effective vaccine preparation was obtained (ALVAC-RG) which induced high levels of immunity in humans (Cadoz *et al.*, 1992) and provided a high level of protection in dogs against challenge with virulent rabies virus, even though the ALVAC-RG virus did not

replicate in the dogs or human subjects (Taylor *et al.*, 1994). The use of ALVAC as a vector for the *env* and *gag* genes of feline leukaemia virus (ALVAC-FL) has also been described (Tartaglia *et al.*, 1993).

Another highly attenuated poxvirus that has been well characterized is modified vaccinia virus Ankara (MVA). This virus was passaged more than 500 times in chick embryo fibroblasts, and is unable to replicate in mammalian cells (Meyer, Sutter and Mayr, 1991). Replication is blocked at the assembly stage so that DNA replication and late protein synthesis occur and large amounts of foreign gene products are produced, similar in amount to wild-type virus (Sutter and Moss, 1992).

It is clear that such non-replicating gene expression vectors provide a safe and effective means to immunize a variety of mammalian species, including humans, since the virus cannot spread beyond the initially infected cells.

CHIMERIC LIVE VACCINES

Candidate live recombinant vaccines expressing foreign genes have been developed from a variety of poxvirus vectors as well as adenoviruses and *Salmonella* spp. For the most part, adenoviruses are currently being explored as potential vectors for human mucosal vaccination and so will not be considered further here. However, there have been some studies in animal species, especially canines, and an excellent review on the subject has been published (Graham and Prevec, 1992). By the same token, the principal interest in *Salmonella* spp. is in the development of multivalent oral vaccines for human use. Although there is also considerable potential for *Salmonella*-based vectors in veterinary medicine, particularly in poultry, the subject is still relatively unexplored. A useful review on *Salmonella*-based vaccines has appeared recently (Chatfield, Dougan and Roberts, 1994). For these reasons, the following discussion will be confined to some remarkable examples of poxvirus vectors for use in veterinary medicine. A more detailed account of the earlier studies in this area was published by Esposito and Murphy (1989).

One of the first vaccinia virus recombinants of veterinary interest to be developed was a potential vaccine against vesicular stomatitis (VSV), which is a contagious disease of horses, cattle and pigs that can cause serious economic losses in North and South America because the lesions may be confused with those of foot-and-mouth disease (FMD). Recombinant vaccinia virus was prepared which expressed the G glycoprotein of VSV at the surface of infected cells. Some protection against intralingual challenge with virulent VSV was observed in cattle 44 days after receiving the recombinant vaccine on days 1 and 29 (Yilma, 1994).

When the glycoprotein gene of another rhabdovirus, rabies, was expressed in a vaccinia virus recombinant, good protection against challenge was found in a variety of animals such as mice, rabbits, skunks, raccoons and foxes (Wiktor *et al.*, 1984; Blancou *et al.*, 1986; Rupprecht *et al.*, 1988). It was possible to immunize raccoons and foxes, but not dogs or skunks, by oral administration of the vaccinia-rabies G protein recombinant (VRG). This has led to a series of detailed studies of the potential for oral vaccination of raccoons and foxes by distribution of baits containing VRG in the wild. Considerable success has been achieved with rabies vaccination of foxes in Europe using this method, and carefully controlled studies of the use of such baits to vaccinate raccoons are under way at several sites in North America (Rupprecht *et al.*, 1988 and 1993; Rupprecht, Hanlon and Koprowski, 1992; Hanlon *et al.*, 1993). The principal concern in the use of such VRG-containing

baits in the field is the possible risk to humans who might become accidentally infected with the vaccinia recombinant. In exceptional circumstances, for example if an immunocompromised person was infected, there might be a risk of a serious vaccinia infection. Alternative poxvirus vectors such as raccoon pox (Esposito, Chandler and Baer, 1989) have been used to create a potentially safer vaccine but insufficient studies have been made of its effects in other species. For this reason the current trials with VRG are being carefully monitored and the risk of human exposure appears to be extremely low. The considerable potential for poxvirus-vectored rabies vaccines to control the unprecedented rabies epidemic in raccoons in North America (Rupprecht and Smith, 1994) needs to be carefully evaluated with regard to risks and benefits. It seems likely, however, that VRG vaccines will soon be licensed for use in the United States.

Unfortunately, a VRG recombinant vaccine that had not been sanctioned by the Argentine authorities was used in Argentina in a field experiment involving cattle (Koprowski *et al.*, 1957). The potential for human exposure to vaccinia virus had not been adequately explained to those who handled the inoculated animals; for example, it is known that vaccinia virus can be isolated from the scabs present on cattle at the site of recombinant virus inoculation (Gillespie *et al.*, 1986). Even though none of the cattle handlers suffered any disease, this incident resulted in a considerable setback to the introduction of recombinant poxvirus vaccines for agricultural purposes, especially in South America (Crawford, 1987).

One of the principal advantages of vaccinia virus, which contributed to the success of the smallpox eradication campaign, is its relative stability. Vaccinia virus preparations will remain viable in conditions which would destroy the infectivity of live vaccines against morbilliviruses such as measles or rinderpest. A highly effective vaccinia recombinant virus expressing the F and H genes of rinderpest has been described for use in cattle (Giavedoni *et al.*, 1991), and this virus will also protect goats against the related morbillivirus, peste des petits ruminants (PPR) virus (Jones *et al.*, 1993). Recently, an alternative chimeric vaccine candidate was described which is based on capripoxvirus, a natural virus infection of cattle, sheep and goats. Live attenuated capripoxvirus preparations are already in use as effective vaccines against sheep and goat pox and lumpy skin disease of cattle, so they are ideal vectors for recombinant vaccines in these species. When the F gene of rinderpest virus was introduced into the capripox vaccine virus at the TK locus, the resultant recombinant virus protected cattle against rinderpest following a lethal challenge and also immunized the cattle against lumpy skin disease (Romero *et al.*, 1993).

In addition to these examples, recombinant vaccines against other bovine diseases have been described. The protection of sheep against bovine leukaemia virus using a vaccinia recombinant expressing the gp51 polypeptide has been described, and this may well be effective in cattle also (Daniel *et al.*, 1993). The use of such poxvirus-vectored rinderpest vaccines could be crucial in the campaign to eradicate this plague from the remaining pockets of disease in Africa and Asia.

The potential of fowl poxviruses as gene vectors for vaccines against a wide range of poultry diseases was recognized more than ten years ago (Boyle and Coupar, 1988). The difficulty of producing such genetically engineered vaccines at an economic cost has hindered their development, however. For vaccines to be effective in the poultry production

industry, they need to be administered by one day of age using mass delivery methods, for example inclusion in the drinking water. There are numerous examples of potential recombinant vaccines against diseases such as avian influenza, Newcastle disease and Marek's disease, but all require wing-web inoculation for maximum efficacy and this may not be practical. An excellent review of potential recombinant vaccines for poultry appeared recently (Boyle and Heine, 1993).

SUBUNIT VACCINES

Live virus vaccines usually induce high levels of immunity which persist in the host. The major concern regarding their use is the possibility of reversion to virulence, and for diseases such as foot-and-mouth disease only inactivated virus is suitable for use as a vaccine. Nevertheless, problems have occurred owing to incomplete virus inactivation, with resultant outbreaks of disease.

An alternative approach is to use subviral components as immunogens in the form of subunit vaccines. Generally, the isolated components of a virus may not be effective in stimulating an appropriate immune response but, if presented in conjunction with an appropriate adjuvant such as alumina gel or 6D-stearoyL-muramyL-dipeptide, a better response can be generated. Potentiation of the vaccine by addition of cytokines or lymphokines is also being studied (Hughes and Babiuk, 1994). For membrane proteins of enveloped viruses, an immunostimulating complex (ISCOM) was developed which, when mixed with the antigen, presents it to the immune system in the form of micelles (Morein *et al.*, 1984; Morein and Simons, 1985).

The advent of expression vectors such as baculoviruses, which can produce enormous quantities of a desired protein in cell culture, has greatly enhanced the possibilities for subunit vaccine development. For example, the baculovirus *Autographa californica* has been developed to express a variety of foreign genes under control of the strong polyhedrin promoter (Miller, 1988). The polyhedrin gene is replaced by the gene encoding the desired protein, and the recombinant baculovirus-infected *Spodoptera frugiperda* cells may produce up to 50 percent of total cell protein as the foreign gene product.

This system has been used to engineer multiple-component proteins of bluetongue virus and, when produced in appropriate quantities, the proteins self-assemble to form non-infectious virus-like particles (Marshall and Roy, 1990; Pearson and Roy, 1993). A further development of this system is to introduce other virus antigens into the bluetongue virus subunits during self-assembly to create protein chimeras which will immunize against more than one disease.

This approach offers considerable potential for the future development of veterinary vaccines.

DNA POLYNUCLEOTIDE VACCINES

Wolff *et al.* (1990) reported that the direct introduction of plasmid DNA into mouse muscle resulted in expression of encoded proteins in the muscle cells. Although the exact process by which transcription occurs in this system has not been completely elucidated, it seems that muscle injection is more effective at inducing an immune response than other routes or tissues that have been tried.

Recently, the application of a biolistic gun (gene gun) that shoots gold particles coated with DNA directly into the muscle, has been described (Williams *et al.*, 1991; Fynan *et al.*, 1993).

This field is just opening up, but the effectiveness of naked DNA in provoking immunity to rabies in mice and virulent

influenza in chickens has been reported (Xiang *et al.*, 1994; Robinson, Hunt and Webster, 1993). The state of the art of DNA vaccination has been well summarized by Ulmer, Donnelly and Liu (1994), Ulmer *et al.* (1993) and Cohen (1993).

POLYPEPTIDE VACCINES

When predicted amino acid sequences from a wide range of pathogenic viruses became available in the mid-1970s, the possibility of using chemically synthesized polypeptides as immunogens was explored. Previous studies on proteins such as lysozyme had established that immune responses could be elicited by short peptides containing about 20 amino acids. It was also clear that peptides corresponding to the aminoterminus or carboxyterminus were frequently antigenic (i.e. elicited antibodies that reacted with the original protein). Numerous examples of viral peptides that served as immunogens were reported (Geysen, Barteling and Meloen, 1985; Shinnick *et al.*, 1983). When it was found that guinea pigs could be protected from foot-and-mouth disease by a short peptide of 19 amino acids corresponding to a portion of the VP1 protein of the virus (Bittle *et al.*, 1982), peptides were hailed as the next generation of foot-and-mouth disease vaccines (Brown, 1985). Subsequently, Di Marchi *et al.* (1986) showed that it was possible to protect cattle from a virulent intralingual challenge with foot-and-mouth disease virus using a peptide of 42 amino acids, but very large quantities of the peptide were required. In general, the peptides were found to stimulate a remarkably strong humoral response even at low doses of peptide, but protection against challenge did not occur except at high doses (5 mg per animal).

Despite the great amount of work done in attempting to improve on these results, for example by coupling the peptide with predicted amino acid sequences designed to stimulate cell-mediated immunity, the potential for such simple peptides as vaccines has not been realized.

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Viral vaccines

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Vaccination is a major weapon in the control of many viral diseases of humans and their domestic and pet animals. There is no doubt that vaccines have made an enormous impact on the health and consequently the productivity of the recipients. Although this chapter concentrates on vaccines which are used in domestic livestock, where necessary it draws analogies with information involving other species. In addition, an overview on the present state of vaccine development must necessarily review what has gone before and, with advantage, should also look to the future. Consequently, this chapter contains references to products that were made in the past, including the way in which they were improved to provide the vaccines in current use, and outlines the possibilities of entirely new products which molecular biology offers.

Although it is always dangerous to make predictions, one can be certain that the methods for preparing vaccines will improve. It is also possible and even likely that the methods will change radically as we learn more about the immune response at the molecular level and apply recombinant DNA technology to produce and present to the host only those parts of the virus that are required to provide protection.

BACKGROUND

Vaccination as we know it today essentially began late in the eighteenth century with the observations of Jesty (see Wallace, 1981; Jenner, 1798) and many others that milkmaids rarely contracted smallpox. The immunity was attributed to the fact that milkmaids contracted cowpox during milking which protected them against the virulent smallpox. Many of the vaccines in use at the present time are based on the same principle, namely that of a weakened or attenuated strain of a virus multiplying sufficiently in the host to elicit a protective immune response without causing clinical disease. Vaccines based on this principle are highly effective, as shown by the eradication of smallpox; the control of many human diseases such as polio myelitis, measles and mumps; and the effectiveness in domestic animals of vaccines against Marek's disease, Newcastle disease and rinderpest.

The second part of our armoury consists of inactivated vaccines, prepared by growing large amounts of the viruses in tissue culture cells or sometimes in the intact animal and then inactivating them either chemically, with agents such as formaldehyde, phenol, β-propiolactone or an imine, or physically, with ultraviolet light. Inactivated vaccines against foot-and-mouth disease, Newcastle disease, poliomyelitis and rabies have been used extensively and with great success.

Together, these vaccines have made very important contributions to animal health worldwide. One of the early vaccines used on a large scale was described by Rosenbusch, Decamps and Gelormini (1948). This vaccine, used against foot-and-mouth disease, was prepared by formaldehyde inactivation of extracts of fresh tongue lesions of artificially infected susceptible cattle. Millions of cattle were immunized with vaccines prepared by this method. It is of interest, in view of later

observations and concern about the innocuity of vaccines prepared by inactivation with formaldehyde, that the authors used an alkaline glycocoll buffer. Recent studies have shown that the virus is very rapidly inactivated at pH 8. This results from the degradation of the RNA within the virus particle by the RNA polymerase (Newman *et al.*, 1994). Rabies vaccines prepared from the brains of sheep and goats artificially infected with the virus are still widely used in humans despite the evidence that such products cause encephalitic reactions.

The discovery of antibiotics in the late 1930s and early 1940s revolutionized the way in which vaccines could be prepared. Viruses had been grown outside the animal body as early as 1930 but these experiments had been done on a small scale. To expand these laboratory observations to a production scale was clearly difficult with the technology available at that time because the problem of ensuring bacteriological sterility would have been overwhelming. The discovery of antibiotics overcame the problem.

The development of large-scale *in vitro* cultivation of foot-and-mouth disease virus by Frenkel (1947) was a major technical achievement. This led to vaccination programmes which have been used to protect millions of animals (and incidentally predated the much publicized polio vaccination programmes by several years).

ATTENUATED VACCINES

Jenner's vaccine against smallpox, referred to above, was quickly applied in many countries but it was not until almost a century later, in the 1880s, that immunization against other infections began. By that time the germ theory of infection was starting to be accepted and, in rapid succession between 1880 and 1885, Pasteur and his colleagues demonstrated the efficacy of vaccines against chicken cholera, anthrax and rabies (Pasteur, 1880 and 1985; Pasteur, Chamberland and Roux, 1881). All three vaccines had been prepared by attenuating the naturally occurring wild-type agents in the laboratory.

Vaccines for several viral diseases have been prepared by growing the wild-type agents in non-natural hosts or in tissue culture cells. For success to be achieved, careful selection of appropriate strains is required. Even then, the selection has to be accompanied by an enormous amount of luck to achieve the correct balance between loss of virulence and the ability to multiply in the host without causing clinical disease. Nevertheless, highly successful vaccines against Newcastle disease and rinderpest have been prepared and used in millions of birds and animals. The selection of a naturally occurring mutant has also been successful in the case of Marek's disease. The virus infecting turkeys was found to be avirulent for chickens and has been used in millions of birds.

CURRENT METHODS OF INACTIVATION

Most inactivated vaccines are produced by reacting the viruses with formaldehyde. This reagent has been used almost exclusively for producing diphtheria and tetanus toxoids since early this century. The reagent was used by Römer in experiments to produce a vaccine against poliomyelitis as early as 1911, but without success. However, it was used successfully by Vallée, Carré and Rinjard (1925) to produce an experimental foot-and-mouth disease vaccine. These researchers, located in Paris, were probably influenced in their choice of reagent by Ramon, who was at that time director of the Pasteur Institute in that city.

Serious concerns about the use of formaldehyde to inactivate poliomyelitis virus completely were voiced following clinical trials of a polio vaccine prepared

in this way by Brodie and Park (1936) in the 1930s. Similar doubts were also expressed later about the foot-and-mouth disease vaccines prepared by the same method. It was generally considered that the preliminary step of adsorbing the virus to alum before adding the formaldehyde was necessary in the inactivation procedure. In fact, the adsorption step may have been responsible for masking the fact that inactivation of the virus was incomplete.

The Cutter incident with an early polio vaccine in the mid-1950s only served to emphasize these concerns and categorical evidence that foot-and-mouth disease vaccines prepared by treatment of the virus with formaldehyde were not innocuous was provided by Brown *et al.* (1963) and Graves (1963). But it needed the outbreaks in Normandy and Brittany in France in 1981, when the causal agent was identified by molecular methods as a virus isolated 16 years previously, to convince manufacturers that formaldehyde inactivation was not safe (King *et al.*, 1981). Subsequent evidence, provided by Beck and Strohmaier (1987) using nucleic acid sequencing to identify the viruses causing several outbreaks in western Europe, pinpointed either spills from factories or vaccines inactivated with formalin as the major causes.

In view of these findings, it is somewhat perplexing, at least to the author, that alternative inactivating agents were not used more generally before these incidents. Firm laboratory evidence had been provided several years previously that acetylethyleneimine was a better inactivant (Brown and Crick, 1958; Brown *et al.*, 1963) and this had been confirmed on a large industrial scale by the Wellcome Laboratories. In recent years, bis-ethyleneimine has replaced acetylethyleneimine because it is safer to handle on a large scale (Bahnemann, 1990).

THE NEW TECHNOLOGY

During the past 40 years, the concepts of molecular biology have been embraced by the entire field of biology and medicine. This has led to a great increase in our knowledge, not only of the molecular structure of many infectious agents but also of those elements which confer protective immunity. More recently, the same concepts have been applied to studies of the immune responses which protect against infection. The objectives of this approach, while clearly of fundamental importance, also have great practical implications for vaccine production, since they will eventually provide products which are safer and more effective and which can be designed to elicit only those responses that are required for protection.

Structure of viruses

The concept that the entire organism is not required to elicit protective immunity was demonstrated a century ago when it was shown that antiserum produced in animals against the toxins secreted by the agents causing diphtheria and tetanus would passively protect against these diseases. The subsequent extension of this work, which showed that these toxins would produce active immunity after suitable inactivation, provided the first clear demonstrations that vaccination with subunits was a practical proposition (Glenn and Hopkins, 1923; Ramon, 1923).

The advances made with viruses depended in the first place on the techniques which became available to grow them in tissue culture in quantities sufficient to allow their purification and characterization. By growing the viruses in the presence of radioactive precursors of nucleic acids and proteins, the individual constituents can be labelled. These analyses have shown that there are many distinct groups of viruses, differing in size and shape, ranging from small

spherical particles of 30 nm in diameter (such as those of foot-and-mouth disease and swine vesicular disease) to bullet-shaped particles measuring 140 × 70 nm (such as that causing rabies) and large spherical particles measuring 300 nm in diameter (e.g. smallpox virus).

In addition, viruses can be grouped according to whether they contain DNA or RNA and whether they possess a lipid envelope. For example, rabies virus possesses such an envelope. By dissolving this envelope in a lipid solvent or a mild detergent, subunits of the virus can be isolated and their potential for eliciting protective immunity tested. More vigorous methods are required to disrupt those viruses not possessing a lipid envelope.

During the same period, major advances were being made in our knowledge of virus multiplication. These studies have provided genetic maps of many groups of viruses, at the same time identifying the genes coding for the proteins of immunological significance. Consequently, when the methods for ligating DNA molecules were described early in the 1970s, the knowledge required for making synthetic vaccines was already available.

The steps involved in the provision of engineered vaccines are:

i) identification of the immunogenic protein;
ii) identification of the gene coding for this protein;
iii) expression of the gene in a suitable vector to provide either a live or a dead vaccine.

i) Identification of immunogenic proteins. The identification of immunogenic proteins is made by dissection of the virus into subunits which are then tested for immunogenicity. With lipid-containing viruses this is a relatively simple procedure, since the envelope can be dissolved in a solvent such as ether or chloroform or a mild detergent such as Tween, releasing the subunits without denaturing the proteins. The separated subunits are also analysed for the proteins they contain. The method which has proved most valuable for analysing proteins is polyacrylamide gel electrophoresis (PAGE), which separates them on the basis of their relative molecular weights. Coupled with examination of the biologically active subunits in the electron microscope, the architecture of a virus can be derived with this method.

ii) Identification of the gene coding for the immunogenic protein. The genome of a virus codes for several proteins in addition to the structural proteins which are involved in the immune response. The relevant gene or genes are identified by expressing the viral nucleic acid in a suitable expression system *in vitro* and then precipitating the products with neutralizing antibody. In this way, the gene coding for the product reacting with the neutralizing antibody can be identified.

iii) Expression of genes. Once a relevant gene has been identified, several vector systems are available for its expression. If the gene is part of a DNA virus genome, it can be ligated directly into the DNA of the vector. If, however, the gene is part of an RNA virus genome, it must first be transcribed into DNA before it can be inserted into the vector DNA.

Several vectors are suitable and these can be used in two ways. In the first, the relevant gene is ligated into the DNA of a bacterium or virus which has been used, or has potential for use, as an attenuated vaccine. Examples of these are the strain bacillus Calmette-Guérin (BCG) (Bloom *et al.*, 1990), avirulent strains of *Salmonella typhimurium* (Dougan and Tite, 1990), adenovirus (Graham and Prevec, 1992), the

poxviruses – notably vaccinia virus (Mackett, 1990) and some members of the herpesvirus family, for example pseudorabies virus (Kit *et al.*, 1991).

Recently, this approach was adapted by Almond and Burke (1990) to make use of the attenuated strains of poliovirus, an RNA virus, taking advantage of the fact that the DNA complementary to the virus RNA is infectious (Racaniello and Baltimore, 1981). This DNA will infect cells to produce poliovirus particles. The DNA corresponding to the gene of interest is inserted into the complementary poliovirus DNA, which then replicates to produce polio virus particles containing the inserted gene product. The recombinant organisms obtained in this way have the potential to be used as live vaccines.

In the second approach, the gene is expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, the baculovirus *Autographa californica* or in mammalian cells, and the gene product is purified for use as a killed vaccine (Brown, 1984; Matsuura *et al.*, 1987). The initial early preference for *E. coli* has been largely superseded because the bacterial vector does not glycosylate the expressed protein. Since many of the proteins of immunogenic importance are glycosylated (e.g. the surface projections of rabies virus) this was a serious and often critical disadvantage. Consequently, more recent developments have been made with eukaryotic vectors.

Recombinant attenuated vaccines

The use of attenuated viruses and bacteria that are already accepted vaccines to carry foreign genes is clearly a concept with many attributes. For example, the great advantages of vaccinia virus (Mackett, 1990) as a vector are its extensive and successful use against smallpox and in the eventual eradication of the disease, together with the fact that its DNA can accommodate several foreign genes. Similar considerations apply to BCG (Bloom *et al.*, 1990) which has been used in tuberculosis prophylaxis for many decades with an impressive safety record, even though there is some debate about its efficacy. In contrast, poliovirus, with its smaller genome and more structurally constrained particle, would be unlikely to accommodate more than one foreign gene.

The use of *Salmonella typhimurium* (Dougan and Tite, 1990) as a carrier is more debatable as it does not possess the proven track record of vaccinia virus and BCG. However, its potential for oral immunization demands that its qualities as a vector should be investigated extensively. Strains of adenovirus (Graham and Prevec, 1992) which have been used as vaccines should also prove valuable since they can be delivered orally, provided the structural constraints imposed by the architecture of the particle do not prove too stringent. Moreover, research done with pseudorabies virus (Kit *et al.*, 1991), a member of the herpesvirus family, indicates that this virus could be a valuable vector for the immunization of animals.

The vaccinia virus system has been studied more extensively than the other candidates, and field trials with the rabies virus glycoprotein recombinant have proved very successful. Of particular interest in the control of rabies has been the successful vaccination of the fox in the wild by using the recombinant vaccine in bait. Experiments with rinderpest virus recombinant in the natural host animal have also been very successful (Yilma *et al.*, 1988). A major issue of debate in the use of such recombinant, however, has been the question of their safety. The innocuity of an organism – and in this instance a new recombinant virus – for the laboratory mouse can be no guarantee of its safety for other species.

It is also well recognized that any

product which is to be inoculated into a healthy animal should ideally not cause any side effects. This position is difficult to achieve and there seems to be no ideal solution. Another issue which has caused concern is whether insertion of a foreign gene alters the tissue tropism of the organism, or even its host range. Again there seems to be no easy solution, particularly since we have little knowledge of the factors which determine host specificity.

Killed vaccines

Proteins produced in *in vitro* systems are essentially equivalent to the subunit vaccines used experimentally several years ago. The crucial problem is to present the expressed proteins to the immune system in the configuration which they have when they form part of the intact organism. Both the subunits derived from the intact organism and the corresponding genetically engineered proteins are much less immunogenic than the intact organism. This is particularly true when the subunits are derived from viruses which do not contain a lipid envelope because their release from the intact organism requires much harsher conditions.

There are, however, lessons to be learned from the experience with the hepatitis B virus surface antigen (Valenzuela *et al.*, 1983). The protein expressed in yeast cells aggregates to form particles which are similar to the 22 nm particles found in the blood of carriers of the disease. These yeast-expressed particles are much more immunogenic than the monomeric protein and they form the basis for the first genetically engineered vaccine. Other evidence also indicates that presentation of an immunogenic protein in a multimeric form on a particulate structure greatly enhances its activity.

Most proteins expressed *in vitro* have a low immunogenicity. By presenting them in a multimeric form, most clearly demonstrated by Morein's experiments with immunostimulating complexes (ISCOMs), their immunogenicity is greatly enhanced (Morein *et al.*, 1990). The principle in this presentation system is the creation of a particle comprising several copies of the protein. The particles are composed of saponin, cholesterol, phosphatidyl choline and the protein under study, the constituents being held together by hydrophobic interactions. The ISCOMS are seen as cage-like structures in the electron microscope, about 40 nm in diameter and comprising 12 nm morphological subunits. Proteins presented in this way not only elicit high levels of antibody but they also stimulate cell-mediated immunity. Moreover, preliminary results indicate that intranasal administration of ISCOMs containing influenza and measles haemagglutinins protect mice against experimental challenge.

Peptides as immunogens

Producing a protein, either *in vitro* or *in vivo*, which has the same amino acid sequence as in the parent organism does not provide any information about the epitopes or short amino acid sequences which elicit the protective immune response. Indeed, the methods which have been used so far to express immunogenic proteins do not even attempt to organize the structure of the protein into the configuration which it has on the parent organism. It is fortuitous if a vaccine produced *in vitro* or *in vivo* is effective because it is improbable that a protein produced in a foreign environment will fold in the way it does in the parent organism. All that has been done so far is to produce immunogenic proteins in a milieu which is different from the native organism. This is not to deny that this is a remarkably sophisticated biochemical achievement but it does not provide any

information about how to achieve the specific immune responses we seek from rationally designed vaccines. These answers can only be obtained by further dissection of the proteins into active peptide fragments.

There are several reasons for pursuing such a strategy (Brown, 1990):

- chemical synthesis allows the production of stable products which do not have the problems associated with materials produced in cells;
- the approach offers the advantages associated with the handling of small molecules compared with much larger protein molecules;
- the information which is accumulating on the interaction of peptides with MHC molecules (Bjorkman *et al.*, 1987; J.H. Brown *et al.*, 1993) suggests that the immune response to proteins will soon be understood at the level of short amino acid sequences.

These considerations indicate that it will be possible to design peptides which will elicit the required immune responses for any pathogen. Clearly, there is still much to be learned about the components required in a totally synthetic vaccine. Immunodominance plays a major part in B cell recognition and this factor is now being seen as important in T cell recognition also. Optimal partnerships of B and T cell epitopes are the clear goal.

Genetic immunization

During the past three years, a quite revolutionary conceptual advance has been made in vaccination. It had always been held that it would be necessary to present the immunogenic protein (or carbohydrate-protein complex) to the host in order to elicit the appropriate immune response. Recent experiments with several viruses have shown that immune responses can be obtained by injecting the DNA coding for the protective antigens directly into the muscle tissue of mice (Ulmer *et al.*, 1993). In the case of influenza, protective immunity was achieved. The proteins coded by the inserted genes are expressed in the muscle and skin cells and are then presented to the immune system. It appears that most of the DNA is degraded but, nevertheless, enough remains for the expression of sufficient protein to stimulate the appropriate immune response. It would be interesting to compare the level of the immune response elicited by this method with that produced by the direct inoculation of the protein for which the DNA codes.

Unlike recombinant proteins made in test tubes, if the proteins were made directly in living animals they would not have to go through the extensive purification steps which are currently demanded. Provided the immune response is sufficient to afford protection, this method appears to provide a major step towards the provision of new vaccines.

SUMMARY

The study of vaccines and vaccination based on the concepts of molecular biology has provided the expectation that new vaccines will be produced which elicit protective immune responses without the disadvantageous side effects of the current products. As we learn more about those parts of a virus which are required to provide immunity and the way in which to present them to the host, it is anticipated that vaccines will consist of small fragments of the agent, synthesized either chemically or biochemically, without the need to grow the agent itself. More specifically, however, it must be recognized that each disease is a separate challenge and the protective immunity which each demands should be considered accordingly. The crucial issue is to identify the immune response which correlates with protection. It should not be forgotten how

effective the empirical approach has been and we should not ignore the lessons that have been learned from these successes.

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Bacterial vaccines

P.D. Walker

Bacterial vaccines can be divided into two categories:

i) Inactivated vaccines which are composed of whole bacterial cells and/or their metabolic products or, alternatively, a purified fraction of the cell.

ii) Live vaccines which are composed of attenuated strains of the parent virulent organisms.

Although in the developed world, particularly with medical vaccines, purified and characterized immunogens are preferred from both a safety and quality control viewpoint (Walker, 1991), in non-domestic animals economic considerations dictate that the product, in addition to being efficacious and safe, should be cost-effective in an animal of finite worth. Under these circumstances, cruder, less purified products of proven efficacy continue to be employed. In addition, protection by vaccination is often multi-factorial, involving a number of antigens, while the use of single entities produced either by conventional purification or genetic engineering should be treated with some caution (Walker, 1992).

Both types of vaccine can either be injected parenterally or administered orally. Inactivated vaccines are invariably administered by injection, although large doses administered orally have also been used successfully. Living vaccines are administered parenterally or, where protection is against enteric diseases, orally. The use of the oral route as a means of stimulating mucosal immunity in general is being increasingly exploited, particularly using genetically engineered organisms such as the *aro* mutants of *Salmonella* spp. as cloning vectors (Hoiseth and Stocker, 1981; Hone *et al.*, 1991). Such vaccines stimulate secretory, humoral and cell-mediated responses. Vaccines are designed to stimulate the normal immunological mechanisms of the host. Following parenteral injection of vaccines, antibodies, mainly IgG, appear in the bloodstream. Vaccines administered orally stimulate local production of antibody (mainly IgA) at the mucosal surface. In general, antigenicity is a function of molecular size. For this reason, bacterial proteins are good antigens, although long chain capsular polysaccharides are also antigenic and have formed the basis of successful vaccines, for example in protection against haemorrhagic septicaemia in cattle (Nagy and Penn, 1976).

In developing a vaccine strategy for the control of disease, the vaccine manufacturer has to be mindful that there are two stages in infectious disease, i.e. establishment of the organism in the host followed by the clinical signs of infection. Although, in practice, these two stages quickly pass from one to the other, from the point of view of the vaccine manufacturer, it is useful to distinguish between them. Vaccines can be designed that either prevent establishment of the organism in the host or prevent the effects of infection. The problems posed to the manufacturer differ depending on which approach is taken (Walker and Foster, 1981).

The effects of infection are caused mainly by toxins elaborated by the organisms which have specific pharmacological effects. Indeed, the results of infection can

be reproduced by injecting sterile filtrates of the organisms concerned. Bacterial toxins are usually secreted into the culture medium and the manufacturer is therefore faced with the problem of producing large quantities of toxins for further processing. These are usually separated from the bacterial cell mass.

On the other hand, if the approach is to prevent establishment of infection then it is the antigens of the bacterial cell which the manufacturer needs to consider. For example it has been shown that in many diseases adherence of the organism to epithelial tissues by specific adherence mechanisms followed by multiplication of the attached organisms is important (Walker and Nagy, 1980). In this connection, the manufacturer must identify these particular bacterial antigens and adjust the growth conditions to ensure the production of large quantities of the appropriate antigen. After inactivation, the organisms are harvested for further processing. Whole culture vaccines may stimulate both types of immunity.

This overview deals with both types of protection using illustrations from veterinary vaccines which are currently in use for sheep, cattle, pigs and dogs or which are under development. A brief section on fish vaccines is included, as aquaculture may be of increasing relevance to developing countries in the future.

PROTECTION AGAINST THE EFFECTS OF DISEASE

The classic example of the use of this type of vaccine protection is that of clostridial toxoid vaccines. The most economically important diseases in sheep, cattle and pigs are caused by clostridial infections (Sterne and Batty, 1975). As a result of the stimulus to research of the potential threat of gas gangrene during the First and Second World Wars, a vast amount of literature exists on the toxins produced by these organisms (Oakley, 1943; Oakley and Warrack, 1953; Brooks, Sterne and Warrack, 1957). The major toxins responsible for disease have been identified and the use of toxoid vaccines to control clostridial diseases is widely practised (Sterne *et al.*, 1962). Bacterial toxins are inactivated to produce bacterial toxoids in such a way that toxicity is lost but antigenicity retained. The usual method of treatment is with formaldehyde (Ramon, 1924).

Toxoids are normally adsorbed on to an adjuvant, usually a mineral salt such as aluminium hydroxide, aluminium phosphate or potassium aluminium sulphate. By far the most potent adjuvants are mineral oils or their derivatives (see Table 2), which enhance immunogenicity by at least an order of magnitude over conventional adjuvants (Freund, Casals-Ariet and Genghof, 1940). However, owing to their production of severe local reactions which may persist indefinitely, they are completely unacceptable for human vaccination and even in veterinary medicine, despite their effectiveness, their use in the developed world is severely limited owing to potential carcass blemish and its effect on the export of meat. Attempts have been made to overcome this problem, for example by the use of the interperitoneal route (Thomson *et al.*, 1969). However, in the developing world it is questionable whether these issues are important. Where production is for a domestic market, the benefits of a more effective and lasting immunity, with all the advantages of reduced handling of animals, must surely outweigh any cosmetic considerations. Furthermore, it is quite possible to identify an injection site such as behind the ear or on top of the skull which is removed at slaughter and will not affect the appearance of the meat.

Adult animals are normally given two injections of toxoid separated by at least 28

TABLE 2

Arithmetic mean antitoxin responses of lambs to 2x2 ml doses of a multicomponent clostridial vaccine precipitated with potash alum and to a 1 ml dose adsorbed with Freund's adjuvant

Weeks after second innoculation	Adjuvant	*Clostridium*				
		perfringens		*septicum*	*novyi*	*tetani*
		beta	epsilon			
Two	Alum	25.4	2.2	1.9	10.2	16.0
	Freund's	46.6	12.9	5.7	13.2	27.8
Ten	Alum	1.1	0.6	-	0.38	0.7
	Freund's	5.6	3.1	0.6	1.3	6.7

days for a primary immunization course. Thereafter, further boosting to the immunity can be given at critical periods of life. One such period coincides with the 14 days preceding parturition to ensure that the maximum amount of antibody is transferred from the serum via the colostrum and milk for the protection of the newborn. Following the disappearance of such protection, the young animal should undergo prophylactic vaccination.

The range of clostridial diseases against which vaccination is practised is shown in Table 3. Both single and combined vaccines are available, with individual manufacturers responding to the needs of the market place. Based on experience, manufacturers have been able to formulate multicomponent products so that there is a balanced response to all the major antigens despite antigenic competition (Table 4). It is clear from the table that, although individual antigenic responses may be lower with a multicomponent than with an equivalent monocomponent vaccine, the responses to the multicomponent vaccine are above the minimum responses required by the regulatory authorities.

In the United Kingdom, in order to provide effective and safe vaccines, manufacturers have had to comply with the conditions laid down in the British Pharmacopoeia (Veterinary) (HMSO, 1985) whose requirements have subsequently been incorporated in the European Pharmacopoeia. These regulations were originally formulated after consultation

TABLE 3

Principal *Clostridia* spp. causing diseases in animals

Species	Disease
Cl. perfringens type A	Wound infections, gangrenous mastitis, enterotoxaemia in nursing lambs in California and Oregon ("yellow lamb")
Cl. perfringens types B, C and D	Enterotoxaemias in various animals including calves, sheep, goats, piglets and foals
Cl. septicum	Malignant oedema of horses, cattle, sheep and swine
Cl. chauvoei	Blackleg in sheep and cattle
Cl. novyi type A	Gas gangrene in cattle and sheep
Cl. novyi type B	Black disease in sheep, sudden death in cattle and pigs
Cl. haemolyticum (*Cl. novyi* type D)	Bacillary haemoglobinuria in cattle
Cl. sordellii	Gas gangrene
Cl. tetani	Tetanus in all species of domestic animals
Cl. botulinum type C and D	Botulism in sheep, cattle, dogs, chickens and wild duck

TABLE 4

Arithmetic mean antitoxin response of groups of rabbits in tests on 10 consecutive batches of combined vaccines compared with tests on 12 consecutive single batches

Vaccines used	Responses in IU per ml to *Clostridium* spp.				
	Cl. welchii beta	*Cl. welchii* epsilon	*Cl. septicum*	*Cl. oedematiens*	*Cl. tetani*
Br. Vet. Codex standards	10	5	2.5	10	3.5
Mean responses to 10 combined batches	55	6	9.2	16	62
Mean responses to 12 single batches	141	15	9	122	-

Note: IU = international units.

with major manufacturers who, based on their own experiences of vaccines that were effective in the field, were able to suggest consensus criteria for the release of products. In the case of clostridial vaccines, with the exception of *Clostridium chauvoei*, in order to release a product a manufacturer has to show that, after two injections given according to the recommended dosage regime in the field, groups of rabbits respond with certain minimum antitoxin titres. Subsequent formal studies have shown that vaccines producing adequate titres in rabbits produce an adequate serological response in the target species (Frerichs and Gray, 1975).

Nevertheless, these criteria have some drawbacks. For example, they only measure antibodies to the major lethal components and not to other toxins and cellular elements which may be important in protection. Thus, they do not distinguish between whole-culture and toxoid vaccines, for example. A further complication is the differences in response by individual breeds of rabbit, which can vary threefold in their response to the same vaccine preparation (Walker and Batty, 1985). In the case of *Cl. chauvoei*, groups of guinea pigs are vaccinated with two doses of the vaccine according to the recommended dosage regime and challenged with a virulent culture 14 days after the second injection. All vaccinates should survive and all controls die within 48 hours.

The problem for the manufacturer is then to produce large quantities of toxins that can be converted into toxoids and, following suitable processing, incorporated into vaccines.

Component manufacture

As a result of developments in fermenter technology, some manufacturers are able to grow clostridial species in fermentation culture to produce high yields of clostridial toxins suitable for toxoiding (Walker and Foster, 1981). Over a period of time, manufacturers have carefully selected those strains which give maximum toxin production in the growth media they have developed. They have, in effect, cloned their strains to give stable high-yielding toxin mutants which enable them to produce consistently high yields of toxins. Strain improvement by mutation and selection forms the basis of a continuing research programme by most manufacturers.

The majority of strains are stable when stored as freeze-dried cultures and this enables large batches of master seed cultures to be stored. By the use of a fresh aliquot of the master seed to prepare working seeds, any deterioration of the strain owing to passage in liquid media can be avoided. Where storage by freeze-drying is not satisfactory, cultures can be stored in liquid nitrogen and fresh ampoules opened as required.

As bacterial toxins are not generally produced in significant quantities in synthetic media, it is necessary to provide a source of peptides. The manufacturer has to select a source of protein for digestion which is readily available, for example meat, casein or soybean. Digests of meat are usually prepared with papain or trypsin and digests of casein with acid or trypsin. For maximum toxin production, digest media need to be supplemented with various amino acids and vitamins; yeast extract provides a convenient, reliable and cheap source for the latter. In the case of certain toxins, it is necessary to regulate carefully the amounts of various inorganic ions present, for example iron in tetanus toxin production (Mueller and Miller, 1954). Glucose is the normal energy source although maltose and sucrose are also used (Moore, 1968; Walker, Harris and Moore, 1971).

Manufacturers have invested heavily in developing media and growth conditions to give maximal toxin production (Thomson, 1979). Published data are available for the growth and toxin production of the Harvard strain of *Cl. tetani* using Mueller medium (Mueller and Miller, 1954). This consists of a calcium caseinate digest and acid hydrolysed casein base supplemented with bullock's heart infusion broth, glucose, vitamins, cystine and iron powder.

The oxidation-reduction potential of freshly prepared digest media after autoclaving is usually sufficiently low to permit the growth of almost all species of *Clostridia* without further additions, particularly when combined with the use of 5 to 10 percent inocula. If necessary, reducing agents such as cysteine hydrochloride can be added prior to inoculation. There is therefore no special requirement for the introduction of gases into the culture.

Clostridia spp. are for the most part saccharolytic, acid-producing organisms and automatic pH control and addition of carbohydrate results in considerable improvements in yield and reproducibility of culture. Nevertheless, it is possible to control these processes manually, albeit at the expense of some efficiency. By using bottled media and working in an incubator, it is possible to control pH and make periodic additions to containers using a pH meter and appropriate solutions. Where the costs of fermenters are unacceptable and adequate labour is available, this can be an effective method of production, particularly for developing countries.

The optimum pH range for maximum toxin production varies with the toxin concerned. For example, maximum production of epsilon toxin by *Cl. perfringens* (*welchii*) type D occurs in the alkaline range while the pH optima for other clostridial toxins are lower.

Toxin production during growth can be monitored by a variety of techniques. These include, where present, the measurement of enzymic activity, for example lecithinase, haemolysis and proteolysis or, alternatively, *in vivo* testing in guinea pigs, mice and rabbits, using death or skin lesions as the indicator. Values of toxin are expressed in terms of indicator effects, for example minimum lethal dose, minimum haemolytic dose or, alternatively, in terms of unit equivalents of standard antitoxin. The Lf dose of a toxin is that dose of toxin which, when mixed with one unit of

antitoxin, flocculates in the least time, i.e. it is the first mixture to flocculate, while the L+ is the smallest dose of toxin which, mixed with one unit of antitoxin, kills 50 percent of injected mice of a designated weight within a designated time (Batty, 1971).

The length of incubation is determined by the particular toxin being produced. *Cl. perfringens* (*welchii*) beta toxin is destroyed by the proteolytic enzymes which the organism produces if incubation continues for too long while others such as epsilon toxin are much more stable.

Bacterial toxins are invariably inactivated with formaldehyde (Ramon, 1924). Although the principal action of formaldehyde on a toxin is to remove toxicity, this is only achieved at the expense of some loss of immunogenicity. For this reason, toxoids are normally standardized in terms of a total combining power (TCP) test. The TCP test involves a partial neutralization of a fixed dose of antitoxin with a series of varying doses of the toxoid being tested. The unreacted antitoxin is then mixed with a fixed dose of toxin equivalent to half the dose of antitoxin used and the whole series of mixtures are injected into mice which are observed for two days. The toxoid present in the mixture that kills half the mice into which it is injected is equivalent to the fixed dose of toxin used (Batty, 1971). This is necessary because, after inactivation, it is no longer possible to measure the activity of the toxoids in terms of indicator effects, although it is possible to carry out an Lf test with certain toxoids. Where there are no appropriate facilities to carry out such tests, developing countries may well have to rely on data from the original testing, for example minimum lethal doses.

Harvesting

For veterinary purposes, crude toxoids are normally used. Crude toxoids are good antigens and are extremely stable under a variety of environmental conditions. The preparation of such toxoids only involves separation from the bacterial cell mass by centrifugation (Walker and Foster, 1981).

Blending

Bacterial toxoids are normally blended into multicomponent vaccines. Very careful formulation is necessary if the host is to produce a balanced response against all components and the vaccine is to pass the criteria laid down by regulatory authorities. Toxoids are normally blended, based on TCP value or some equivalent, and the appropriate unit equivalents added to the final vaccine. In practice, the manufacturer will take into account the testing of the toxin before inactivation (Lf, L+, Lv, Lh, etc.) as well as the TCP and, where appropriate, the Lf of the toxoid. Bearing in mind the loss of immunogenicity on inactivation if the toxoiding process is allowed to proceed too far, the ratio of TCP to Lf declines. A fine balance has to be struck between a high TCP-Lf ratio, on the one hand, and minimum residual toxicity and freedom from any tendency to reversal, on the other.

After blending and the addition of the adjuvant, the vaccines are ready for issue when certain statutory regulatory tests have been completed.

Use of vaccines

The aim of the manufacturer is to provide protection both to the adult animal and to the offspring by the passive transfer of antibody. In the case of the adult animal, providing it has been given primary and secondary injections with appropriate booster doses, it should remain protected for life. In an adequately primed animal, any subsequent infection will result in an anamnestic response which will afford immediate protection (Chadnik, Watson and Hepple, 1959). In the case of the

offspring, the length of protection will be determined by the persistence of antibody titres which will, in turn, be determined by the amount and titre of the colostral antibody ingested. As the decay of passively transferred antibodies is known (roughly 16 percent per week), it is relatively easy to predict how long protective levels are maintained. Protective antibody levels are known for several antitoxins, for example 0.1 unit for *Cl. perfringens* epsilon and tetanus and 0.5 units for *Cl. perfringens* beta. Tables 5 and 6 illustrate this by reference to two commercial vaccines, A and B.

Table 5 shows the difference between the performance of the two commercial vaccines, A and B, when the *Cl. perfringens* epsilon antitoxin titres to the first and second injections in previously unimmunized sheep are compared. It can be seen with vaccine A that after a single injection 90 percent of the animals gave a measurable titre compared with 65.5 percent with vaccine B. The group geometric mean (GMT) titre achieved after a single injection with vaccine A is about eight times that of vaccine B. These differences in the titres are also seen after the second injection (Table 5), and with vaccine B two animals still have no measurable titre. The responses in the ewes (Table 6) are reflected in the antitoxin titres obtained in the lambs subsequent to parturition and, hence, the length of protection afforded to the lambs during the early weeks of life (Table 7).

It can thus be seen that, even with two vaccine preparations released on the basis of the Codex requirements, there are differences in potential protection of both the adult and the newborn.

While toxoid vaccines are normally effective, there are examples of breakdown in the field, usually in cases where high levels of challenge lead to large quantities of toxins which overwhelm the antitoxic immunity. For this reason some manu-

TABLE 5

Distribution of *Cl. perfringens* epsilon antitoxin titres in the sera of 8-month sheep immunized with two commercial multicomponent clostridial vaccines, A and B

Serum titres *(IU ml^{-1})*	No. of sera (%)	
	Vaccine A	Vaccine B
42 days after first injection		
5	13 (43)	1 (3.0)
1-5	9 (30)	8 (28.0)
0.1-1.0	5 (17)	10 (34.5)
<0.1	3 (10)	10 (34.5)
Total	30	29
Group GMT	**1.84**	**0.21**
14 days after second injection		
10	13 (43)	2 (6.5)
1-10	16 (53)	16 (59.0)
0.1-1.0	1 (4)	8 (28.0)
<0.1	0	2 (6.5)
Total	30	29
Group GMT	**9.04**	**1.39**

Note: GMT = geometric mean titre.
Source: Adapted from Kerry and Craig, 1979.

TABLE 6

Distribution of *Cl. perfringens* epsilon antitoxin titres in the sera of ewes immunized with two commercial multicomponent clostridial vaccines, A and B, following the second injection and bled 2 to 5 days after lambing

Serum titres (*IU ml⁻¹*)	No. of sera (%)	
	Vaccine A	Vaccine B
10	13 (38)	3 (9)
1-10	19 (56)	20 (61)
0.1-1.0	2 (6)	7 (21)
Total	34	33
Group GMT	**6.72**	**1.66**

Note: GMT = geometric mean titre.
Source: Adapted from Kerry and Craig, 1979.

TABLE 7

Distribution of *Cl. perfringens* epsilon antitoxin titres in the sera of 2- to 5-day lambs born to ewes immunized with two commercial multicomponent clostridial vaccines, A and B

Serum titres (*IU ml⁻¹*)	No. of sera (%)	
	Vaccine A	Vaccine B
5	19 (61)	4 (13)
2-5	6 (19)	8 (26)
1.0-2.0	4 (13)	3 (10)
0.5-1.0	1 (3)	4 (13)
<0.5	1 (3)	12 (39)
Total	31	31
Group GMT	**7.48**	**1.25**

Note: GMT = geometric mean titre.
Source: Adapted from Kerry and Craig, 1979.

facturers do not remove cells from all the components and leave them as anacultures so as to obtain both antibacterial and antitoxic immunity.

A typical example of this was seen in Denmark in vaccination against the disease piglet enteritis, caused by *Cl. perfringens* type C. This disease is a haemorrhagic necrotizing enteritis of young piglets which, over a period of ten years, has spread from small foci to cover large areas of Jutland and Zeeland. Initial studies on this disease by Hogh (1974) showed that the causative organism was *Cl. perfringens* type C and that the disease could be controlled by administration of high-titre horse antisera containing beta antitoxin and also by vaccination with vaccines containing beta toxoid. Using a vaccine which contained both cells and toxoid (Xento, Wellcome), successful protection was demonstrated (Hogh, 1974).

Some years later a combined *Escherichia coli/Cl. perfringens* type C toxoid vaccine (Xentocol, Wellcome), specifically designed for the market to control both *E. coli* diarrhoea and *Cl. perfringens* type C enteritis, was introduced. In many herds

the vaccine was used successfully to control *E. coli* and piglet enteritis. However, in a number of problem herds the vaccine was totally ineffective in controlling piglet enteritis.

Table 8 shows the results of a comparative trial that was carried out using two product formulations and a control group. It can be seen that removal of the cells of *Cl. perfringens* resulted in a considerable loss of efficacy. The results indicated that, under conditions of high challenge, both antibacterial and antitoxic immunity were required to contain infection (Hogh, 1988). Electron microscopy studies on the disease showed a pattern of adhesion of *Cl. perfringens* type C to the jejunal villi in piglets identical to that of *E. coli* infection in the same species (Walker and Nagy, 1980). The initial phases of the disease were characterized by colonization of the villi owing to adhesion and multiplication of the organisms. Unlike *E. coli*, the adhesive factors are not characterized but clearly were present on the cells of *Cl. perfringens* in Xento vaccine and probably play the same role as the "K" antigens of *E. coli*.

The lessons of these results are clear. Protection is multifactorial and, under extreme conditions of challenge, vaccines based on more simplified entities break down.

The combination of *Cl. perfringens* type C and *E. coli*, i.e. a combination of anaerobic and aerobic vaccines, represents a further development in the marketplace. This is a response both to the needs of the market and the desire to obtain a competitive edge. In this respect it is worth noting two other developments in this area.

A combination clostridial and *Pasteurella* vaccine (Heptavac-P) was introduced by Hoechst (United Kingdom) to provide simultaneous protection against clostridial and respiratory diseases, particularly in young lambs. A somewhat different approach is the combination of clostridial vaccines with an anthelmintic, levamosole, introduced by the Tasman Veterinary Laboratories (New Zealand). This combination allows simultaneous protection against clostridial infections and parasites although, as dosing with anthelmintics is very dependent on the weight of the animal, dosage regimes can be difficult.

PROTECTION AGAINST ESTABLISHMENT OF INFECTION

The importance of the cellular component as a supplement in traditional toxoid vaccines has already been discussed. Cellular antigens are important in the establishment of an organism in the host. Antibodies interfering with establishment

TABLE 8

Comparison of *Cl. perfringens* beta toxoid vaccines and an identical vaccine containing *Cl. perfringens* type C cells in pigs on a problem farm in northern Jutland

Vaccine	Necrotic enteritis			
	Litters	Piglets	No. dead	%
Cl. perfringens beta toxoid	21	239	83	34.7
Cl. perfringens beta toxoid plus cells	22	249	21	6.8
Control	20	217	93	42.9

will prevent infection. In general, cellular antigens can be divided into cell-wall and capsular types. Until fairly recently, most whole-culture vaccines were relatively undefined and consisted of suitably inactivated cells standardized on the basis of cell numbers (approximately 10^{10} organisms per ml), as measured by opacity. Such vaccines undoubtedly act by stimulating bactericidal antibodies directed against the cell and, as such, tend to be sero-specific. When the number of important serotypes in a species is limited, the formulation of vaccines is relatively simple. However, in species where a large number of serotypes are important, it is necessary to blend a multiplicity of serotypes into the final vaccine.

Toxicity resulting from endotoxin components of the cell wall of Gram-negative bacteria which can result in adverse reactions in the adult and abortion in pregnant animals becomes important as the numbers of organisms are increased. In the case of *E. coli* vaccines for immunization against diarrhoea in animals, these consisted of a mixture of the predominant "O" serotypes prevalent in the disease. In the case of *Pasteurella* vaccines for the treatment of respiratory diseases, these again consisted of inactivated cells of the predominant serotypes of *Pasteurella haemolytica* and *Pasteurella multocida.*

Similar inactivated whole-culture vaccines have also been used for the control of leptospiral infections. *Leptospira* spp. are well known as causal agents of disease in the dog (Broughton and Scarnell, 1985). The importance of *Leptospira* spp. as a causative agent of disease, particularly in cattle, has been recognized in recent years. The problem has become acute owing to the transfer of leptospirosis from cattle to humans during handling. The organism is shed in urine and is a potential source of infection. Vaccines have been developed from inactivated whole cultures to prevent shedding of the organism into the environment (Broughton *et al.*, 1984). Results show that, in unvaccinated cattle, leptospiral organisms are isolated frequently from the urine whereas, in vaccinated cattle, there is virtually no excretion (Walker, 1987).

While the transfer of *E. coli* bactericidal antibodies via the colostrum and milk to the suckling animal undoubtedly affords some protection, the approach to vaccination against *E. coli* was revolutionized by the identification of the antigens responsible for adhesion of the organism to the intestinal villi. The adhesion of organisms to the intestinal villi is the first stage in *E. coli* infection and, if this stage can be blocked by antibodies, infection is prevented. K88ab and K88ac were identified as important adhesins for the disease in piglets (Smith and Lingood, 1971) and early work in pigs showed that experimental vaccines prepared from purified K88 antigens were effective in preventing disease in piglets after oral challenge (Jones and Rutter, 1972).

The first available commercial vaccines were based on partially purified K88 antigens of *E. coli* (Nagy *et al.*, 1978). During growth, the K88 antigen is secreted into the growth medium and can be selectively removed from the supernatant by adsorption on to aluminium hydroxide, leaving behind endotoxin in the supernatant.

Rather than depend on anti-adhesion alone, the early vaccines comprised cells of *E. coli* supplemented with measured quantities of K88ab and K88ac. In this way, both the anti-adhesive and bactericidal effects of antibodies were present. Sows or gilts were given two injections of the vaccine, with the second dose timed to be given two weeks prior to parturition, to achieve maximum levels of antibody in the colostrum and milk for a passive transfer to the piglets. To piglets of

vaccinated gilts following oral challenge, such vaccines gave significant protection against mortality, diarrhoea and excretion (Nagy *et al.*, 1978). While a conventional *E. coli* K88-negative vaccine afforded significant protection over the controls, the vaccine supplemented with measured quantities of K88 antigen is even more significantly protective.

As time passed, further adhesins such as K99 and 987P were recognized as being important and it became necessary to formulate multicomponent vaccines (Orskov *et al.*, 1975; Moon *et al.*, 1977; Smith and Huggins, 1978). The protection afforded by such vaccines is shown in Tables 9 and 10. Experiments similar to those for the original K88 vaccines were performed and it can be seen that significant protection is afforded against mortality (Table 9) and diarrhoea (Table 10) with all four serotypes (Nagy and Walker, 1983; Nagy, Mackenzie and Painter, 1985).

The identification of the surface antigens responsible for adhesion in *E. coli* represents a move away from the traditional vaccine to a more defined product. Other envelope antigens which may be important in infection have been the subject of increasing interest.

Treponema hyodysenteriae is the aetiological agent of swine dysentery, a disease occurring in post-weaning pigs, ususally in fattening units where pigs are kept in large numbers (Alexander and Taylor, 1969; Taylor and Alexander, 1971; Harris *et al.*, 1972; Hughes, Olander and Williams, 1975). After infection with the organism, swine develop a mucohaemorrhagic diarrhoea characterized by extensive necrosis of the mucosal epithelium of the colon and caecum. This results in dehydration, emaciation and rapid weight loss followed, in severe cases, by death. Recent studies have shown that, following a single-dose parenteral immunization with an inactivated culture of *T. hyodysenteriae* emulsified in oil, swine are protected against experimental challenge.

A field trial indicated that this vaccine confers significant benefits. In one fattening unit, swine dysentery was reduced by 50 percent and the severity of infection by 50 percent, while vaccinated piglets showed a 10 percent increase in weight gain over controls in the same period after a single injection of the vaccine (Fernie, Ripley and Walker, unpublished results). As mentioned previously, there is increasing resistance to oil-based vaccines because of carcass blemish and its effect on the export of meat. For this reason, the vaccine has not been commercially developed.

To obtain further data on the protective antigens with a view to formulating a more non-reactive product, research has centred on the envelope proteins of *T. hydosyenteriae*. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, it has been shown that polypeptides with a molecular weight of between 30 000 and 36 000 were the predominant ones detected by porcine immune serum and were not present on non-pathogenic strains of this organism. These unique antigens may play a role in the virulence of *T. hyodysenteriae* and form the basis of a new effective non-oil-based vaccine (Chatfield *et al.*, 1988).

A similar outer membrane protein (68 kDa) was demonstrated to be an important antigen in protection against atrophic rhinitis in pigs caused by *Bordetella bronchiseptica*. There is a strong correlation between antibody titres to this antigen and protection in pigs. The antigen is present in virulent strains of *B. bronchiseptica* but absent in non-virulent strains. Monoclonal antibody produced against this antigen passively protects mice from aerosol challenge with the organism (Montaraz, Novotny and Ivanyi, 1985; Kobisch and Novotny, 1990).

TABLE 9

Mortality rates 24 hours after challenge in litters from vaccinated and non-vaccinated gilts after orogastric challenge with 10^{10} *Escherichia coli* plus types 24 hours after birth

Vaccine	*E. coli* challenge cultures			
	K88ab	K88ac	K99	987P
	(died/challenged) (%)			
None	19/36 (53) (n=5)[a]	38/58 (66) (n=10)	10/34 (29) (n=5)	33/41 (80) (n=7)
5:1[b]	0/39 (0) (n=5)	3/36 (8) (n=5)	2/37 (5) (n=5)	3/35 (9) (n=5)
Reduction in mortality rates *(%)*	100	88	83	90

[a] n = number of litters used.
[b] Vaccine 5:1; partially purified K88ab, K88ac, K99, 987P of *E. coli* (100 units each).

TABLE 10

Diarrhoea rates 24 hours after challenge in litters from vaccinated and non-vaccinated gilts after orogastric challenge with 10^{10} *Escherichia coli* plus types 24 hours after birth

Vaccine	*E. coli* challenge cultures			
	K88ab	K88ac	K99	987P
	(diarrhoea/challenged) (%)			
None	46/54 (85) (n=7)[a]	47/58 (81) (n=10)	34/54 (62.9) (n=8)	50/52 (96) (n=9)
5:1[b]	1/51 (1.9) (n=7)	2/53 (3.7) (n=7)	20/57 (35.1) (n=7)	22/48 (46) (n=7)
Reduction in diarrhoea *(%)*	97.8	95.4	44.1	52.1

[a] n = number of litters used.
[b] Vaccine 5:1; partially purified K88ab, K88ac, K99, 987P of *E. coli* (100 units each).

In an attempt to improve the performance of *Pasteurella* vaccines, considerable work has been carried out using the specific pathogen-free (SPF) lamb model developed at the Mordun Institute (Gilmour *et al.*, 1975). Such lambs are highly susceptible to challenge with *Pasteurella* spp. when given in conjunction with PI 3 virus. Even in the absence of death, lung lesions develop after a few days and can be scored at post mortem examination.

For a number of the A biotypes, significant protection has been shown in this model using heat-killed cells or sodium salicylate extracts of the organisms as vaccines. However, vaccines containing extracts or heat-killed cells of serotype A2, which is responsible for the majority of outbreaks in sheep, have been less effective (Gilmour *et al.*, 1983; Donachie *et al.*, 1986).

Studies have indicated that organisms grown *in vivo* produce additional antigens to those grown *in vitro*. *Pasteurella haemolytica* A2 cells recovered from the

pleural fluid of lambs with pasteurellosis were found to express previously unidentified proteins, and two of these with molecular weights of 70 and 100 kilodaltons (kDa) could be induced *in vitro* by restricting iron availability in the growth medium.

It was subsequently shown that significant protection against *P. haemolytica* A2 infection was conferred on SPF lambs by a vaccine containing extracts of *P. haemolytica* A2 cells grown under iron restriction, compared with a vaccine made in exactly the same way from cells grown in an iron-replete medium. This was confirmed by comparing the antibody responses seen previously in SPF lambs which were immune after recovery from A2 infection. Using immunoblotting, it was demonstrated that there were strong responses to iron-limiting proteins of 70 and 35 kDa and a weaker response at 100 kDa (Gilmour *et al.*, 1991).

In the case of *Pasteurella multocida*, an alternative approach has been to concentrate on the toxins produced by the organism. Some success has been reported in immunizing pigs against experimentally induced progressive atrophic rhinitis caused by this organism, using a formalin-detoxified purified toxin. This approach may well be valid for control of disease in lambs (Chantler, Rutter and Mackenzie, 1986; Fogel, Neilsen and Joral, 1989).

Surface appendages known as pili (fimbria) have also been shown to be important in the pathogenesis and protection against ovine footrot. Ovine footrot results in separation of the horn from the underlying soft tissues of the foot (Beveridge, 1941). Separation of the horn leads to abscess formation and an extremely painful condition in which the sheep have difficulty in walking and grazing. Although the flora of the foot is very complex, it has been shown that the key triggering organism for infection is *Bacteroides nodosus* (Egerton, Roberts and Parsonson, 1969). A culture of scrapings from the infected area reveals a very distinctive rhizoidal colony (Thorley, 1976), the growth of which is highly pilated. On subculture in the laboratory a number of non-pilated smooth colonies are generated, and it has been demonstrated that the most effective vaccines are those made from pilated cultures (Egerton and Thorley, 1981; Thorley and Egerton, 1981). The organism is divisible into a number of serotypes based on pili agglutination and, in Australia, a number of serotypes have been identified as being important (Claxton, 1981). Multivalent vaccines incorporating whole cultures of a mixture of serotypes have been shown to be highly effective both in experiments involving challenge and in the field.

In summary, progress has been made in recent years to identify those antigens which are important in the pathogenic process and to ensure that, under fermentation conditions, these antigens are produced for vaccine production. During production, in addition to controlling pH and temperature, it is necessary to be able to measure and control aeration of the culture. The degree of aeration is dependent on the growth rate of the organism. The growth of *E. coli* strains requires high rates of oxygenation whereas strains of *Leptospira* spp., which grow much more slowly, have relatively low oxygen requirements. For several species, it is necessary to supplement the air or oxygen with carbon dioxide. Provision must be made for the sterilization of the incoming air and outgoing effluent; the latter presents a number of problems when high aeration rates are necessary.

The majority of these problems are minimized by growing the organism on solid surfaces, although this is achieved only at the expense of efficiency as compared with fermenter-grown material.

Individual bottles of nutrient agar are inoculated with the appropriate organism and incubated for one to three days. Following incubation and growth, the organisms can be removed from the surface of the culture and put into a resuspending fluid by agitation. Where the antigens are secreted into the supernatant fluid, there has been some attempt to purify them but the marketplace does not allow the extensive purification required for its human counterpart.

LIVE VACCINES

For many diseases, investigators believe that cell-mediated immunity (CMI) is necessary to provide solid long-term protection against infection. This has led to the use of live attenuated strains as vaccines because, by comparison, killed vaccines generally do not elicit such strong CMI responses.

Live attenuated vaccines in the veterinary field include *Salmonella dublin*, *S. cholera suis*, *Brucella abortus* and anthrax spore vaccine. The attenuated *S. dublin* and *S. cholera suis* strains are avirulent rough variants of the smooth virulent strain and, as such, are unable to synthesize a number of polysaccharide side chains required for virulence. However, in the present climate of legislation, a more rationally modified strain harbouring well-defined, attenuating genetic lesions is to be preferred. Examples of the latter are strains harbouring defined mutations in genes in the prechorismate pathway rendering them deficient in the ability to synthesize certain essential aromatic compounds. In addition, it is preferable that strains used for live vaccines should harbour at least two stable attenuating mutations which map for separate regions of the chromosome, thus limiting the danger of reversion to virulence. It has been shown that such strains, for example *aroA*, *aroC* or *aroD*, are admirably suitable for this purpose (Hoiseth and Stocker, 1981; Hone *et al.*, 1991).

In the case of enteric pathogens, mucosal immunity is regarded as an important factor in protection. In this respect, orally delivered vaccines are superior and the use of live oral vaccines to protect against salmonellosis in cattle provides a good example of modern developments.

Using a double *aro* mutant of *Salmonella typhimurium* to immunize cattle orally, it has been shown that there is significant protection against a live challenge with this organism. Of eight cattle immunized orally with approximately 10^{10} of the mutant seven days after birth using an antacid mixture, seven were completely protected against a challenge of approximately 10^{8} organisms three weeks later. All four controls showed significant symptoms and, along with the remaining vaccinate, had to be killed (Jones *et al.*, 1991).

More recently, this approach has been extended to the use of *S. typhimurium* G30 as a vector to express the fimbrial antigens K88 and K99 of enterotoxigenic *E. coli* (Stevenson and Manning, 1985; Attridge *et al.*, 1988). Oral administration of 10^{11} vaccine organisms to adult pigs elicited significant serum antibody responses to the respective fimbrial antigens (Morana *et al.*, 1994).

Live attenuated organisms have also been used in the case of *Pasteurella* spp. Passaged in the laboratory, cultures of *Pasteurella* spp. disassociate, throwing off mutants that have lost their ability to produce capsular polysaccharides. Such mutants are non-pathogenic but highly immunogenic when injected subcutaneously into the animal. Combined vaccines comprising mixtures of the living attenuated strains can be formulated and are highly effective against challenge in the SPF lamb model (Walker, 1987). Again, these studies are limited by the use of a naturally occurring avirulent strain. Such

strains will not be favoured because of potential reversion and it will be necessary to develop rationally attenuated strains harbouring well-defined attenuating genetic lesions such as the *aro* mutants for *Salmonella* spp.

Although reservations regarding the use of naturally occurring mutants or strains attenuated in the laboratory as vaccines have been stressed, the contribution that such vaccines have made in controlling economically important diseases in the field should not be forgotten. In the case of anthrax, the live attenuated spore vaccine derived from an avirulent non-capsulated variant of *Bacillus anthracis* has had a pronounced effect in limiting economic losses resulting from anthrax and an indirect effect in reducing human incidence (Sterne, Nichol and Lambrechts, 1942; Turnbull, 1991). Similarly, the *B. abortus* S19 strain attenuated by temperature has been highly effective in controlling brucellosis.

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Mycoplasmal vaccines

A. Provost

Compared with other sectors of microbiology, it is unfortunate to have to admit that the range of available vaccines for mycoplasmal diseases does not provide entire satisfaction for a number of reasons, among which are their poor safety record for recipient animals and a weak, short-lived resistance to natural disease after vaccination.

This disappointing situation can be explained on the following grounds:

- Imprecise knowledge of the actual pathogenic processes in mycoplasmal diseases.
- Insufficient knowledge of the precise role of the different antigens present in the mycoplasmal organism in both the pathogenesis and immune mechanisms; for example, in the case of *Mycoplasma mycoides* subsp. *mycoides SC*,[1] the function of galactan, the polyosidic outer capsule, is unclear.
- A lack of progress in developing simple, cheap and reliable potency tests for vaccines, taking account of the strong host species specificity of the mycoplasms.
- An almost total ignorance of the nature of the protective mechanisms in resistant animals, either after recovery from the natural disease or after vaccination. In no case is there a strict correlation between protection and the presence of circulating antibodies. The situation in contagious bovine pleuropneumonia (CBPP) is such that complement fixing, agglutinating, growth and/or metabolism inhibiting antibodies to *M. mycoides* are no proof of resistance to a challenge. Conversely, resistance can occur without them. The same events exist in enzootic pneumonia of pigs, owing to *M. hyopneumoniae*, and in *M. gallisepticum* infection of poultry. A possible exception, found in humans, is the presence of growth-inhibiting antibodies to mycoplasms in bronchial washings. This is an avenue which should be pursued in animal mycoplasmosis. Cellular-type immunity proves equally ineffective for any correlation with protection.
- Only partial protection against natural or experimental challenge can be induced – partial being understood in two ways: either only a reduced proportion of the total number of animals are protected, or the protection leads to an abortive or less severe disease. This occurrence may happen frequently following the use of inactivated vaccines but is also encountered with live vaccines.
- Unforeseeable reactions following vaccination, either locally at the site of injection (called Willem's reaction in the case of CBPP live vaccine) or by the potentiation of other diseases after the use of an inactivated vaccine (as observed in human primary atypical pneumonia and in enzootic pneumonia of pigs).

In the field of animal diseases, there is only one condition for which vaccination

[1] In this document, *Mycoplasma mycoides* subsp. *mycoides* (SC_1 bovine biotype) will be referred to as *Mycoplasma mycoides* (abbreviated as *M. mycoides*).

is currently in use, namely CBPP. Experimental vaccines have been produced against swine pneumonia but other strategies are also used to control the disease. An inactivated vaccine against contagious caprine pleuropneumonia (CCPP) has been studied in Kenya and is reported to have given valuable results in the field; it is not known, however, whether it is produced on a large scale. Live vaccines have been developed for avian mycoplasmosis and have been produced commercially on a temporary basis but, owing to some disappointments (including a lack of safety for non-vaccinated birds), their use tends to be ignored and control is confined to a combination of means centred on the treatment of fertile eggs (dipping, heating) to evade vertical transmission of the agent.

CBPP VACCINE: GENERAL CONSIDERATIONS

Types of vaccine

Vaccination against CBPP has been implemented by the following methods:

- *Willem's method,* using the pleuritic or pulmonary "lymph", or the secondary lymph resulting from a subcutaneous Willem's reaction induced artificially. The traditional African procedure (subcutaneous inoculation of a macerated lung lesion into the nasal mucosa) is an improvised and related variation of the procedure rediscovered in Europe in the 1850s.
- *Vaccines prepared from broth cultures of naturally attenuated strains* (e.g. the Australian strain V_5 and the Sudanese strain F), some of which are freeze-dried.
- *Cultures of* M. mycoides *attenuated by subculture,* varying in number of serial passages according to the virulence of the original strain, the culture medium, the sensitivity of local cattle and the inoculation site. These vaccines have been called incorrectly "Bennett vaccines". At present, strain T_1 is one of the two available. There have been recent advances in freeze-drying, prolonging the storage life of vaccine and facilitating its distribution in the field.
- *Egg culture vaccines,* employed successfully during the 1960s on account of their good immunogenicity and availability in a freeze-dried form. However, they have been held responsible for pulmonary complications in certain circumstances and have now been abandoned.

Inactivated vaccines of various types are of only poor immunogenicity or lead to an unacceptable reaction at the site of inoculation.

Fundamental rules

Vaccines against CBPP need to comply with three fundamental rules, derived from experience:

- *Viability.* Only vaccines prepared from the specific live mycoplasma possess adequate immunogenicity, engendering a resistance of the premunition type. The vaccine strain can be recovered from lymph nodes several months after vaccination and antigens may persist for up to 204 days.
- *Virulence.* Following the observations of scientists working in 1906, which were confirmed in Kenya in 1921 and a few years later in the Sudan in 1929, subcultures in broth lead to a certain decrease in the pathogenicity of *M. mycoides.* For any given strain, there are a certain number of *in vitro* subcultures which will attenuate the culture.
- Attenuation is characterized by the ability of the particular strain to produce at the site of injection only a minor reaction (localized oedema, which heals spontaneously) after subcutaneous or intradermal inoculation.

The actual technique of inoculation varies from one laboratory to another. This attenuation is not fixed, for further subcultures lead to a progressive decrease in virulence, with the disappearance of immunogenic properties. Therefore, it is necessary to reach a delicate compromise between retention of immunogenicity and minimal local reactions acceptable to cattle owners. Subculturing should be avoided.

- The attenuation of cultures to a greater or lesser extent by *in vitro* subculturing has provided the foundation for vaccination policy during the past 80 years. It is only the approach to the problem that has varied.
- *Vaccination route.* It is usual to follow the rule of the permissible or acceptable vaccination site. This requires dense connective tissue of a low reactivity to limit adverse local and generalized reactions which may sometimes be fatal. For this reason, it has long been advisable for the vaccine to be inoculated into the skin or the subcutaneous tissue of the tip of the tail. Nevertheless, for practical purposes and to avoid loss of the tail after a severe reaction to the vaccine, the current method is to inoculate the vaccine subcutaneously on the flank posterior to the shoulder blade, necessarily using a vaccine of acceptable or no residual virulence.

Occurrence of local postvaccinal reactions

At the end of the last century it was believed that, for a vaccination to be effective, a local lesion was needed – a circumscribed Willem's reaction. It was then thought inevitable that there would be a certain number of vaccination complications, ranging from extensive postvaccinal swelling to death, which could affect 2 to 3 percent of the vaccinated animals.

Towards 1930, scientists first started to claim that there was no relationship between the residual pathogenicity of a vaccine strain of *M. mycoides* and its immunogenicity. The years that followed seemed to disprove this. Most researchers and field observers shared the view that protection was transient and weak in the absence of a local postvaccinal reaction.

This fact was widely recognized at the time, but was difficult to explain. Among the factors involved might have been poor cultures, faulty storage of vaccine and only partial vaccination in a heavily contaminated herd. The local reaction was proof of the viability of the vaccine and, in its absence, there were doubts about the vaccine's efficacy. To this may be added the lack of uniformity in methods of experimental infection of cattle, leading to differing evaluations of the resistance conferred by one or other of the various procedures.

However, research conducted in Australia between 1960 and 1970 clearly demonstrated that there was no correlation between the intensity of the vaccinal reaction and the quality of protection. Excellent resistance to experimental infection developed without any local reaction provided that the minimum required number of live mycoplasmas were inoculated (10^7 per dose for strain T_1)

If untoward local reactions of the Willem's type occur (i.e. extensive oedema starting 10 to 12 days after vaccination) antibiotic treatment (spiramycine, tylosin, tilmicosin) can be advocated.

General vaccination precautions

Vaccination against CBPP is never harmless. Apart from the local, and even generalized, reactions which may be produced, vaccination may potentiate latent infections or infestations in cattle existing under the precarious physiological balance prevalent in tropical conditions (e.g. trypanosomiasis and piroplasmosis).

Factors to take into account:

- *Breed* (where the term "breed" is often used incorrectly for species). In general, cattle breeds are more susceptible to vaccination accidents than zebu, at least in Africa. Dairy breeds are usually more susceptible than beef breeds but exceptions occur in both cases.
- *Group*. Within the same species and same breed, there are individual variations in sensitivity to vaccination (and also to infection). Such variations are a hindrance to vaccination campaigns because of possible unpredictable reactions.
- *Sex*. Pregnant females in the terminal stage of gestation should not be vaccinated.
- *Age*. The inoculation of calves under three months is usually followed by insignificant local reactions but such animals may develop arthritis, synovitis and heart valve lesions which can be fatal, at least with the most virulent strains. In practice, calves should not be vaccinated under six to eight months of age.

The level of protection induced

The vaccination of cattle against CBPP usually elicits temporary serological conversion, detectable by various standard serological tests. It takes approximately six to eight weeks to return to a serologically negative level.

The establishment of resistance is slow, at three to four weeks, and the level of resistance conferred by a CBPP vaccine can be demonstrated by testing a group of animals but not individuals. The effect of vaccination is to raise and consolidate the average level of resistance in a herd rather than to provide individual protection. Clearly, it cannot protect 100 percent of vaccinated animals. Among those vaccinated, there is a variation in resistance to infection, ranging from excellent immunity (mycoplasmocidal antibodies) to simple clinical resistance accompanied by encapsulated lung lesions from which *M. mycoides* can be recovered.

With any CBPP vaccination, there is the risk of creating semi-resistant cattle which can become chronic carriers following natural infection, thus promoting the continuance of the disease. Hence the importance of the rule of disease control which states that CBPP vaccination *must be extensive in an area and continuous in time* to be effective. By contrast, partial and unrenewed vaccination of a susceptible herd may simply maintain the disease at a low level. Failure to observe this rule has resulted in numerous disappointments in the past.

In practice, there is considerable discrepancy between resistance as assessed in the laboratory and that which operates in the field. A vaccine capable of protecting only 70 percent of cattle experimentally may still give excellent results in the field.

CBPP VACCINE: GENERAL MANUFACTURING AND CONTROL PROCEDURES

Source materials

***Strain of* M. mycoides**. The strains of *M. mycoides* used for vaccine production are identified by documents which provide information on their origin and the manipulations they have undergone. The strain selected must have been shown to be suitable for the region and the type of bovines for which it is intended, when administered by the inoculation route prescribed by the manufacturer. Before use, the strain must first have been tested to show that the resulting vaccine is safe and confers a protection lasting at least one year in cattle. One such vaccine with these characteristics is the strain T_1-44.

Strain T_1 was isolated in 1961 at the East African Veterinary Research Organisation, from a clinical case of CBPP in what is now the United Republic of Tanzania. From the

outset, it appeared to be of only moderate virulence. It has been cultured in embryonated hens' eggs, first on the chorio-allantoic membrane for six passages and then in the yolk sac. Attenuated from the ninth egg passage, it has been used mainly in East Africa for preparing freeze-dried egg culture vaccines with good immunogenic qualities.

Unfortunately, following the use of egg culture vaccines there were a number of cases of postvaccinal lung lesions and numerous local reactions, leading to the suspicion that such vaccines may be dangerous. Their use was therefore suspended in 1962. Since then, it has been shown that the lung lesions may have been caused by biologically active substances present in the egg material. The intrinsic qualities of strain T_1 were not doubted, however, so it was decided to prepare liquid vaccines from broth cultures of the 44th egg passage of the strain. This material is stored in freeze-dried form.

In parallel with these studies, the possibility of combining the strain T_1 with cell-culture rinderpest vaccine to obtain a combined vaccine has also been investigated. Mutants of the strain T_1-44 that are resistant to streptomycin (present in the culture fluid of rinderpest virus) have been developed at the Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT) Laboratory at Maisons-Alfort in France and the Farcha Laboratory in Chad. The Maisons-Alfort mutant (T_1-SR), obtained by three passages in a liquid medium containing increasing concentrations of streptomycin, has been studied under experimental conditions at Dakar, Senegal, for its immunogenic properties.

Thus, two lines of T_1 are now available: the original strain T_1-44 and the strain T_1-SR, which has undergone some additional passages in broth. Streptomycin appears to act as a growth factor for the latter strain, although there is no question of streptomycin-dependence. The immunogenic properties of both strains are identical, but the strain T_1-SR has produced fewer local postvaccinal reactions in humpless cattle breeds.

Both strains may be obtained from the Director of CIRAD-IEMVT, 10 rue Pierre Curie, 94704 Maisons-Alfort, Cedex, France. This is the FAO reference centre for mycoplasmas of ruminants.

Culture medium. The culture medium used contains serum or some other product of animal origin, which must be:

- either heated at 56°C for at least 30 minutes before being added to the medium, or treated by some other procedure that is equally effective in destroying contaminant microbial agents;
- submitted to appropriate tests to show that the material is free from contaminants.

In general, culture media contain:

- a basic medium consisting of meat extract (e.g. infusion or papain digest of beef heart) and / or peptone (trypose, tryptone);
- an extract or autolysate of brewer's yeast (commercially available or prepared on the spot), which provides growth factors (group B vitamins);
- glycerol, oleic acid, palmitic acid, glucose, sometimes a buffering system, a small amount of DNA and, if the vaccine is produced in a fermenter, also an antifoaming agent;
- 10 percent blood serum, preferably from adult horses, which must comply with the standard mentioned above.

If the strain T_1-SR is used, streptomycin is added at 200 mg per litre of medium. The final pH is adjusted to 8.1. A satisfactory formulation is Medium F-66 of the Farcha Laboratory.

After clarification, it is advisable to sterilize the medium by membrane filtra-

tion rather than by autoclaving. It should be used as soon as possible after pre-incubation for 24 hours at 37°C to verify the absence of bacterial contamination.

Seed lot system. Vaccine production is founded on a seed lot system. A seed lot may be part of a culture which has served for vaccine production, has been shown to be safe for cattle when administered under natural conditions and is capable of conferring an immunity that lasts for at least a year. A seed lot obtained from such a culture must not be submitted to more than three additional passages.

Seed lots[2] are freeze-dried and stored at a temperature not higher than -20°C.

Tests on seed lots. Each seed lot must be free from extraneous agents and must satisfy the standards of identity and safety in laboratory animals. In addition, a vaccine prepared from the seed lot must be tested for safety and efficacy in cattle.

Cattle used for this testing must be representative of the cattle to be vaccinated; they must come from an area free from CBPP and must be at least 24 months old and in good health. The cattle should be identified in an indelible manner and kept in quarantine under veterinary supervision for at least four months. If any clinical signs of disease are present, the animal concerned should not be used until the cause of the abnormality has been determined and recognized as having no effect on the correct conduct of the tests. Serum should be taken from each animal at the start and end of the quarantine period to be tested for CBPP antibodies. Only serologically negative cattle should be used.

- *Safety testing.* A quantity of vaccine containing at least ten times the number of mycoplasmas present in a recommended dose should be inoculated subcutaneously on the flank posterior to the scapula in each of at least ten cattle. The seed batch is satisfactory if the cattle fail to develop any unexpected clinical reaction during a period of observation lasting for at least four weeks and if no lung lesion is found on post mortem examination at the end of this period.
- *Efficacy testing.* A quantity of vaccine containing the same number of bacteria as the recommended dose should be inoculated subcutaneously on the flank posterior to the scapula in each of at least ten cattle. A similar number of cattle should be kept as a control group. After a minimum of two months have elapsed, all cattle should be submitted to challenge infection by contact with infected cattle (i.e. cattle infected by the introduction of lung lesion homogenate through an endobronchial tube). At least one infected animal should be used for each group of three cattle under test. The duration of the test is three months and any donor animal infected by intubation that dies during this period must be replaced. The seed batch passes the test if the animals inoculated with vaccine prepared from the batch do not develop a clinical or a serological reaction (i.e. an anamnestic response), post challenge, if they are free from lesions of pleuropneumonia at slaughter and if the tracheobronchial lymph nodes are free from *M. mycoides*. At least 80 percent of the unvaccinated control animals should have typical lesions at post mortem examination.

[2] A seed lot prepared jointly by the IEMVT and the Pan African Veterinary Vaccine Centre (PANVAC) is now stored at both centres for free distribution to vaccine manufacturers.

Production precautions

Inoculation and culture. The sample of the vaccine strain used for inoculating the culture vessels or the fermenter should not

have been submitted to more than two passages in broth from the seed lot.

It is best to inoculate the production medium, pre-incubated at 37°C, with a culture of the vaccine strain in the logarithmic phase of growth (36 to 40 hours culture from agitated medium), using one part of culture to ten parts of the culture medium.

After a stationary phase of incubation at 37°C, the medium is aerated (by a magnetic stirrer if flasks are used, or by injection of air if a fermenter is used).

The culture is stopped at peak growth, usually at 65 to 77 hours after inoculation. This time has to be determined precisely by each producing laboratory, with regard to local conditions, by establishing a growth curve for a culture of the vaccine strain in medium and with vessels identical to those used for production. This recommendation is important because continuation of culture after the growth peak leads to a considerable fall in pH and a diminished number of live mycoplasma.

Harvesting and freeze-drying. After a culture has been stopped, it is essential to harvest immediately, add stabilizer (for the preparation of the final product in bulk), fill the final containers and freeze-dry the product. Storage of the harvest or the final product in bulk, even in a cold chamber, has an adverse effect on the number of live mycoplasma in the vaccine. These operations are depicted in Figure 1.

The bulk suspension is prepared from a single harvest. Once transferred to the tank from which the final containers are filled, it becomes the final product in bulk. This final product is transferred quickly to the final containers and prepared for freeze-drying. At this point it becomes a batch of vaccine.

Distribution into the final containers and the subsequent freeze-drying must be done as soon as possible after the final bulk product has been prepared. After freeze-drying of the vaccine, the containers are hermetically sealed under vacuum or under dry nitrogen free from oxygen. In case of failure, all containers sealed under vacuum should be tested for airtightness and any defective container rejected.

If part of a vaccine batch is to be freeze-dried at some other time, it is best to freeze the vaccine within the final containers. After freeze-drying, it should be submitted to the control tests described below.

Freeze-drying procedure. A freeze-drying process of long duration is used where the vaccine is not exposed to temperatures above 0°C while it still contains more than 3 percent residual moisture. This can only be done in freeze-driers of the shelf type, not in the centrifuge type. Under these conditions and with a satisfactory stabilizer, the loss of viable units should not exceed 1 $\log_{10}$. In other words, with a harvest containing 10^{10} viable units per ml, concentrated freeze-dried vaccines are obtained, and their storage is considerably simplified. Operating with modern freeze-driers, less than 1.5 percent residual moisture can in fact be attained.

Control tests

Samples are taken from each vaccine batch or each distribution batch for the tests given below. The tests are performed on the vaccine reconstituted to a volume which will depend on the initial concentration of the final product in bulk. After reconstitution, samples should be stored at 4°C and tested within an hour.

Identity tests. A test of identity has to be performed on at least one labelled container of each distribution batch by using a technique suitable for identifying the presence of *M. mycoides*.

A simple and satisfactory test is growth inhibition on agar gel, using filter paper

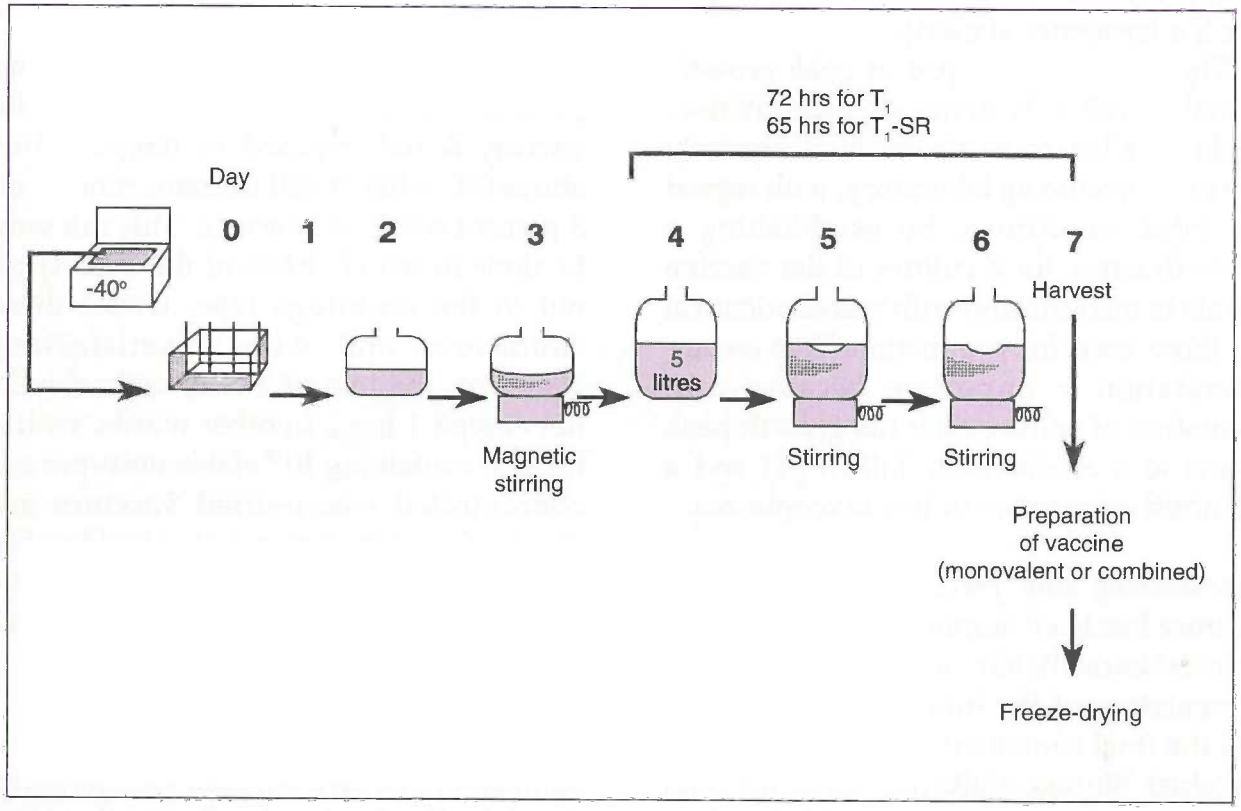

FIGURE 1
Procedure for producing CBPP vaccine from strains T_1 and T_1-SR

discs impregnated with a serum containing a high titre of antibodies to *M. mycoides* subsp. *mycoides* (SC bovine biotype). Another procedure is to place antiserum in wells punched into the agar culture. A zone of inhibition measuring at least 2 mm will be present after 48 to 72 hours of incubation. This test has the advantage of identifying other mycoplasmas (which grow within the inhibition zone) and/or bacteria present as contaminants. More refined tests (Western Blot, dot immunobinding) can also be considered.

Sterility tests. Freedom of the vaccine from bacteria and fungi is assessed by tests made according to the recommendations of the World Health Organization's Standards for Biological Substances No. 6 (general standards concerning the sterility of biological substances).

Determination of the mycoplasma content. An organism count should be made on each batch of vaccine (or each distribution batch) to determine the number of viable *M. mycoides* per dose for cattle. This assay is done by the following procedures:

- An initial titration is performed on a mixture of at least five bottles selected at random. This mixture can also serve for testing safety and efficacy in cattle if necessary.
- Second and third titrations are each performed on the mixed contents of three bottles.
- The titre of the vaccine is the geometric mean of the three titres thus obtained.

Each titration uses a system of vaccine dilutions and the appropriate number of tubes of growth medium sufficient to estimate the 50 percent end point by a standard statistical procedure. A series of tenfold dilutions, with ten tubes of medium for each dilution, or any other dilution system which provides equal precision, is used. The tubes are incubated for at least ten days at 37°C and are shaken lightly each day to aerate the medium. The minimum titre per vaccine dose must be at least 10^7 viable mycoplasmas.

It is recommended that production laboratories take into account possible problems arising from local transport conditions and therefore supply vaccines with titres of at least 10^8 mycoplasmas per dose.

Safety tests. These tests are performed on each batch of vaccine by inoculating guinea pigs and mice. The procedure generally used consists of injecting 0.5 ml of reconstituted and undiluted vaccine intraperitoneally into each of two guinea pigs, the same dose intramuscularly into two more guinea pigs and 0.1 ml intraperitoneally into six mice. The animals are kept under observation for three weeks and then killed for examination. The batch of vaccine is considered satisfactory if the animals remain healthy and if they have no pathological lesion post mortem.

Tests for safety and efficacy in cattle. These tests are done with a mixture of vaccine from at least five bottles selected at random and as stipulated in the section Seed lot system, p. 68.

A safety test is obligatory for each vaccine batch. Because of the complexity and cost of an efficacy test, this may be performed occasionally (at least once a year) for a given batch of tested seed culture or for a vaccine derived from culture which has undergone no more than three passages from the seed culture, provided that all the arrangements for biological security of the installation and manipulations have been observed.

Stability test. It is desirable to conduct a test of stability on samples of vaccine batches to obtain experimental data to predict the effect of storage temperature

and the expiry date. This data should be given on the label and in the accompanying information leaflet.

In the absence of data on long-term stability, it is recommended that accelerated degradation tests should be conducted on the vaccine to arrive at recommendations for storage conditions and expiry date. The information provided by such tests must be confirmed as soon as possible by observations on vaccine stored under the recommended conditions. Storage under these conditions must guarantee that the titre of mycoplasmas in the vaccine up to the expiry date is not less than 10^7 viable mycoplasmas per vaccine dose.

Stability tests may also be made to provide a foundation for the information accompanying each container. These tests estimate the fall in titre of mycoplasmas once the product has been reconstituted for use and after storage of the intact product under the temperature conditions which may be encountered in the field. Information should also be provided for vaccine kept in ice boxes (0° to 10°C) and vaccine kept at different ambient temperatures between 20° and 30°C (or even 40°C).

Storage conditions

While awaiting dispatch from the production establishment or storage depot, the final containers of vaccine should preferably be stored in the dark and at a temperature of -20°C or lower within rooms provided with continuous temperature-recording equipment.

During storage and transport of the vaccine, it is strongly recommended that temperature indicators be used (heat-sensitive strips or paint) to check quickly the temperature at which the vaccine has been exposed.

The expiry date should not exceed two years from the last determination of the mycoplasma count, provided that the vaccine has been stored continuously at a temperature of -20°C until delivery. Producers with large stocks of vaccine should retest the titre before extending the authorized storage period. On the other hand, no vaccine should be used later than six months after its supply by the production establishment or storage depot, and then only if the containers have been stored at temperatures of less than +10°C.

The half-life of vaccine freeze-dried to a residual moisture content of 1.5 percent is as follows:

- 4 days at 45°C;
- 1.6-2.8 weeks at 37°C;
- 2.8-4.6 weeks at 28°C;
- 30-40 weeks at 4°C.

These figures mean that, when tested during production, a vaccine that contains a concentration of 10^8 viable units per dose (which is on the low side and encountered in batches of only moderate quality) can be kept for 2 to 4 months at 28°C, 1.5 to 3 months at 37°C and 12 to 14 days at 45°C.

The storage life of reconstituted ready-to-use vaccine must not exceed the time during which the mycoplasmal count is equal to or greater than 10^7 viable organisms per vaccine dose at the specified temperatures, this having been verified experimentally.

It has been shown experimentally that reconstitution of freeze-dried vaccine in the field to give the number of doses specified by the producer is best done with a molar solution of magnesium sulphate. Under these conditions there is no fall in mycoplasma titre for four hours at 37°C. In no case should the vaccine be reconstituted with distilled water, tap water or surface water, even after boiling.

CONCLUSIONS

As stated in the introduction, any improvement in mycoplasmal vaccines is

hampered by little or no information on the function of the essential immunogens of the organism, the lack of a precise understanding of the pathogenesis of mycoplasmal diseases and the insufficient knowledge of factors governing resistance to challenge or natural disease. This means that subunit vaccines, and even possibly recombinant vaccines, are still far from successful development.

Some simpler avenues appear worth exploring. New adjuvants, namely immunostimulating complexes (ISCOMs), could be studied to enhance the immunogenicity of inactivated vaccines; such steps are under way for CBPP. Another approach would be to explore the value of local immunity. In the case of CBPP, "local" means the application of the immunizing agent to the nasal mucosa by means of a vaccine spray. Preliminary tests have already been shown to be successful in some cases of enzootic pneumonia, CCPP and *M. pneumoniae* in hamsters.

Similar experiments conducted 20 years ago in Nigeria and Chad proved to be very successful with CBPP. Such an approach would now seem to be worth pursuing further.

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Protozoal and rickettsial vaccines

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THE NEED FOR VACCINES

Tick-borne protozoal and rickettsial diseases affect most domestic animal species and have been a major constraint to the development of livestock industries in developing countries in the tropics and subtropics. Although tick-borne diseases (TBDs) are important in all domestic animals, this review will be concerned mainly with vaccines against the economically important tick-transmitted diseases of cattle caused by the protozoal parasites *Babesia bovis* and *Babesia bigemina* (babesiosis, red water or tick fever), *Theileria parva* (East Coast fever) and *Theileria annulata* (tropical theileriosis), as well as the rickettsial organisms *Anaplasma marginale* (anaplasmosis) and *Cowdria ruminantium* (cowdriosis, heartwater). Vaccines against coccidiosis have been reviewed by Danforth and Augustine (1989).

Babesiosis caused by *B. bovis* and *B. bigemina* is present in many countries between 40° N and 32° S (McCosker, 1981). The most important vectors are ticks of the genus *Boophilus* (Friedhoff, 1988).

East Coast fever is a disease limited to countries in eastern, central and southern Africa where the principal vector, *Rhipicephalus appendiculatus*, is present (Norval, Perry and Young, 1992). In these countries, it is of major importance because of the high morbidity and mortality it causes. Tropical theileriosis is transmitted by a number of species of the genus *Hyalomma* which have a wide distribution along the Mediterranean littoral, in the Near East and throughout Asia (Pipano, 1989).

Anaplasmosis has the widest distribution of all economically important TBDs and is transmitted biologically by at least 20 tick species. Mechanical transmission by biting flies and unsanitary veterinary practices are also important in certain areas (Palmer, 1989).

Heartwater is important in cattle and is also a major cause of losses in sheep and goats. This disease is transmitted by ticks of the genus *Amblyomma* and occurs mainly in sub-Saharan Africa, although it has also been introduced to islands in the Atlantic and Indian Oceans as well as the Caribbean. It is regarded as the most important TBD in South Africa and, next to East Coast fever, the most important one in Africa (Uilenberg, 1983; Bezuidenhout, 1989). The presence of heartwater and its vector in the Caribbean poses a threat to livestock production in the tropical and subtropical parts of the American mainland (Barre *et al.*, 1987).

Up to 500 million cattle the world over are exposed to one or more of the TBDs but this figure is not a true reflection of the number at risk to disease. Indigenous breeds of cattle often have a certain degree of natural resistance to these diseases and the consequences of infection are not as serious as when exotic *Bos taurus* breeds are involved. In addition, a state of enzootic stability frequently develops whereby local cattle become naturally infected at an early age when there is some passively acquired or innate immunity. These cattle are resistant to subsequent challenge (de Vos, 1992).

Cattle, particularly of exotic breeds, are at risk to TBDs, especially under the following circumstances.

- The importation of susceptible cattle into endemic regions. Mortality rates under these conditions can exceed 50 percent in regions endemic for babesiosis (McCosker, 1981) and the same may apply in areas infected with East Coast fever and heartwater.
- The spread of ticks and TBDs into previously uninfected areas. An example of this was the loss of over one million cattle owing to TBDs following the disruption of tick control measures in Zimbabwe (Lawrence, Foggin and Norval, 1980).
- The introduction of TBDs into a disease-free vector population – as happened when East Coast fever was introduced into southern Africa in 1896. This introduction resulted in the loss of an estimated 1.4 million cattle and took 50 years to bring under control (Lawrence, 1992).
- Increases in tick transmission rates in endemic areas caused by, among others, ecological factors. Most outbreaks of babesiosis in Australia occur in cattle bred in the endemic area (Callow and Dalgliesh, 1980).

Immunization and vector control are two options which need to be considered in all these situations. Provided there are suitable facilities and assuming commercial vaccines are not available, the local production of vaccine may be the only sustainable means of control in the country concerned.

HISTORY OF VACCINE PRODUCTION AND FUTURE PROSPECTS

B. bovis and *B. bigemina*

Methods used to immunize cattle against babesiosis have been described or reviewed (Callow and Dalgliesh, 1980; Pipano, Frank and Shkap, 1991; de Vos and Jorgensen in FAO, in press). In many countries, early attempts to vaccinate cattle against *B. bovis* and *B. bigemina* infections involved the use of blood from naturally or artificially infected carriers. This carrier-donor method, also known as premunition, has several major limitations, including unreliable potency, unpredictable reactions and the risk of contamination (Callow, 1984; de Vos and Jorgensen, 1992). During the past 30 years, more sophisticated techniques were developed to produce live vaccines from splenectomized donors, which had predicably low levels of virulence (Callow and Dalgliesh, 1980). The inherent disadvantages of vaccine containing parasites derived from the blood of animals are well documented, including the risk of reactions, contamination, sensitization against blood groups and the need for cold chain transportation (Callow and Dalgliesh, 1980; Wright, 1991). In spite of these disadvantages, virtually all operational centres producing *Babesia* vaccines are at present using variants of this technique to produce chilled or frozen live vaccines. *In vitro* culture methods reviewed by Pudney (1992) have been used to produce *B. bigemina* parasites for vaccine (Jorgensen *et al.*, 1992) and are also suitable for producing *B. bovis* parasites. However, these culture techniques are still at the developmental stage and not widely used for the production of vaccine.

Other products used in attempts to immunize cattle against babesiosis include antigens extracted from parasites or parasitized blood. Cell culture-derived exoantigens of *B. bovis* and *B. bigemina* have been extensively studied and proposed for use as vaccine in developing countries (Montenegro-James, Kakoma and Ristic, 1989). Unfortunately, the level and duration of protection conferred by these antigens against heterologous challenge are less than those of live vaccines (Timms *et al.*, 1983).

Progress to overcome the limitations of vaccines based on blood or blood extracts

through the development of synthetic or recombinant *Babesia* vaccines, were reviewed by Wright *et al.* (1992) and Dalgliesh (1993). Preliminary results suggest that vaccines based on single antigens do not confer the desired level or duration of protection. It is likely that a vaccine containing several recombinant antigens will be needed to induce adequate protection (Pipano, Frank and Shkap, 1991). Even a multicomponent recombinant vaccine may not provide long-term protection against field strains of *Babesia* which have been shown to be capable of considerable genetic variation (Dalrymple *et al.*, 1992; de Vos and Jorgensen, 1992). As yet, no recombinant vaccine for bovine babesiosis has been registered for use in any country and it seems unlikely that such a vaccine will be available in the near future. When one does become available, it will almost certainly be marketed internationally and in competition with vaccines produced locally.

T. parva and *T. annulata*

As reviewed by Purnell (1977), Irvin and Morrison (1989), Pipano (1989) and Lawrence (1992), a variety of products and procedures have been or are being used to immunize cattle against East Coast fever and tropical theileriosis. Early attempts involved the use of infected blood and tissues. In the case of East Coast fever, the results were very variable but the blood of donor calves infected with low-virulence stocks of *T. annulata* was used for many years as vaccine (Pipano, 1989). Subsequently, a vaccination technique was developed which involved infecting cattle with sporozoites from ticks and then mitigating the clinical responses by chemotherapy (FAO, 1984; Irvin and Morrison, 1989; Dolan and McKeever, 1993). This "infection and treatment" method using *T. parva* sporozoites is used in some countries in East and central Africa, mainly on an experimental or field trial basis. A ground-up suspension of infected adult *R. appendiculatus* (ground-up ticks supernatant [GUTS]) is used to initiate the infection which is then controlled by treatment with oxytetracycline. Depending on the *T. parva* stocks used, sporozoite vaccine has proved effective under laboratory conditions and in field trials, providing long-lasting immunity against homologous and heterologous challenge (Burridge *et al.*, 1975a and 1975b). However, antigenic heterogeneity of *T.parva* isolates has been a major obstacle to the widespread application of this method of immunization (Irvin and Morrison, 1989). The use of a mixture of stocks is necessary to provide wide-spectrum cover against field challenge, especially where *T. parva lawrencei* is involved. In general, good protection is afforded by a vaccine made up of *T. parva* (Muguga), *T. parva* (Kiambu 5) and buffalo-derived *T. parva* (Serengeti transformed). However, because animals immunized in this way may become carriers to the infection and thus may transmit the parasite to the resident population, there is an understandable reluctance to implement immunization programmes with stocks exotic to the region or country (Irvin and Morrison, 1989).

Despite this drawback as well as the lack of attenuated, immunogenic stocks and high cost, the "infection and treatment" method is still the principal means of immunizing cattle against *T. parva*. *T. parva* (Boleni) has been used in extensive field trials in Zimbabwe. It is of low pathogenicity and treatment of reactions is rarely necessary. An application has now been made to have vaccine containing this stock registered for more general use in Zimbabwe (Pegram, personal communication, 1994). There is evidence that *T. parva* (Boleni) will also protect cattle against some highly pathogenic isolates but this

feature has not been exploited in practical disease control strategies in other parts of Africa. The infection and treatment method has also been used to prepare a *T. annulata* sporozoite vaccine (Pipano, 1989) but it has not been evaluated under field conditions. Immunological differences between field isolates of *T. annulata* do not appear to be as important as in the case of *T. parva*.

In vitro techniques for the culture of schizonts of *Theileria* spp. were reviewed by Brown (1979; 1981) and Pipano (1989). *T. annulata* schizonts can readily be propagated in culture and the pathogenicity of the parasites decreases during prolonged maintenance in culture. This provided the basis for developing a safe, culture-derived vaccine for the control of tropical theileriosis (Pipano, 1989; Singh, 1990). This vaccine is currently in use or in different stages of development in several countries in Asia and around the Mediterranean (Dolan and McKeever, 1993). Unfortunately, attempts to develop a similar vaccine for *T. parva* gave disappointing results. Histocompatability is seen as one of the main obstacles to the successful transfer of *T. parva* schizonts from infected cultures to recipient hosts.

The hazards involved in using the sporozoite vaccine and the stock-specific nature of the immunity conferred have led to intensive efforts to identify parasite antigens which may be targets for protective immune responses, particularly against *T. parva* infection (Dolan and McKeever, 1993). Of sporozoite antigens identified as candidates for inclusion in a vaccine, a gene that codes for a 67 kilodalton (kDa) major surface protein has been isolated, sequenced and expressed. It provides partial protection in cattle against homologous challenge (Musoke *et al.*, 1992). Two sporozoite antigens of *T. annulata* defined by different monoclonal antibodies have also been studied as vaccine candidates. These antigens have been expressed in *Escherichia coli* and stimulate the production of neutralizing antibody in cattle (Hall and Baylis, 1993).

Anaplasma marginale

A variety of procedures have been used to immunize cattle against anaplasmosis (McHardy, 1984; Palmer, 1989). These procedures involve the use of live organisms such as attenuated *A. marginale* or *A. centrale* and killed organisms or extracts of infected blood.

A stock of *A. marginale* has been attenuated by irradiation and passage in sheep and deer (Palmer, 1989). Vaccine containing this stock was marketed as Anavac and used to immunize cattle in Latin America and the United States (Corrier, Johnson and Wagner, 1985). It provided substantial protection against challenge with most heterologous isolates. In general, use of the vaccine had no or very little clinical effect although severe morbidity and mortality have been reported, especially when older bulls and cows were immunized (Palmer, 1989). Use of attenuated *A. marginale* is the most effective means of immunization against anaplasmosis but this vaccine is apparently no longer available. Attempts to attenuate a stock of *A. marginale* by passage in sheep in Australia were unsuccessful (Jorgensen *et al.*, 1993).

Infection with *A. centrale*, an organism originally isolated in South Africa (Potgieter, 1979), provides partial cross-immunity against *A. marginale* challenge. It is usually mildly pathogenic, particularly in young animals. These features have led to the use of *A. centrale* as vaccine in a number of countries in Africa, Asia, South America and in Australia. Cross-immunity between *A. centrale* and *A. marginale* is adequate if challenge is moderate as in Australia (Callow and Dalgliesh, 1980), but may be insufficient against virulent heterologous challenge (de Vos and

Jorgensen in FAO, in press; Palmer, 1989). Despite this limitation and the risk of reactions in older animals, use of *A. centrale* appears to be the only choice available to most developing countries where anaplasmosis is endemic. An advantage of *A. centrale* is its apparent non-transmissibility by the common vectors of *A. marginale*, with the exception of the African tick *Rhipicephalus simus* (Potgieter and van Rensburg, 1987).

A non-living vaccine based on a lyophilized preparation of *A. marginale* organisms administered with adjuvant has been available for many years as Anaplaz (Fort Dodge Laboratories) in the United States and Latin America (Brock, Kliewer and Pearson, 1965). It induces partial protection against virulent heterologous challenge but the level of protection is less than that provided by live *A. marginale* vaccine (McHardy, 1984; Palmer, 1989). Neonatal iso-erythrolysis has also been reported following use of this vaccine owing to the induction of iso-antibodies to blood group antigens (Palmer, 1989). Attempts to develop a vaccine containing highly purified *Anaplasma* spp. particles have been successful (Luther *et al.*, 1989; Montenegro-James *et al.*, 1991) and a vaccine is available in Louisiana, in the United States, which does not induce sensitization against blood groups. In general, the limitations of non-living *Anaplasma* vaccines (difficulty of manufacturing them, their relative ineffectiveness and the risk of contamination with erythrocyte stroma) render production of this type of vaccine impractical in most developing countries. However, some of these vaccines are or were commercially available in Central and South American countries.

The ability to induce protective immunity with killed *Anaplasma* organisms led to further studies on purified antigens. Two surface proteins (36 and 105 kDa) induce a protective immune response in calves to homologous and heterologous challenge (Palmer, 1989; Tebele, McGuire and Palmer, 1991; McGuire *et al.*, 1992). Future work will include the evaluation of recombinants of these proteins (McGuire *et al.*, 1992).

Cowdria ruminantium

Animals which recover from heartwater acquire an immunity to the disease. This knowledge has formed the basis of an "infection and treatment" method of immunization involving the inoculation of animals with *Cowdria* sp. and treatment of ensuing reactions with tetracycline (Uilenberg, 1983). This procedure is used routinely to immunize cattle in South Africa (Oberem and Bezuidenhout, 1987; Bezuidenhout, 1989). It has been standardized to include the use of blood of sheep acutely infected with a stock of *Cowdria* sp. known to cause well-defined febrile reactions, thus allowing the optimum time of treatment to be determined. Because of the limited viability of the organisms in the unfrozen state, cryopreservation is necessary (Oberem and Bezuidenhout, 1987). The innate resistance of young animals to the clinical effects of *Cowdria* infection are well documented and have been exploited by recommending immunization at a young age. Disadvantages of this procedure include the risk of reactions in older animals, the large volume of the inoculum required (5 to 10 ml) and the need to use the intravenous route of inoculation (Uilenberg, 1983).

A supernatant of ground-up infected nymphal *Amblyomma hebraeum* ticks (GUTS) has also been used to immunize animals against *Cowdria* sp. (Oberem and Bezuidenhout, 1987; Bezuidenhout, 1989). Details of the production of this vaccine, including the infection of larval ticks, the preparation of infective nymph supernatant and the dilution and storage of the supernatant were reviewed by

Bezuidenhout (1989). The immunity conferred by this vaccine was similar to that of the blood vaccine but an unacceptably high number of animals, particularly goats, developed severe allergic or shock reactions (van der Merwe, 1987).

Considerable progress has also been made in recent years with the development of *in vitro* methods for propagating *Cowdria* sp. and a great number of stocks have been grown successfully (Bezuidenhout and Brett, 1992). One roller bottle (surface area 800 cm^2) with bovine or ovine endothelial cells can yield enough organisms to infect 20 000 animals. There is also evidence of attenuation *in vitro* without loss of immunogenicity (Jongejan, 1991). Future work will be aimed at optimizing culture conditions for organism yield, improving viability following freezing and thawing (Bezuidenhout and Brett, 1992) and overcoming the effect of antigenic differences between isolates (Jongejan *et al.*, 1993).

Attempts are being made to identify and express immunodominant *Cowdria* proteins (Barbet *et al.*, 1992). The ultimate goal of the Heartwater Research Project at the University of Florida, United States, is to develop a recombinant vaccine delivered by a live virus vector.

PRODUCTION OF VACCINES

The following methods of vaccine production are at present being used in developing countries or are likely to be within the means of countries where reliance on enzootic stability is not an option and immunization is the only viable alternative. As discussed in the previous section, the vaccines all have the drawbacks inherent in the production and use of live products.

Babesia bovis and *Babesia bigemina*

Selection, attenuation and storage of stocks. It is tempting to use *Babesia* isolates from the country in which the vaccine is to be produced. Such isolates can be obtained by feeding infected *Boophilus* spp. ticks on a susceptible bovine host or by inoculating it with blood from a long-standing carrier (Callow, 1984). The major limitation of using a carrier is the risk of contamination. Techniques for collecting, purifying and attenuating *B. bovis* and *B. bigemina* stocks have been described (de Vos and Jorgensen in FAO, in press).

Attempts to develop attenuated stocks of *B. bovis* from local isolates are not always successful (de Vos and Jorgensen, 1992). For this reason, it may be worthwhile to import a stock that is known to be protective, of low virulence and free from contaminants. Australian stocks have been shown to be protective in several countries and vaccines containing these stocks have been used with beneficial results in Africa, Asia and South America (de Vos and Jorgensen, 1992).

The attenuation of a *B. bovis* stock is usually achieved by rapid syringe passage through splenectomized calves (Callow and Dalgliesh, 1980; de Vos and Jorgensen in FAO, in press; Pipano, Frank and Shkap, 1991). The mechanism by which attenuation is achieved is not fully understood but appears to be the result of a selective enrichment of avirulent parasite subpopulations. Other methods used to attenuate *B. bovis* include irradiation (Wright, Goodger and Mahoney, 1980), *in vitro* culture techniques (Yunker, Kuttler and Johnson, 1987) and the use of alternative hosts, but these methods give less reliable results than passage in splenectomized calves. Rapid passage of *B. bigemina* in splenectomized calves is not recommended. A stock of this parasite with generally low virulence may be obtained after a series of slow passages in non-splenectomized calves (Callow and Dalgliesh, 1980).

Vaccine stocks of *B. bovis* and *B. bigemina* can be stored for lengthy periods as

cryopreserved stabilates of infected blood. Dimethyl sulphoxide (DMSO) (Mellors *et al.*, 1982), glycerol (Dalgliesh, Jorgensen and de Vos, 1990) and polyvinyl pyrrolidone (PVP) (de Vos and Jorgensen in FAO, in press) have all been used successfully to cryopreserve *Babesia* spp. parasites.

Supply of infective material using donor calves. Cryopreserved master seed of an attenuated stock is used to initiate a primary infection in a susceptible, splenectomized donor. The initial *B. bovis* parasitaemia is often low but adequate to initiate a series of four to five passages, thus allowing a continuous supply of vaccine over a three- to five-week period. *B. bovis* vaccine stocks should not be passaged more than a total of 30 times to reduce the risk of losing immunogenicity (Callow and Dalgliesh, 1980; Bock *et al.*, in press; de Vos and Jorgensen, 1992). Inocula containing about 1×10^{10} parasites are ideal for passaging and will result in parasitaemias in excess of 1×10^{8} per millilitre within four to five days. Up to 25 000 doses of vaccine can be obtained from a calf weighing 150 to 200 kg. *B. bigemina*-infected blood is obtained from individually infected calves; serial passing is not recommended because of possible selection for virulence.

Supply of infective material using parasites grown in vitro. Available technology allows *B. bovis* and *B. bigemina* to be maintained in continuous culture (Pudney, 1992). These culture systems have the advantage over the calf-donor system of minimizing the risk of contamination and limiting the number of animals required. A relatively robust method for producing *B. bigemina* vaccine in sealed flask suspension cultures was developed by Jorgensen *et al.* (1992) and may be a viable option in countries where insufficient numbers of donors are available for the production of vaccine *in vivo*. Some studies found that neither the virulence nor the immunogenicity of *Babesia* vaccine stocks were appreciably modified by maintenance in culture (Timms *et al.*, 1983; Jorgensen, de Vos and Dalgliesh, 1989). However, recent work using the polymerase chain reaction (PCR) of polymorphic genetic markers has shown that proportions of *B. bovis* subpopulations do change with culture *in vitro* (Bock *et al.*, in press; Lew, unpubl.).

Theileria parva

Isolation, storage and characterization of stocks. Vaccine should be prepared using master seed of well-characterized *T. parva* stocks to account for the antigenic heterogeneity of this species. If there is a requirement for local stocks, these can be obtained by using bait or naturally infected cattle or by collecting and feeding infected ticks from the field. Procedures used to isolate stocks of *T. parva* have been documented in the literature (FAO, 1984; Purnell, 1977). However, considerable expertise is required in the isolation and characterization of isolates. Species identification is based on examination of some or all of the following: geographical distribution, vector specificity, morphology, host specificity, pathogenicity, serology, cross-immunity and DNA probes. Stock characterization traditionally involves cross-immunity studies, among others. Several monoclonal antibodies and DNA probes have also been used to divide stocks of *T. parva* into groups (Minami *et al.*, 1983; Conrad *et al.*, 1987; Allsopp and Allsopp, 1988) but more work is necessary to determine whether polymorphisms detected correlate with differences in cross-protection (Irvin and Morrison, 1989).

Preparation of ground-up tick supernatant. Details of the preparation of GUTS are available in the literature (FAO, 1984). The process involves the inoculation of cattle with *T. parva* seed material. Approximately ten days after inoculation, the cattle are

infested with non-infected nymphal ticks. Engorged ticks are harvested and allowed to moult. The level of infection can be determined by examining the number of infected acini in the salivary glands of some of the prefed adult ticks. The remainder of the adult ticks are fed on the ears of rabbits, detached after four days, washed, disinfected and ground up in a suitable medium. Ground-up tick tissue is centrifuged at a low speed and the supernatant collected, mixed with glycerolized medium, equilibrated and cryopreserved. One litre of frozen vaccine can be prepared from 10 000 adult ticks and this will provide 1 000 1-ml doses of vaccine.

Theileria annulata

Isolation of stocks. Isolation of *T. annulata* parasites from the field was reviewed by Pipano (1989). Isolates can be obtained by inoculating cattle with the blood of infected animals or by exposing the cattle to infected ticks. An alternative and more elegant technique is to infect peripheral blood leucocytes *in vitro* with sporozoites obtained from macerated infected ticks.

Propagation of schizonts in vitro. Techniques used to initiate and maintain *T. annulata* cultures are well documented (Brown, 1979; Pipano, 1989). Briefly, lymphocytes are collected from the blood or organs of infected cattle and grown in monolayer or suspension cultures. Alternatively, normal peripheral blood lymphocytes can be infected *in vitro* with sporozoites obtained from infected ticks (Brown, 1979). Standard culture procedures are used and a wide variety of culture media are suitable. Yields of up to 9×10^7 schizont-infected lymphocytes can be obtained from a stationary culture vessel containing 100 ml of medium, and even more from roller bottles.

Continuous propagation of *T. annulata* in lymphoid cell cultures eliminates other tick-borne agents such as *Babesia* spp., *Anaplasma* spp. and *Eperythozoon* spp. Theoretically, however, some viral infections can be introduced into cultures from ticks or cattle used as a source of infected lymphocytes.

Partial or complete attenuation of *T. annulata* is achieved by prolonged cultivation *in vitro* (Pipano, 1989). Complete attenuation (no clinical manifestation and no detectable tissue schizonts or erythrocytic merozoites) has been reported after 600 to 900 days in culture (Pipano, 1989; Singh, 1990). Partially attenuated schizonts have also been used for vaccination (Zablotsky, 1988).

When the desired degree of attenuation is reached, schizont-infected cells can be cryopreserved with a high degree of survival upon reconstitution (Wathanga, Jones and Brown, 1986). DMSO or glycerol can be used as cryopreservative. Master seed stock is usually prepared and used to initiate a series of passages in culture to yield parasites for vaccine. There is little information on the selective effect of long-term passaging of schizonts on immunogenicity but there is evidence that attenuated schizonts are less infective and less protective than virulent schizonts. Therefore, it is recommended that production cycles be restarted periodically from the same master seed (Pipano, 1989).

Anaplasma spp. using *A. centrale* as vaccine

Selection and storage of stock. Only one stock of *A. centrale* exists. The history of its isolation in South Africa in 1911 was recorded by Potgieter (1979). This stock has been used to initiate vaccine production programmes in several countries (Potgieter, 1979; Callow and Dalgliesh, 1980; Pipano *et al.*, 1986; Abdala *et al.*, 1990). Master seed of *A. centrale* is prepared and stored in the same way as that of *Babesia* spp.

Supply of infective material. Infective blood

containing suitable numbers of *A. centrale* organisms for the production of vaccine can be obtained in much the same way as *Babesia* parasites. Blood from acutely infected splenectomized calves is most suitable for the purpose although, in some laboratories, the blood of carrier-donors showing quantifiable rickettsemia is used and reported to be effective (Potgieter, 1979). *A. centrale* vaccine can be prepared in frozen or chilled forms in the same way as *Babesia* vaccines (de Vos and Jorgensen in FAO, in press). In some countries, for example Australia, mixed *Babesia/ Anaplasma* vaccines are produced (Callow and Dalgliesh, 1980).

Cowdria ruminantium

Selection and storage of stocks. The isolation and characterization of *Cowdria* stocks have been reviewed in the literature (Uilenberg, 1983; FAO, 1984; Oberem and Bezuidenhout, 1987; Bezuidenhout, 1989). Isolates can be obtained from both the vertebrate host and the tick vector by subinoculation of infected blood, organ emulsions or supernatant of ground-up, engorging *Amblyomma* ticks. Stocks vary considerably in virulence and there is some evidence of immunological differences (Jongejan *et al.*, 1993). However, immunity does not depend on the virulence of the isolate. It is important to select a stock which is as mild as possible but which shows adequate cross-protection against local isolates. Furthermore, it must also be susceptible to treatment with available anti-*Cowdria* drugs. Only one stock (Ball 3) is used in South Africa. It generally causes a marked febrile response some days before the onset of other clinical signs.

Stabilate of the stock used for production of vaccine is produced in the same way as the vaccine itself (see below) and stored at -70° or -196°C. Infective material has also been freeze-dried successfully if kept below -18°C (du Plessis *et al.*, 1990).

Production of infective material from donor sheep. The method used in South Africa has been described in some detail (FAO, 1984; Oberem and Bezuidenhout, 1987; Bezuidenhout, 1989). Briefly, susceptible sheep are inoculated with thawed stabilate and bled nine to ten days later at the height of the febrile reaction. The stabilate is usually prepared from the blood of reacting sheep but tick-derived stabilates will fulfil the same purpose. Similarly, goats and cattle can also be used as donors. Blood from reacting animals is collected directly into an equal volume of buffer to which DMSO has been added. The diluted blood is then snap-frozen and stored in liquid nitrogen. Up to 4 000 doses of vaccine can be prepared from one sheep.

VACCINE DISPENSING AND DISPATCH

Dilution of vaccine concentrate

Some vaccine starter materials, notably *Theileria* GUTS and blood infected with *Babesia* spp. and *Theileria* spp., are very concentrated and can be diluted to increase the vaccine yield. The appropriate medium to be used as diluent depends on the starter material and whether the vaccine is to be frozen or not. Details of media used in the production of TBD vaccines have been reviewed (FAO, 1984; Bezuidenhout, 1989; Pipano, 1989; de Vos and Jorgensen in FAO, in press).

Viability of *Babesia* and *Anaplasma* organisms can be maintained in chilled form by storing infected blood at 2° to 4°C. Even at this temperature, infectivity is lost exponentially, declining by about one log unit during the first week of storage (Callow, 1984). Chilled *Babesia* and *Anaplasma* vaccines usually contain 0.5 to 1×10^7 organisms per dose or about 100 times the minimum infective number required for subcutaneous inoculation. The required number of organisms is obtained by diluting the infected blood with a suitable medium. Chilled *T. annulata*

vaccine can also be prepared from culture material, with the recommended number of infected cells being 0.5×10^7 (Pipano, 1989).

The minimum volume of a dose of vaccine should preferably not be less than 2 ml to allow for inaccuracies in vaccination equipment and procedures. An exeption is *T. parva* sporozoite vaccine where the recommended dose is 1 ml to allow for inoculation over the parotid lymph node.

Vaccine dispensing

Techniques for producing frozen vaccines using DMSO as a cryoprotectant were described by Mellors *et al.* (1982) and Abdala *et al.* (1990). Vaccine with glycerol as cryoprotectant has also been produced (Dalgliesh, Jorgensen and de Vos, 1990) and is known to remain viable for at least eight hours after thawing. Frozen vaccine is dispensed in cryovials suitable for low-temperature storage.

Dispatch and cold chain

The types of transport networks and their efficiency vary greatly between countries and the choice depends largely on availability. Rapid, reliable means of communication and transport are desirable for frozen vaccine and essential for chilled products. Frozen vaccine can be transported in vacuum-insulated containers with a refrigerant (liquid N_2 or solid CO_2). Vaccine should not be thawed until just before use and, once thawed, must not be refrozen. Chilled *Babesia, Anaplasma* and *Theileria* vaccines have a very short shelf-life, even when packed in ice, and may become ineffective if transport to the destination exceeds 48 hours.

QUALITY ASSURANCE

Because of the live nature of TBD vaccines, quality control is essential to ensure potency and to minimize the risk of contamination. Most of the early *Babesia* and *Anaplasma* vaccines produced by the carrier-donor method and the *T. parva* vaccines produced with tissue suspensions had variable potencies, thus bringing these vaccines into general disrepute. In some countries in the tropics and subtropics, the risk of contamination is also very real. Quality assurance therefore needs to be an integral part of any TBD vaccine production programme covering all operating procedures.

The standards of quality assurance of *Babesia, Anaplasma* and *Theileria* vaccines have been addressed by the Office internationale des épizooties (OIE, 1991). However, these standards only cover the main aspects. Quality control of *T. annulata* vaccine in Israel is based on the procedures for testing live vaccines described in the United States Code of Federal Regulations for Animals and Animal Products. In Australia, procedures for the production of *Babesia* and *Anaplasma* vaccines are being modified to comply with the Australian Code of Good Manufacturing Practice for Veterinary Preparations. Compliance with these or similar codes may not be possible in all developing countries. However, the potential consequences of providing substandard or contaminated vaccine should be considered. The contamination of one batch of *Babesia/Anaplasma* vaccine in Australia with enzootic bovine leucosis (Rogers *et al.*, 1988) resulted in the payment of US$1.5 million in compensation.

Quality control should be performed at both the preproduction and postproduction stages. Preproduction quality control is particularly important in the case of chilled vaccine but is, in itself, not adequate to guarantee potency and purity. It should include facilities, the documentation of standards and procedures for obtaining and quarantining suitable donor animals and the preproduction testing of animals, master seed and other starting materials used in the production of vaccine.

It should also include environmental monitoring to ensure that there is only a minimal risk of infectious challenge in the region where the production facility is located.

Postproduction quality control should be aimed at determining potency, purity and, where relevant, virulence. Potency and virulence are monitored by inoculating groups of susceptible cattle and then monitoring the reactions. Dalgliesh, Jorgensen and de Vos (1990) described a method for testing the potency of frozen *Babesia* vaccine. Similar procedures have been described for *Theileria* spp. (FAO, 1984) and *Cowdria* sp. (Bezuidenhout, 1989). Each batch should also be tested for freedom from contaminants, with the choice of tests depending on the diseases suspected and the type of vaccine concerned. Tick-derived sporozoite vaccines naturally do not need to be scrutinized as intensely as vaccines produced from the blood of infected animals but there is a risk of other TBDs, including Crimean Congo haemorrhagic fever.

FACTORS CRITICAL TO THE PRODUCTION OF TBD VACCINES IN DEVELOPING COUNTRIES

- Availability of stocks known to be protective against local challenge and showing other desirable traits.
- Availability of disease-free donor animals as a source of infected blood, infected ticks and other starter materials. This may require the animals to be bred under tick- and arthropod-free conditions for the specific purpose of vaccine production.
- Availability of suitable facilities to maintain disease-free animals and to perform the necessary procedures (splenectomies, feeding of ticks, collection of blood).
- Laboratory facilities and expertise to monitor donor cattle and starter materials (infected blood, culture material, infected ticks) for quality control purposes.
- Suitable laboratory facilities and services, including reliable electrical supplies and low-temperature storage facilities.
- Access to foreign capital for the purchase of equipment, reagents and other supplies needed for production and quality control.
- Access to reliable transport networks.
- A core of dedicated, trained staff.
- Access to training opportunities, procedures and proven master seed.

KEY LABORATORIES PRODUCING TBD VACCINES

The following laboratories are involved in the production of TBD vaccines and may be able to provide suitable master seed, details of production procedures and quality control as well as training opportunities.

Babesia spp.

- Tick Fever Research Centre, Wacol, Queensland, Australia
- Kimron Veterinary Institute, Bet Dagan, Israel
- Veterinary Research Institute, Onderstepoort, South Africa
- Central Veterinary Laboratory, Lilongwe, Malawi
- Instituto Nacional de Tecnologia Agropecuaria, Rafaela, Argentina
- Miguel C. Rubino Laboratory, Pando, Uruguay

Theileria parva

- Central Veterinary Laboratory, Lilongwe, Malawi
- ILRAD, Nairobi, Kenya

Theileria annulata

- Kimron Veterinary Institute, Bet Dagan, Israel
- Animal Disease Research Laboratory, NDDB, Anand, India

Anaplasma

- See *Babesia* spp. list.

Cowdria spp.

- Veterinary Research Institute, Onderstepoort, South Africa

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Multicellular parasite vaccines

J.B. McKeand and D.P. Knox

THE NECESSITY FOR ANTIPARASITE VACCINES

Diseases caused by multicellular parasites are a major cause of mortality and morbidity in humans and domestic animals throughout the world. Jenner's discovery that cowpox virus was effective in immunization against smallpox stimulated research into vaccination against most major pathogens including multicellular parasites. Unfortunately, the successes achieved against viruses and bacteria have not been paralleled in the control of diseases caused by multicellular parasites. At present these are controlled by chemotherapy, although it is now apparent that resistance is an inevitable consequence of prolonged chemical application. Anthelmintic resistance has been reported against all the broad-spectrum agents currently available (Prichard, 1990). For example, benzimidazole resistance has been found in *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis* nematode infections in sheep and goats throughout the world, while levamisole and morantel resistance has been recorded with increasing frequency in Australasia and South Africa (Prichard, 1990; Jackson, 1993). Of concern is the fact that resistance to the relatively recently developed ivermectin has been experimentally selected in *H. contortus* (Egerton, Suhayda and Eary, 1988) and has now been reported in the field in *H. contortus* in both sheep (van Wyck and Malan, 1988) and goats (Jackson *et al.*, 1991). There is widespread acaricide resistance among the important cattle ticks, *Boophilus microplus* and *Hyalomma anatolicum anatolicum* (Wharton, 1976) and an increasing incidence of insecticide resistance is evident in the sheep blowfly, *Lucilia cuprina* (Arundel and Sutherland, 1988).

Although the rapid spread of drug resistance in parasite populations can to a degree be reduced by strategic management procedures, these can impose considerable restraints on land use, especially on marginal pastures. The cost of developing novel chemical control agents is prohibitive and considerable effort is now being directed towards prolonging the efficacy of the currently available drugs, with a particular emphasis on minimizing the effects of drug resistance. Furthermore, there is now widespread public concern regarding the effects of chemical residues in both animal products and the environment. There is clearly a requirement to develop novel control methods for parasitic disease.

Recently, biological control strategies, such as the use of nematophagous fungi, have been evaluated for the control of gastrointestinal nematodes of domestic animals (Waller and Larsen, 1993) but most research has been directed at the development of antiparasite vaccines. Despite decades of intensive work, only two commercially available multicellular parasite vaccines exist. These are based on radiation-attenuated larvae for the control of *Dictyocaulus viviparus* in cattle (Jarrett *et al.*, 1958) and *Dictyocaulus filaria* in sheep (Sharma, Bhat and Dhar, 1988). It would

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appear that the antigenic complexity of multicellular parasites has provided an effective barrier to the development of useful vaccines. To compound this, it is often the case that protective antiparasite immune responses are absent or deficient in immature animals. The reasons for this apparent unresponsiveness are unclear but, given that production losses primarily accrue owing to the infection of young stock, studies must be undertaken to identify the cause of this and to develop vaccines which are effective in this group.

Recent advances in immunology, protein chemistry and recombinant DNA techniques have enabled a more accurate identification of potential host-protective immune responses and the parasite antigens which stimulate them. In order to understand the problems and difficulties associated with the development of vaccines against multicellular parasites, this review provides a historical account of the work that has been undertaken and then continues with a description of recent advances which suggest that the development of subunit parasite vaccines may be an achievable goal. Numerous kinds of parasite preparations have been used to immunize animals. These range from the use of attenuated whole parasites, through crude extracts of dead parasites to specific purified molecules.

VACCINATION WITH RADIATION-ATTENUATED PARASITES

Parasites attenuated by irradiation do not reach patency but can stimulate protective immune responses without significant parasite-induced pathology. Immunization with irradiated third-stage larvae (L3) of the bovine lungworm, *D. viviparus*, is a highly successful example of vaccination using this method of attenuation. Natural infection with this parasite induces high levels of resistance in calves (Poynter and Cauthen, 1942; Jarrett, McIntyre and Urquhart, 1954). Vaccination studies using two doses of 10 000 40 kilorad- (400 joules per kilogram-) irradiated L3 showed that calves could be significantly protected against challenge using attenuated larvae (Jarrett *et al.*, 1958). This vaccine was developed commercially and has been used successfully for the control of dictyocaulosis in the United Kingdom and other parts of Europe ever since. This pioneering breakthrough led to several attempts to develop similar vaccines against other important helminth species.

In India, a successful irradiated larval vaccine was developed for the control of the pathogenic ovine lungworm, *D. filaria* (Sharma, Bhat and Dhar, 1988). Vaccination of dogs against the intestinal nematode *Ancylostoma caninum* was similarly effective (Miller, 1971). Unfortunately, this vaccine failed commercially because of its respiratory side-effects, short shelf-life and, in some cases, lack of sterile immunity (Miller, 1978). *Schistosoma* spp. are trematodes of importance in humans and animals in tropical regions and successful immunization has been achieved using irradiated cercariae in laboratory models (Simpson *et al.*, 1985). Furthermore, zebu cattle were significantly protected against *S. bovis* challenge following vaccination with irradiated schistosomula or cercariae of the homologous species (Bushara *et al.*, 1978).

Other attempts to immunize animals with irradiated parasites have met with less success. Mature sheep (> six months) were protected against homologous challenge using irradiated L3 of either *H. contortus* (Urquhart *et al.*, 1966b) or *T. colubriformis* (Gregg and Dineen, 1978). However, as in natural infection, immature sheep (< three months) failed to develop immunity following vaccination (Urquhart *et al.*, 1966a; Gregg *et al.*, 1978).

VACCINATION USING MATERIAL FROM DEAD PARASITES

Attempts to vaccinate against parasitic helminths using somatic extracts from dead organisms were first made in the 1930s but achieved limited success (Chandler, 1932). However, some subsequent attempts have given protection: for example, extracts of fourth-stage larvae of *T. colubriformis* induced significant levels of protection in guinea pigs against homologous challenge (Rothwell and Love, 1974) and worm burdens in sheep immunized with sonicates of adult stage *H. contortus* were significantly lower (63 percent) than burdens found in challenge controls (Adams, 1989). Somatic extracts derived from onchospheres of the cestodes *Echinococcus* sp. and *Taenia* sp. were also found to be potent sources of host-protective antigens (Rickard and Williams, 1982; Xilinas, Papavasiliou and Marselou-Kinti, 1976). These results appear to be the exception rather than the rule, since in most other species success has been limited when somatic helminth extracts were used for immunization (Clegg and Smith, 1978).

Similar to the situation with helminths, somatic extracts of ectoparasites have induced variable degrees of protective immunity (Willadsen, 1980). Salivary gland extracts induced protective immunity to *B. microplus* (Brossard, 1976) and *Amblyomma maculatum* (McGowan *et al.*, 1981) in cattle and also in *Dermacentor andersoni* (Wikel, 1981) in guinea pigs. Subsequently, Opdebeeck *et al.* (1988) vaccinated calves with crude soluble and membrane extracts from the midgut of partially engorged female *B. microplus* and observed up to a 98 percent reduction in tick numbers following challenge. Sheep produce antibodies to *L. cuprina*, the primary agent responsible for fly strike in Australia (O'Donnell *et al.*, 1980) and vaccination of sheep with soluble extracts of third-instar larvae stimulated antibodies which inhibited larval growth and development *in vitro* (O'Donnell *et al.*, 1981). Larvae which were harvested from sheep previously vaccinated with soluble components of second-instar larvae were 58 percent smaller than larvae from control sheep (Johnston, Kemp and Pearson, 1986).

VACCINATION WITH MATERIAL FROM LIVE PARASITES

Although some studies using crude extracts of dead parasites gave protection, few of these studies were taken further owing to the difficulty in defining the protective components within these complex extracts, and attention increasingly focused on less complex parasite compartments which may comprise components essential to parasite survival within the host.

Sarles and Taliaferro (1936) first suggested that products released from helminths *in vivo* were involved in host immunity when they observed immune precipitates at the mouth, excretory pore and anus of *Nippostrongylus brasiliensis* parasites which had been incubated with serum from immune rats. It was thought that the antigen/antibody complexes formed at these parasite orifices might act by blocking the activity of secretions essential to parasite survival. Several attempts to immunize laboratory animals with these excretory-secretory (ES) antigens have produced encouraging results. For example, protective immunity was achieved in mice using the ES products of first-stage larvae of *Trichinella spiralis* (Vernes, 1976) as well as in guinea pigs using the ES products of fourth-stage *T. colubriformis* (Rothwell and Love, 1974), the ES products of third- and fourth-stage *Ascaris suum* (Stromberg and Soulsby, 1977) and the ES products of adult *D. viviparus* (McKeand *et al.*, 1995).

Immunization using ES materials has not always been successful, however, as

demonstrated by Neilson (1975) who immunized lambs with the ES products of L3 and L4 *H. contortus* and found no differences in faecal egg counts or worm burdens between vaccinates and challenge controls. The lambs used in this study, however, were less than three months old and may have been immunologically unresponsive. Alternatively, the result may have reflected differences in the immunogenicity of ES in laboratory animals compared with that in natural hosts.

Materials released from ectoparasites have also been proposed as major immunogens. Fly strike is initiated by enzymes secreted by the fly larvae on to the sheep's skin and sheep exposed to several infestations strongly recognized antigens derived, and probably released from, the larval gut and salivary glands (Skelly and Howells, 1987). It has been postulated that inflammation induced by larval ES products plays a central role in wound formation and exudation and that these, in turn, may reduce larval establishment (Sandeman, 1990). More refined vaccination studies in which the potential of secreted larval enzymes as vaccine candidates have been indicated are outlined later.

Despite all these studies, until recently no one has been able to identify and isolate protective components within these fractions and incorporate them into commercially viable vaccines. With the advent of improved immunochemical methods for identifying protective antigens and the ability to produce these in large quantities using recombinant DNA technology, it now appears possible to develop subunit parasite vaccines. The likely steps in subunit parasite vaccine development are summarized (Figure 2).

DEVELOPMENT OF SUBUNIT MULTICELLULAR PARASITE VACCINES

Historically, the selection of protective antigens has been based on extracting native antigen on a small scale from parasites harvested from donor animals or from parasites maintained *in vitro*. This would clearly be unsuitable for commercial vaccine production. Emery and Wagland (1991) estimated that, to obtain sufficient nematode antigen to vaccinate one sheep, parasites would have to be harvested from three donor animals! The problem of antigen production can now be resolved by using peptide synthesis and/or recombinant DNA technology. The main challenges are to identify appropriate individual protective antigens suitable for incorporation into subunit vaccines while maintaining their immunogenicity.

Antigen selection

While many parasite proteins are antigenic, the immune responses to these antigens may not be protective (O'Donnell *et al.*, 1989). Parasite antigens can be classified as conventional or covert. Conventional antigens are recognized by the host during the course of a natural infection and, as vaccine components, would augment natural immunity. However, mutations in the genes coding for these antigens may be selected by the pressure associated with immunity in vaccinated hosts and could restrict their long-term use. On the other hand, covert antigens are hidden from the host immune system during natural infection and, in the absence of selection, are less likely to exhibit antigenic variation. The main disadvantage of using covert antigens is that repeated vaccination may be necessary as specific host immune responses would not be boosted during subsequent natural infections. However, if animals are protected during the very susceptible period in early life, as for example in haemonchosis in sheep, subsequent exposure to natural challenge may maintain protective levels of immunity.

Antigens can be selected on either an empirical or a rational basis. The empirical

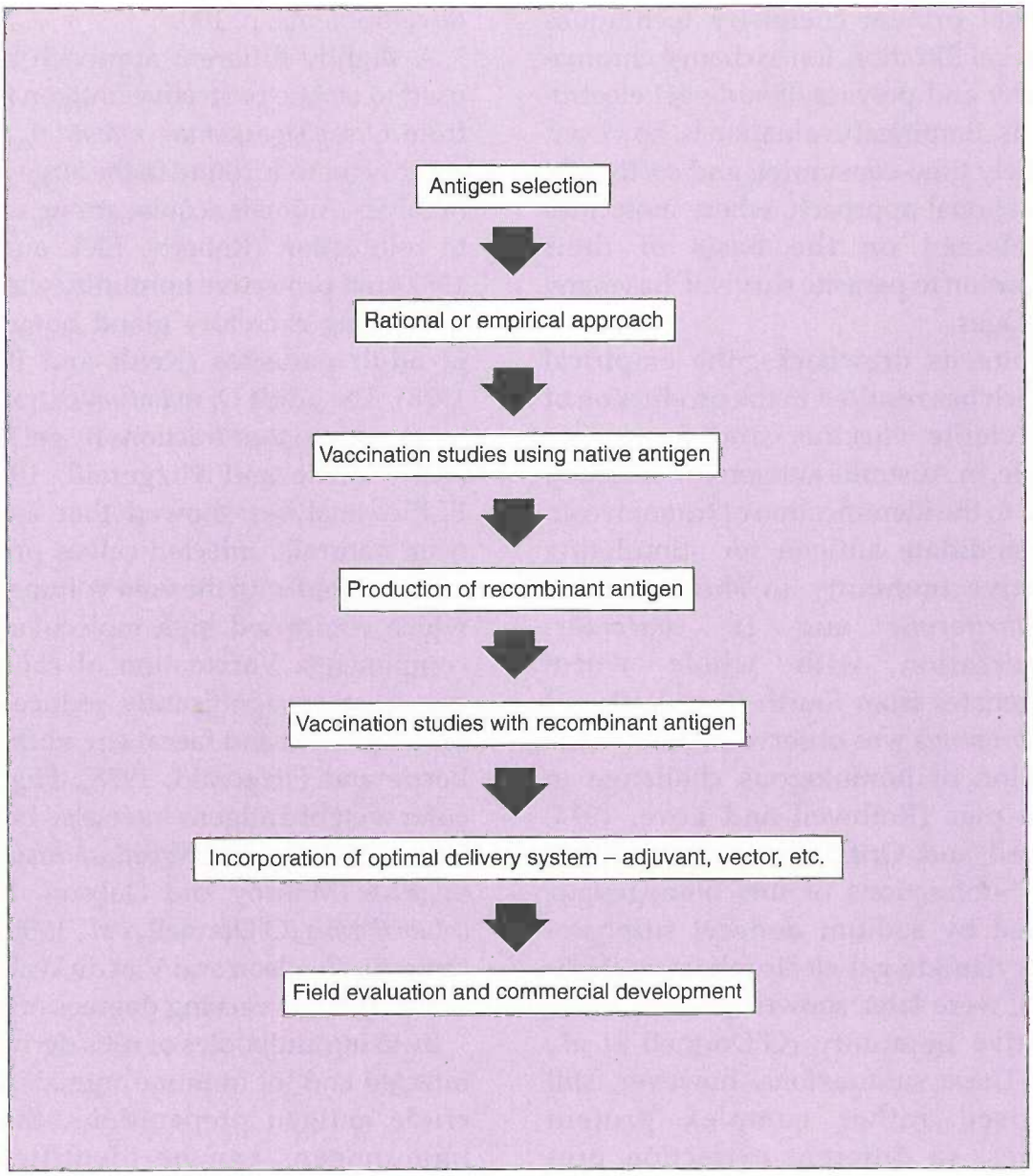

FIGURE 2
Steps in the development of subunit parasite vaccines

approach relies on the fractionation of parasite material followed by successive immunization studies. These extracts provide the starting material for progressively more refined fractionation and the ultimate definition of specific protective components. Fractionation has normally been achieved using conventional protein chemistry techniques such as gel filtration, ion exchange chromatography and polyacrylamide gel electrophoresis. Empirical evaluation is, however, extremely time-consuming and costly so a more rational approach, where molecules are selected on the basis of their contribution to parasite survival, has many advantages.

Despite its drawbacks, the empirical approach has resulted in the production of some viable vaccine candidates. For example, in Australia antigen fractionation has led to the identification of tropomyosin as a candidate antigen for stimulating protective immunity in sheep against *T. colubriformis* and *H. contortus*. Immunization with whole worm homogenates from fourth-stage larvae of *T. colubriformis* was observed to accelerate expulsion of homologous challenge in guinea pigs (Rothwell and Love, 1974; Rothwell and Griffiths, 1977; Rothwell, 1978). Subfractions of this homogenate, obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), were later shown to induce host protective immunity (O'Donnell *et al.*, 1985). These subfractions, however, still comprised rather complex protein mixtures, so different extraction procedures were then evaluated to reduce their complexity (O'Donnell *et al.*, 1989). Using phosphate-buffered saline/sodium deoxycholate (PBS/NaDOC) to make somatic extracts of *T. colubriformis* larvae, a simple antigen mixture was produced which consisted of four protein components, one of which, a 41 kilodalton (kDa) protein, induced 43 to 51 percent protection in guinea pigs (O'Donnell *et al.*, 1989). Partial amino acid sequence analysis of this protein indicated that it was tropomyosin. Progress towards a recombinant vaccine based on parasite tropomyosin is outlined in the section Multicellular parasite vaccines: recent developments, p. 100.

A slightly different approach has been used to isolate protective antigen fractions from *Oesophagostomum radiatum*, a pathogenic nematode found in the large intestine of calves. Animals acquire strong resistance to reinfection (Roberts, Elek and Keith, 1962) and protective immunity was stimulated using excretory gland homogenates of adult parasites (Keith and Bremner, 1973). The adult *O. radiatum* extracts were resolved into four fractions by gel filtration (East, Berrie and Fitzgerald, 1989) and ELISA analyses showed that antibodies from naturally infected calves predominantly reacted with the void volume fraction which comprised high molecular weight components. Vaccination of calves with this fraction significantly reduced worm establishment and faecal egg output (East, Berrie and Fitzgerald, 1988). High molecular weight antigens have also been used to vaccinate against *Nematospiroides dubius* in mice (Monroy and Dobson, 1987), *T. colubriformis* (O'Donnell *et al.*, 1985) and *H. contortus* (Neilson and Van de Walle, 1987) in sheep, with varying degrees of success.

By using antibodies or cells derived from infected and/or immune animals to probe crude antigen preparations, the major immunogens can be identified. This strategy can be augmented by comparing the responses of individual hosts which are defined as susceptible or resistant to infection. Monoclonal antibodies derived from immune mice were used to define and purify the major immunogens of the small intestinal nematode, *T. spiralis*. This work led to the purification to

homogeneity of the first single, protective antigen of a nematode, a 48 kDa protein (Silberstein and Despommier, 1984). Initially, three proteins were isolated from extracts of infective larvae by immunoaffinity chromatography (Silberstein and Despommier, 1984). When used to immunize mice, one of these proteins gave significant protection at very low (0.1 D1 µg) doses. Unfortunately, this protein was ineffective in the natural pig host (Gamble, Murrell and Marti, 1986).

In another study, sera from infected sheep, which were defined as resistant or susceptible to *O. circumcincta*, were used to identify a 31 kDa molecule present in Triton X-100 extracts of L3. This molecule was recognized preferentially by resistant animals as early as three weeks after experimental infection and was present in and secreted by third-stage larvae (McGillivery *et al.*, 1990). Lambs immunized with the purified protein were significantly protected against homologous challenge as judged by faecal egg counts and adult worm burdens compared with challenge controls (McGillivery *et al.*, 1992). Homologous antigens have also been identified in *T. colubriformis* and *H. contortus* (McGillivery *et al.*, 1990).

Recently, more rational approaches to vaccine design have produced encouraging results. One approach has been to identify and isolate antigens from the intestinal luminal surface of blood-feeding parasites. It has been suggested that the induction of systemic antibody responses against the gut antigens of blood-sucking parasites may cause sufficient damage to impair parasite survival. This response is "artificial" as these gut antigens are not recognized by the host during the course of natural infection, i.e. the antigens are covert. Lambs have been immunized successfully against haemonchosis, using antigens fractionated from the intestinal luminal surface of adult *H. contortus* (Munn, Greenwood and Coadwell, 1987; Munn and Smith, 1990). A major immunogen of this preparation was an integral membrane glycoprotein of molecular weight 110 kDa, termed H11 (Munn, 1988; Smith and Munn, 1990). Immunization of sheep with microgram amounts of essentially pure H11 stimulated substantial protection against challenge infection in several breeds of sheep (Munn, 1988; Tavernor *et al.*, 1992a and 1992b; Munn *et al.*, 1993a). This immunity was closely correlated with H11-specific circulating IgG levels (Munn and Smith, 1990) and vaccination was equally successful in young lambs (< two months, Tavernor *et al.*, 1992a) as in older lambs (> eight months, Tavernor *et al.*, 1992b). With respect to the age-related unresponsiveness of young lambs to vaccination with irradiated L3 (Urquhart *et al.*, 1966b), the results obtained with the H11 antigen would suggest that this would be an ideal vaccine candidate for the control of this disease. Sequence analysis of full-length cDNAs encoding H11 has shown the protein to be homologous to mammalian amino peptidases. Amino peptidase activity was subsequently found in native pure H11 and enzyme activity was inhibited by immunoglobulin from H11-vaccinated sheep (Munn *et al.*, 1993b). A full-length, enzymically active H11 cDNA has been produced in the baculovirus expression system (Munn *et al.*, 1993b) and its ability to protect lambs against haemonchosis is currently being evaluated. Recent work has indicated that integral gut membrane preparations from adult *H. contortus* contain other protective antigens apart from H11. For instance, substantial protection against *H. contortus* was induced in lambs hyperimmunized with an integral membrane extract in which the characteristic H11 doublet was not usually observed (Smith, 1993).

A similar approach has led to the

isolation of several protective antigens from blood-feeding arthropods. For example, cattle are protected against the tick *B. microplus* by using components isolated from the tick gut (Johnston, Kemp and Pearson, 1986; Kemp *et al.*, 1986; Willadsen, McKenna and Riding, 1988; Opdebeeck *et al.*, 1988). The number of ticks engorging, their average weight and egg-laying ability were all diminished in those ticks feeding from immunized cattle (Willadsen and McKenna, 1991). Triton X-100 extracts of the gut of first-instar larvae of *L. cuprina* were used to vaccinate sheep, and challenge larvae obtained from these sheep had their growth reduced by more than 40 percent when compared with larvae from challenge controls. In addition, immune sera from vaccinated sheep inhibited the growth of larvae *in vitro* by more than 50 percent indicating that the effect was antibody-mediated (East *et al.*, 1993). Gut membrane preparations have also given promising results with sucking lice, flies and mosquitoes (Schlein and Lewis, 1976).

The lumen of the blowfly gut is lined with a periotrophic membrane which, in addition to being essential to digestion, acts as a molecular sieve preventing intact antibody molecules coming into contact with underlying gut epithelial cells (East *et al.*, 1993). This range of functions, crucial to insect survival, attracted attention to the periotrophic membrane as a source of potential protective antigens. Periotrophic membranes, harvested *in vitro* from *L. cuprina* larvae, were used to immunize sheep and resulted in a 30 percent reduction in the weight of established larvae compared with those obtained from challenge control animals (East *et al.*, 1993).

Another source of potential vaccine candidates are ES products which are thought to assist in tissue invasion, parasite feeding and evasion of host effector mechanisms. ES products may be derived from the nematode cuticle or from specialized excretory-secretory organs and represent the major antigenic and functional challenge to the host (Maizels and Selkirk, 1988; Lightowlers and Rickard, 1988). In terms of so-called functional molecules, several classes of enzyme have been identified in ES products. These include proteinases which may facilitate penetration of host tissues (Matthews, 1982; Dalton and Heffernan, 1988) or act as anticoagulants (Hotez and Cerami, 1983), acetylcholinesterases (AChE) (Rhoads, 1984) and superoxide dismutases (Rhoads, 1983; Knox and Jones, 1992). Some of these have the potential to modulate host immune responses, for example secreted proteinases have been ascribed a role in cleaving surface-bound immunoglobulin in several helminth systems (Auriault *et al.*, 1981; Chapman and Mitchell, 1982; Smith *et al.*, 1993). AChE may interfere with hosts' immune mechanisms by breaking down host acetylcholine (ACh), which is known to stimulate potential effector mechanisms such as neutrophil-mediated antibody-dependent cellular cytotoxicity (Gale and Zhigelboim, 1974), neutrophil chemotaxis (Hill *et al.*, 1975) and mast cell histamine release (Kaliner and Austen, 1975). Although not an enzyme, a γ-interferon analogue has been identified in *T. colubriformis* ES products (Dopheide *et al.*, 1991). Owing to these putative functional roles, ES products have received close attention as a source of protective antigens.

Secretions produced during the moult from third- to fourth-stage larvae of *Ascaris suum* induced significant protection against challenge in guinea pigs, while soluble proteins produced during culture of L2, L3, L4 and adult worms were ineffective (Stromberg and Soulsby, 1977). Metabolites secreted by *H. contortus* during the moult from the third to fourth larval stages were also effective in sheep (Ozerol

and Silverman, 1970) and the ES products of adult *D. viviparus* induced significant levels of protection in immunized guinea pigs (McKeand *et al.*, 1995).

Attention is now focusing on defining the precise nature of the polypeptides which comprise nematode ES products. The application of molecular biology and increasingly specific immunological methods to the study of parasite ES products has enabled the definition of a number of novel proteins with protective properties. Recently, the dominant glycoprotein present in ES products derived from the parasitic stages of *T. colubriformis* has been cloned and sequenced and shown to induce protective immunity and have significant homology to the porcine intestinal peptide, valosin (Savin *et al.*, 1990). Valosin can modulate several aspects of gastrointestinal function and, by secreting a homologue protein, the parasite itself may alter its local environment.

AChE is secreted by several nematode species and is immunogenic in a number of host/parasite systems (Lee, 1970; Edwards, Burt and Ogilvie, 1971; Jones and Ogilvie, 1972; Ogilvie *et al.*, 1973; McKeand *et al.*, in press). For example, sheep infected with *T. colubriformis* produce antibodies to worm AChE; however, AChE purified from this parasite failed to induce a protective immune response in vaccinated guinea pigs (Rothwell and Merritt, 1975). In the *D. viviparus* system, calves naturally or experimentally infected with this parasite also produce antibodies which bind and inhibit parasite AChE activity (McKeand *et al.*, in press) and *D. viviparus* ES preparations enriched for AChE activity protected guinea pigs against subsequent challenge compared with adjuvant controls (McKeand, 1992).

Glutathione S-transferase (GST) appears to be one of the major detoxification enzymes of parasitic helminths and has received much attention as a candidate protective antigen (Brophy and Barrett, 1990). GST is recognized by antibodies from mice infected with the trematode *Schistosoma japonicum*, and the development of protective immunity in various mice strains has been positively correlated with the levels of specific anti-GST antibody (Smith *et al.*, 1986). Recombinant GST, prepared from *S. japonicum* and *S. mansoni*, was subsequently observed to confer partial, but not significant, protection in rats and hamsters (Balloul *et al.*, 1987). However, vaccination of genetically susceptible mice with a β-galactosidase fusion protein of this enzyme gave significant protection against infection with *S. japonicum* (Smith *et al.*, 1986). Affinity-purified GST from *Fasciola hepatica* did not significantly protect rats against challenge (Howell, Board and Boray, 1988), but sheep immunized with purified GST were significantly protected (57 percent reduction in fluke numbers) (Sexton, Milner and Paraccio, 1990). The genes encoding *F. hepatica* GSTs have now been isolated with a view to testing a recombinant vaccine (Wijffels, Sexton and Salvatore, 1992). In contrast to these trematode systems, antisera raised against GST purified from adult *H. contortus*, while inhibiting enzyme activity *in vitro*, failed to affect survival of the parasite *in vivo* (Sharp *et al.*, 1991).

Proteinases are secreted by *L. cuprina* larvae on to the sheep's skin to enable the larvae to feed and it is these enzymes that are thought to initiate wound formation. It has been observed that proteinases released by these flies can be inhibited by sheep plasma proteinase inhibitors and that the proteinase inhibitors α2-macroglobulin and antithrombin III reduced larval growth *in vitro* (Bowles, Feehan and Sandeman, 1990). Furthermore, antiproteinase antibodies inhibited larval growth *in vitro* (Sandeman, 1990). In common with many helminth parasites,

blowfly larvae produce a multiplicity of secreted proteinases, each of which may need to be inhibited by the host immune response to impair larval growth adequately (East and Eisemann, 1993).

Surface antigens of parasitic nematodes are often highly antigenic (Maizels and Selkirk, 1988). Cetyltrimethylammonium bromide (CTAB) surface extracts of *T. spiralis* larvae induced significant levels of protection against reinfection in mice (Grencis *et al.*, 1986). However, sheep immunized with surface extracts of *H. contortus* L3 were as susceptible as challenge control animals (Turnbull *et al.*, 1992) and cuticle collagens from the third and fourth stages of the same parasite failed to induce significant protection against homologous challenge (Boisvenue *et al.*, 1991).

MULTICELLULAR PARASITE VACCINES: RECENT DEVELOPMENTS

The isolation of protective antigens in sufficient quantities for practical use in vaccine development has, until recently, been restricted by a lack of available native parasite material. The development of recombinant DNA techniques for the *in vitro* expression of foreign genes in eukaryotic and prokaryotic cells has provided an alternative strategy which has recently stimulated extensive research into antiparasite vaccines. While there are now many examples of expression systems for parasite proteins, there are only a few reports of recombinant parasite proteins which induce significant levels of protective immunity. The steps in recombinant vaccine development are illustrated by the following successful examples.

Immunization of sheep using T. colubriformis *tropomyosin recombinants.* As discussed earlier, the *T. colubriformis* muscle protein, tropomyosin, was shown to be a potentially useful protective antigen in the guinea pig when given in native form (O'Donnell *et al.*, 1989). Using oligonucleotide probes based on partial amino acid sequence data of tropomyosin, the gene encoding this protein was isolated from a cDNA expression library prepared from fourth larval-stage mRNA (Cobon *et al.*, 1989). A 27 kDa subunit of *T. colubriformis* tropomyosin, expressed as a β-galactosidase fusion protein in *Escherichia coli*, produced accelerated worm expulsion following challenge in guinea pigs. When the *T. colubriformis* tropomyosin DNA was used as a hybridization probe, the gene encoding a related antigen was isolated from mRNA prepared from adult *H. contortus*. The expressed gene product significantly protected immunized sheep against *H. contortus* challenge (Cobon *et al.*, 1989).

Immunization of sheep with recombinants of T. ovis *onchosphere ES antigens.* A similar approach has shown promise in experiments with *Cysticercus ovis*, the intermediate stage of *T. ovis*, in sheep. Previous experiments indicated that the ES products of hatched and activated *T. ovis* onchospheres contained potent host-protective antigens. Several of the ES components, ranging from 47 to 52 kDa in size, were found to be recognized strongly by sera from resistant sheep (Rickard and Bell, 1971a and 1971b; Rickard and Adolph, 1977) and lambs immunized with a polyacrylamide gel-purified preparation of these components were significantly (98 percent) protected against challenge (Johnson *et al.*, 1989). Rabbit antibodies, specific for the 47 to 52 kDa region, were eluted from Western blots and used to probe a cDNA expression library prepared from mRNA extracted from hatched and activated onchospheres (Johnson *et al.*, 1989). β-galactosidase fusion proteins, prepared from selected immunopositives, were found to be antigenic in sheep but

were not protective. Subsequently, one of the proteins was prepared as a fusion with *S. japonicum* glutathione S-transferase and was found to protect sheep significantly against challenge (Johnson *et al.*, 1989).

Recombinant proteins have also been assessed in other *Taenia* species. Three fusion proteins, derived from *T. taeniaeformis* onchosphere cDNA and expressed in a pGEX plasmid vector, gave a 95 percent reduction in total metacestode recoveries in Wistar rats (Ito *et al.*, 1991)

***Immunization of ruminants using recombinant forms of membrane gut proteins of* B. microplus**. As discussed previously, in infections in which immunity develops slowly or poorly, artificial immunization with hidden, or covert, antigens may be used to circumvent a lack of responsiveness. Recent developments using recombinant gut antigens from the blood-sucking tick *B. microplus* in cattle have been promising.

Cattle acquire limited levels of resistance following prolonged natural exposure to *B. microplus* (Wagland, 1975). Protective immunity has been achieved using antigens fractionated from gut membrane preparations of engorged female ticks (Willadsen, McKenna and Riding, 1988), and a membrane-bound glycoprotein (Bm86) expressed on the surface of tick gut digestive cells was shown to be a highly effective immunogen (Willadsen *et al.*, 1989). The gene coding for this antigen was subsequently isolated from a cDNA expression library prepared from adult *B. microplus* and a fusion comprising 599 amino acids of Bm86 and 651 amino acids of β-galactosidase was expressed in *E. coli* as inclusion bodies (Rand *et al.*, 1989). Ticks engorging on cattle vaccinated with these inclusion bodies were significantly damaged as a result of the immune response to the cloned antigen (Rand *et al.*, 1989).

The above examples have attracted considerable commercial support and are currently undergoing development.

PROBLEMS OF SUBUNIT VACCINE DEVELOPMENT

Laboratory models versus natural host/parasite systems. Potentially useful antigens are often selected on the basis of protection trials conducted in laboratory animal models. In general terms, laboratory animals expel the parasite more rapidly than does the natural host and infection is often terminated prior to patency. Protection observed in model systems is therefore often only an acceleration of an already efficient antiparasite response and does not necessarily reflect utility in the natural host. Caution must therefore be taken when extrapolating from the laboratory model to the definitive host.

Recombinants expressed in the wrong conformation. The protective properties of a recombinant protein will be influenced by the tertiary structure which can, in turn, be altered depending on the fusion partner. It also depends on whether or not the native protein is glycosylated. This cautionary note is exemplified by work on the protective onchosphere antigens of *T. ovis* in which protective immunity was only attained after expression of the recombinant molecule as a GST fusion protein (Johnson *et al.*, 1989).

Solubilization of the relevant antigens. The solvent used to extract parasite proteins can be crucial to the isolation of the appropriate protective antigens. For example, work performed on the cestode *Taenia pisiformis* indicated that sodium deoxycholate, but not PBS, extracts made from onchospheres of the parasite conferred high levels of protection to rabbits (Rajasekariah, Rickard and O'Donnell,

1985). Similarly, DOC-PAGE, but not SDS-PAGE, gel cut-outs of *T. taeniaeformis* onchosphere antigens protected mice against challenge (Lightowlers, Rickard and Mitchell, 1984). In comparison, sheep have been successfully immunized against challenge with *Echinococcus granulosus* using onchosphere antigens which had been solubilized in SDS (Dempster *et al.*, 1992) and against *T. ovis* using homogenized SDS-polyacrylamide gels containing homologous antigen (Harrison *et al.*, 1993). The variation produced by the different extraction buffers may be caused by the disruption of conformational epitopes or by the lack of solubilization of effective components.

Presence of non-peptide epitopes. B cell epitopes are often conformational and may not be peptide in nature. These epitopes will be difficult to reproduce using conventional molecular biology or peptide synthesis techniques. As the antigen binding site of an antibody molecule is complementary in physical structure to the antigen against which it is raised, the antigen binding site can, itself, be used as antigen to raise anti-idiotype antibodies which mimic the shape of the original antigen. For example, an anti-idiotype vaccine which mimics the glycan component of a 38 kDa surface component of *S. mansoni* stimulated protective immunity in rats against homologous challenge (Grzych *et al.*, 1985). This approach is very laborious, however, and would probably only be considered if the protective epitope was non-peptide or demonstrably conformational and could not be produced by the variety of recombinant DNA approaches now available. In addition to containing carbohydrate epitopes, glycosylation is also likely to affect the protective properties of proteins by modifying protein folding, so that recombinants will be more appropriately expressed in systems which glycosylate the recombinant protein such as mammalian cell lines and viral vectors.

Accessibility of antigen to the immune system. In order to have an effect on an invading parasite, host effector mechanisms must be directed against antigens which are accessible to this response. For example, *Haemonchus* spp. and *Boophilus* spp. ingest host blood so that antigens on the gut surface are bathed in blood components such as antibody. The extent to which this approach can be applied to non-bloodsucking parasites such as *Ostertagia* spp. and *Trichostrongylus* spp. remains to be defined. Furthermore, because covert antigens will not be exposed during subsequent natural infection it is presumed that booster vaccination would be required until natural immunity develops or live vaccine vectors may be used to enable persistence of the vaccine components within the host.

Genetic variation in the vaccinated population. Once a vaccine has been developed, it is essential that it is effective in most, if not all, of the target population. The major histocompatibility complex (MHC) and other, as yet undefined, background genes within a population influence the capability of individuals to respond immunologically to specific antigens. This in turn will establish the success or failure of any vaccine (Kennedy, 1990). For example, a variable responsiveness was encountered when synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum* were used to vaccinate mice and it was observed that both antibody and T cell responses to this antigen were H-2^b-restricted (Del Giudice *et al.*, 1986; Togna *et al.*, 1986). Thus, it can be anticipated that subunit vaccines which stimulate elements of the natural immune response to the parasite will have to comprise a variety of peptides to maintain

long-term field utility. Furthermore, the induction of natural immune responses will be profoundly influenced by the way the antigen(s) is/are presented to the immune system.

Assisting antigen presentation

The rapid developments in recombinant DNA technology and protein chemistry should provide antigens in quantity for experimental immunization studies. Individual molecules are likely to have reduced immunogenicity and will have to be presented to the host immune system in the context of an adjuvant or a live vaccine vector. Moreover, the method of antigen presentation can be optimized if the relevant effector arms of the immune response are defined.

Adjuvants

Adjuvants non-specifically stimulate immune responses to antigen. The understanding of how adjuvants work has increased rapidly and many of the adjuvants now available are recognized to stimulate different types of responses (see chapter by Bomford, Adjuvants in veterinary vaccines, p. 277, and Bomford, 1989).

Adjuvant selection, and hence vaccine efficacy, can be improved if the precise immunological mechanisms involved in parasite killing or expulsion are defined. For example, aluminium hydroxide preferentially stimulates T helper type 2 (Th_2) lymphocytes so that a humoral response, particularly IgG_1 and IgE, is favoured (Smith, 1992). In contrast, Freund's complete adjuvant, which contains bacterial components, is a strong promoter of cellular responses. Freund's incomplete adjuvant, which lacks the mycobacterial component, stimulates only a humoral response. Unfortunately, Freund's adjuvants have several adverse side-effects and are not acceptable for use in veterinary vaccines. However, because of their general efficacy, they continue to be used in experimental trials to establish antigen utility, after which less toxic adjuvant mixes may be attempted.

A novel adjuvant which is now licensed for use in domestic animals is that based on immunostimulating complexes (ISCOMs). These complexes are most readily formed with antigens which possess a hydrophobic transmembrane region (Bomford, 1989), suggesting that they may be particularly useful for the formulation of helminth vaccines based on integral membrane proteins. So far, there has been little work detailing the potential of helminth or ectoparasite subunit vaccines in the context of any novel adjuvant preparations.

Vectors and antigen delivery

The chronicity associated with many parasitic infections suggests that antigen may need to be administered over a prolonged period to stimulate protective immunity. Antigens administered orally with an avirulent virus or bacterial vector will have the potential to stimulate a vigorous cell-mediated and persistent immune response (Murray, 1989). These vectors also have the potential to stimulate IgA precursor B cells in gut-associated lymphoid tissue, a possibility for overcoming immune unresponsiveness at this site. Virus vectors, such as vaccinia and herpesviruses, have the advantage that the antigen(s) are processed in their native form (Murray, 1989). So far, vaccinia has been used successfully as a vector for a cloned 28 kDa surface antigen of *S. mansoni* (Simpson and Cioli, 1987). Enterobacteria, such as *E. coli* and *Salmonella* spp., have been used as vaccine vectors in non-parasite systems, although use of the latter may lead to public concern.

Vectors may be designed so that the antigens they encode are expressed in

combination with the appropriate recombinant cytokine(s) in order to stimulate the desired immune effector arm. For gastrointestinal nematodes, antigen and cytokine genes could be expressed in parallel in a bacterial or viral vector suitable for oral vaccine delivery.

Bacterial toxins also have considerable potential for the oral presentation of antigen. For example, cholera toxin has a high affinity for specific receptors on the surface of the intestinal epithelium and stimulates both secretory IgA and serum IgG antibody responses with prolonged immunological memory (McGhee *et al.*, 1992). These responses would be appropriate in vaccination against gastrointestinal nematodes, although the problems of toxicity may have to be overcome.

CONCLUSIONS

The optimism expressed when recombinant DNA technology was first developed perhaps overestimated the capabilities of these techniques and failed to recognize the complex nature of the host-parasite interaction and the difficulty in maintaining immunogenicity. A major problem has been the inability to identify the relevant antigens from complex preparations in the face of multiple immune responses, not all of which are protective. These responses still have to be unravelled for all multicellular parasitic infections. A substantial amount of parasitological research is now directed at defining the precise immune responses relevant to the elimination, or otherwise, of parasites. Although beyond the scope of this vaccine review, it appears that, of the many immune effector mechanisms involved in the host-parasite interaction, only some are relevant to the development of protective immunity. Nearly all the studies described in this review selected antigens on the basis of humoral immunity, and there has been little detail given regarding the essential cellular arm of the immune response. In terms of vaccine development, this reflects the route that research has taken in the past. More recently, detailed analyses of the cellular responses against helminths have identified responses which are protective or those which may be immunopathological or immunorepressive (Smith, 1992). By measuring mitogen- or antigen-specific *in vitro* propagation of immune cell lines, including T cell subsets, or measuring the types of cytokines released or expresssed by these cells, cellular responses can be dissected for each parasite infection. Parasite antigens also trigger mast cell degranulation with the associated release of mast cell proteinases, histamine and various cytokines. In several cases, these responses appear to be central to worm expulsion, and assays which detect antigen-specific mast cell proteinase release may be used to identify which antigens stimlate these cells (Jones, Huntley and Emery, 1992). Thus, it can be seen that basic parasite immunology should go hand in hand with vaccine research in order to define which kind of responses should be aimed for when identifying vaccine candidates.

As outlined above, delivery systems and adjuvants can now be selected on the basis that they can optimize or augment the required effector arm of the immune system. Nevertheless, by obtaining information on putative functions for specific parasite components or realizing their contribution to parasite survival, rational subunit vaccines may be designed without the need for a full understanding of the host parasite interaction.

Pioneering advances have been made in the last decade, most notably in the definition of candidate antigens for protection against *T. ovis* and *H. contortus* in sheep, but the identification of protective antigens and evidence for their efficacy in immunization trials is only the start of the

process. Field trials and the incorporation of the antigens into a commercially viable vaccine are expensive and time-consuming and will rely heavily on commitment from large pharmaceutical companies which must accept the vaccine as a potential marketable commodity. It should be noted that a vaccine which only reduces nematode egg output might reduce pasture contamination to a level where production losses are balanced by stock management savings. Thus, at least with veterinary vaccines, it may not be imperative to induce sterile immunity completely and, when considering the most appropriate vaccine development strategy, full account must be taken of the relative prevalence and epidemiology of the parasite.

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Poultry vaccines (1)

Conventional vaccines

J.B. McFerran

Vaccination of poultry presents a number of special problems. The first is the limited life of the meat-producing chicken, which is often as short as 35 days from hatching to slaughter. These birds have a low individual value and any vaccine must therefore be relatively cheap. They are kept in very large numbers at a very high density. Houses often contain 10 000 to 30 000 birds and sites often have between 50 000 and one million birds. The problem is compounded because these birds have probably originated from eggs produced by a number of breeding farms. These breeding farms may well be in a different region (or even country) and may have a different antibody status and disease history. Therefore, the chickens hatching from these eggs will have considerable variations in maternal antibodies and, in some cases, chicks will have been vertically infected with viruses such as chick anaemia virus (CAV) or avian encephalomyelitis. This problem is becoming more acute as hygiene and disease precautions are increased in the breeding farms, for example to control *Salmonella* spp. If all the breeding farms are infected with a virus such as CAV before they start producing eggs, it is not a problem. However, if some are not infected, then two problems can arise: the first is if the chicks are placed in an area where CAV is endemic, in which case the susceptible chicks originating from parents without antibody become infected; the second is when one of the parent flocks becomes infected. This flock will produce infected and diseased chicks and they will infect the susceptible chicks in the hatcheries.

Poultry are infected with a number of immunosuppressive viruses, of which the most important is infectious bursal disease (IBD) virus, which has virtually a world-wide distribution. These viruses have three important effects: they can cause disease in their own right; they can compromise the birds' immunological response to other vaccines; and they can reduce the birds' resistance to other organisms so that a severe reaction to vaccination with an attenuated vaccine can occur as a result of other agents combining with the vaccine to induce disease.

The disadvantages of large-scale poultry production can in part be overcome by having an "all-in all-out" system, where a site is completely depopulated and is properly cleaned and disinfected before the next intake of day-old chicks. It is relatively easy to clean and disinfect (e.g. by formalin fumigation) environmentally controlled houses with impervious walls and concrete floors but it is virtually impossible to disinfect open-sided houses with earth floors. In addition, commercial pressures often lead to bad microbiological practices. It is not uncommon for organizations which do depopulate and clean houses to have large sites where the last houses to be depopulated are still being cleaned when chicks are filling the first houses. Even worse, in some areas litter is reused many times and young chicks are brooded in houses still containing older birds.

Most commercial poultry organizations are highly integrated with considerable movement between farms. Thus, laying flocks will have regular egg collections, with transport moving from farm to farm;

breeders and broilers are often weighed regularly by the organization's advisory staff; there is movement and contact by catching and vaccination teams, etc. While a reasonable disinfection of staff can be carried out, it is more difficult to ensure that tools and equipment (e.g. weighing scales, electrician's tools, egg trolleys) are safe. These factors make the spread of disease relatively easy.

From a disease control viewpoint there are three main types of poultry: i) commercial flocks, usually kept under intensive husbandry methods; ii) local small backyard flocks, which in most areas are unvaccinated; and iii) hobby birds. The latter range from Psittacines (especially dangerous because they can be infected with virulent Newcastle disease virus) to racing pigeons. Wild birds also pose a problem as they are capable of carrying infection long distances and may indeed be hosts of certain viruses. For instance, wild ducks are often infected with paramyxoviruses, including Newcastle disease, myxoviruses and the egg drop syndrome, adenovirus.

Humans are probably one of the most important vectors for spreading infection, second only to the bird. It is possible to reduce the risk of introducing disease by keeping all but essential people out of poultry houses and by ensuring that those who enter take precautions (e.g. by using special outer clothing kept on the farm for each essential visitor).

It is unlikely that organisms will spread from hobby birds or backyard flocks by aerial routes because the number of birds involved is too small. Some viruses can spread from commercial farm to commercial farm by wind owing to the large concentration of birds (e.g. Newcastle disease and Marek's disease viruses). Other routes are also important, such as untreated water (e.g. spreading influenza, Newcastle disease, egg drop syndrome, salmonellosis), feed (e.g. paramyxovirus type 1 [pigeon Newcastle disease], *Salmonella* spp., IBD) and transport.

Given the above considerations, it can be seen that the chances of poultry disease spreading are very high. In addition, the poultry industry is truly international, with birds (and advisory staff) moving freely between countries. The speed of the spread of "new" diseases in recent years underlines this. Diseases such as runting-stunting, egg drop syndrome, turkey rhinotracheitis virus, virulent forms of Newcastle disease, Marek's disease and IBD as well as antigenic variants of infectious bronchitis (IB) have all spread rapidly around the world. The emergence of other new diseases such as chick anaemia, may be the result of changes in management rather than the spread of virus, but this is unclear at present.

VACCINATION

Clearly, vaccination is essential to commercial poultry production. However with the birds' short life, high-density conditions and variable antibody levels, it is very difficult to achieve full protection and it is essential that every effort be made to prevent the introduction of infection or carryover of infectious agents from flock to flock. To some extent, the degree of resistance induced by any vaccine is subject to the level of challenge present and, if that challenge is great enough, vaccines are unlikely to give protection.

The ideal vaccine should:

- be able to be given by mass application;
- be cheap;
- be safe;
- be capable of inducing a strong immunity even in the presence of maternal antibody;
- be capable of inducing both local and general immunity;
- induce an immune response that can be distinguished from that induced by

infection or maternal derived antibody;
- require only one dose to give lifelong immunity;
- be packaged in different sizes (e.g. while 1 000-dose vials are appropriate for use in commercial flocks, they are not appropriate for use in a small backyard flock).

Such a vaccine does not exist at present.

Successful vaccination against many organisms depends on stimulating both local immunity at a mucosal surface and general humoral antibody. The local immunity is of major importance for those agents which affect mucosal surfaces – for example IB – and is of special importance in meat-producing birds which are housed at very high densities, have a short life span and require quick immunity.

Serology

There are a wide range of tests used to monitor vaccination and also for diagnosis. The modern tests, for example ELISA tests, are expensive and it is important to use them appropriately and not just to accumulate data. They can be used to monitor the efficiency of vaccination where immunity (general) is measured by an antibody response. Titres should not only be high but, even more important, be in a close range. It is important to remember that if 10 percent of the birds have low titres or no antibody, this can be translated into 2 000 to 3 000 birds dying in a house. There is no easy method of measuring local immunity. This is unfortunate, since local immunity is often very important, as for example in IB. Other uses for serology are to determine if a flock has been infected. Thus, if birds in the rearing period develop antibody to CAV, there is no need to vaccinate. As already discussed, it is the flocks without antibody that pose the problems.

Determination of antibody titres in breeding flocks can allow the breeding flocks to be batched into groups so that the progeny will have similar titres and, consequently, the timing of vaccination can be better judged. This has been used in the control of IBD. The difficulty is that there is often a wide variation in antibody titres in birds in any one flock.

Vaccine administration

There are a number of commonly used methods of vaccination. Meat-producing chickens are normally handled twice – at birth and at death. Therefore, vaccination is either done individually in the hatchery (e.g. Marek's vaccination) or else mass application methods are used. The easiest method is to give the vaccine in the drinking-water, yet this is most difficult to do properly. There is always a percentage of birds that will not drink. Although this percentage can be reduced by ensuring the birds are thirsty, they must not be stressed and the water must be turned on immediately afterwards, with no danger of airlocks occurring. Furthermore, the vaccine may not be uniformly available in the drinking-water because of physical problems such as uneven troughs, or vaccine may be lost as a result of adherence to rusty containers or feed which has fallen into the drinkers. Vaccine can also be inactivated by chemicals (e.g. chlorine) in the water or by heat. Such problems can in part be overcome by adding protein (e.g. dried skim milk powder) to the vaccine and by giving the vaccine in two parts (McFerran *et al.*, 1972). It is also essential to keep the birds at a comfortable air temperature. This may require raising the temperature in cold areas or vaccinating early in the morning in hot regions.

Partly to overcome the problems of water administration and partly to induce local immunity, spraying has been widely used. The use of a coarse spray (in reality a mass eye and nose drop application) to immunize day-old chicks which possess

maternally derived antibody against diseases such as IB, has been routine in hatcheries in some areas for 25 years. This has given excellent immunity, lasting the life of the meat-producing chicken. Even in the case of emergency vaccinations of antibody-free chicks with Newcastle disease virus, excellent immunity was provided with acceptable side-effects (McFerran, 1992). Severe side-effects did occur when the spray was too fine and virus penetrated the lower respiratory tract. Results similar to those given by coarse spray have been obtained using the labour-intensive method of beak dipping.

Aerosols, generated using an electrically powered sprayer, or a fine spray from a hand operated pump have been used to revaccinate birds. In some instances, this has been done every 12 weeks to ensure that virtual continuous local immunity is maintained at mucosal surfaces.

One constraint on the use of spray vaccines in some areas is that other micro-organisms infecting the birds, such as *Mycoplasma* spp. and *Haemophilius* spp., can produce severe side-effects. Therefore, in certain organizations or regions it is inadvisable to use spray vaccination.

In theory, the large cleft in the oral palates of fowl and turkeys should allow orally administered vaccine to come in contact with the upper respiratory tract and achieve the same results as spray vaccination. However, in practice, spray vaccination is much more effective in stimulating local immunity in the respiratory tract as well as being less labour-intensive. In part, the increased immunity following spray vaccination may be the result of stimulation of the Hardarian gland by the spray-applied vaccine.

Injection (including wing-web stabbing) is still widely used. Virtually all egg-producing birds and many meat-producing chicks are vaccinated at one day of age with Marek's vaccine. This is a quick and cheap method of application. Breakdowns occur when the vaccine is deposited in the feathers rather than under the skin in a quest for speed and when a blocked needle or bacterial contamination of the apparatus goes unnoticed.

Inactivated vaccines, often with an oil adjuvant, are widely used. They tend to be expensive to buy and deliver but have been very successful in controlling disease in adults. While they are of value in protecting chicks against disease through maternally derived antibody, recent experience has shown (as in the case of IBD) that virulent strains can break through the maternal antibody protection and active immunity must therefore be induced.

SOME CONVENTIONAL VACCINES

There are a wide variety of conventional vaccines available. Brief descriptions of some are given here, concentrating on unusual aspects or problems and potential future developments.

Marek's disease

Marek's disease has been controlled by using type 1 attenuated vaccines (either artificially attenuated or naturally occurring strains) or the type 3 turkey herpesvirus (THV). In some areas, a type 2 naturally occurring attenuated strain was necessary. In some genetic lines, if the birds are infected with both the serotype 2 and the lymphoid leukosis virus early in life, there is an enhancement of lymphoid leukosis. In the 1980s, very virulent strains of Marek's disease arose, and control was achieved using a mixture of vaccine – either types 2 and 3 or types 1 and 3. There appears to be a true synergism between different strains, especially between serotypes 2 and 3, and polyvalent vaccines have been found to provide better

protection against tumour development (Witter *et al.*, 1984). However even these vaccines have not been totally successful and preliminary results suggest that, in order to ensure maximum resistance to Marek's disease, it may be necessary to select a vaccine appropriate for the predominant B-haplotype of the chicken flock (Bacon and Witter, 1993).

Infectious bronchitis

The envelope of IB virus possesses surface projections comprising two glycopolypeptides, S_1 and S_2. While both are important in the immune response, it appears that the S_1 glycopolypeptide is the major antigenic component.

Protection is given against the homologous serotype as well as, to a varying degree, against other serotypes. This has resulted in the use of a number of different attenuated vaccines in some areas. Recombination among field and vaccine strains possibly occurs and is responsible for producing new "variant" strains (Kusters *et al.*, 1990). Because of the possibility of recombination and the fact that attenuated strains can revert back to virulence, a new vaccine serotype should not be introduced into an area until there is clear evidence that its use that is essential.

There have been two approaches to immunizing egg-producing birds. The first is to prime the birds with an attenuated vaccine (around four weeks when maternal antibody levels have fallen) and then with an inactivated oil-adjuvanted vaccine or a less attenuated vaccine around 18 weeks to produce high levels of circulating humoral antibody. The second is to prime the birds and then commence spray vaccination at 18 weeks, followed thereafter every eight to ten weeks with an attenuated vaccine. The aim here is to keep a high degree of local immunity in the gut and respiratory tract.

Infectious bursal disease

Both attenuated and inactivated vaccines exist fro IBD. Attenuated vaccines have been divided into three broad categories based on virulence: i) virulent or "hot"; ii) intermediate; and iii) "mild" or avirulent. There is some discussion as to whether some of the "hot" vaccines are any more virulent than the intermediate ones. The suggestion is that some of the "hot" strains spread better between birds and can therefore infect birds that were not initially vaccinated either because they did not drink or because they have high maternal antibody levels. It has been suggested that virulent viruses can overcome virus-neutralizing antibody titres of 1.500, intermediate vaccines can overcome titres of 1.250 and avirulent viruses can overcome titres of 1.100 or less (Lukert and Saif, 1991). However, given the wide variation of titres in broiler chickens, these figures are at best only a guide as to the optimum time to vaccinate.

In view of the wide antigenic variation within IBD viruses (McFerran *et al.*, 1980) it is surprising that only one subtype vaccine (the Delaware) has been necessary. There has been a major upsurge in virulence of IBD viruses throughout the world, and the policy adapted in many areas of only vaccinating the breeders and relying on maternal antibody to protect the broilers has proved flawed. In many areas, it has been necessary to use intermediate or hot vaccines in broiler flocks to control the losses. Even when flocks are clinically normal, IBD virus can attack the bursa and cause lesions. In these circumstances, the subclinical infections can cause up to a 10 percent reduction in income (McIlroy, Goodall and McCracken, 1989).

Mycoplasma gallisepticum

Although *Mycoplasma gallisepticum* has been eradicated from commercial poultry

in many areas, it is still widely distributed, especially in areas where multi-age sites and open-sided houses with earth floors make eradication very difficult. Under these conditions, vaccination of commercial egg-producing birds to control falls in egg production is indicated. Bacterins, which are inactivated suspensions of whole *M. gallisepticum* (Mg^1) organisms in oil emulsion, are apparently effective in controlling disease but have little benefit in eliminating infection in multi-age sites. Recently, inactivated *M. gallisepticum* bacterin mixed with 0.2 percent iota carrageenan as an adjuvant induced resistance to air sacculites in chicken challenged with the virulent MGR strain (Elfaki *et al.*, 1992).

Live vaccines are also used to control *M. gallisepticum*. The F strain is a naturally occurring strain of relatively low virulence for chickens but of a higher virulence for turkeys. It will displace more virulent field strains from multi-aged sites but vaccinated birds become permanent carriers (Kleven, Khan and Yamamoto, 1990). A temperature-sensitive mutant which is avirulent has recently been described (Whithear *et al.*, 1990).

Fowl cholera

Fowl cholera is caused by *Pasteurella multocida*, which can be serologically subdivided by differences in capsular antigens and cell wall lipopolysaccharide antigens. Using a passive haemagglutinin test, five capsular serogroups (A,B,D,E and F) can be identified; however, these capsular antigens do not appear to play a role in protection. *P. multocida* can also be subdivided into 16 serotypes using a heat-stable somatic antigen (Brogdens and Rebers, 1978). Both inactivated and live *P. multocida* vaccines are available. Inactivated vaccines are whole cell products of a number of strains (often including "autogenous" local strains) emulsified in an oil adjuvant. The attenuated strains have a range of virulence, although two doses of inactivated vaccine during rearing usually provide adequate immunity as long as the appropriate strains are included in the vaccine. Attenuated vaccines are widely used in some areas, especially in meat turkeys. Live vaccines often cause chronic fowl cholera. This effect can be minimized by first giving an inactivated vaccine.

Infectious coryza

Infectious coryza is caused by *Haemophilus paragallinarum*, the isolates of which it is possible to divide into three serogroups with at least seven serovars by using the HI test (Kume *et al.*, 1983). Vaccines consist of inactivated whole-cell bacterins emulsified in oil adjuvant or absorbed on to aluminium hydroxide. The immunity induced by these vaccines is serogroup-specific. Breeders and commercial layers are routinely vaccinated in many countries, using two injections during rearing at least four weeks apart. Using chemical mutogens, Blackall *et al.*, (1993) have produced non-pathogenic mutants which may be of value as attenuated vaccines.

Coccidiosis

A widespread and serious disease caused by *Eimeria* spp. is coccidiosis. It has been controlled in the past by the continuous administration of chemotherapeutic agents to the flock's feed. However, problems with the development of resistance and the need to withdraw the agents for a specified period before slaughter have led to the development of vaccines.

At present, vaccines are used mainly in layers and breeders. They contain attenuated oocysts of multiple species of *Eimeria*. Attenuation has been achieved by a number of methods such as adaptation to growth in avian embryos or by selecting strains with a shortened prepatent period in the precocious strains.

A second approach has been to define coccidial immunogens which could be used as subunit vaccines. There are, however, many species of *Eimeria,* with little or no cross-immunity between species.

NEW TECHNICAL DEVELOPMENTS

In 1982, Sharma and Burmester inoculated embryos with THV in an attempt to circumvent the massive Marek's disease challenge that day-old chicks faced on some sites. These chickens developed a persistent THV infection and were resistant to early challenge with pathogenic Marek's disease. The vaccine was inoculated into the allantoic sac; and it was found that the best results were obtained in 17- to 18-day-old embryos. Inoculation at 18 days would coincide with the transfer of eggs from the setting to the hatching machines.

Sharma (1985) extended this work to the investigation of protection against IBD. He found that low virulent vaccine strains could induce immunity without effecting hatchability or survival of the chicks. However, maternal antibody did neutralize strains of low virulence. Recently, the use of an IBD commercial vaccine-antiserum mixture to inoculate 18-day-old embryonated eggs has been described. This preparation induced immunity in chicks produced from eggs with maternal antibody to IBD (Whitfill *et al.*, 1993). Embryos originating from specific pathogen-free (SPF) turkeys were successfully inoculated with marble spleen disease and Newcastle disease vaccines and the poults were resistant to subsequent challenge (Ahmad and Sharma, 1993).

This work has been developed commercially and an automatic egg injection system is now in operation (Marchant, 1993), injecting THV and SB-1 Marek's vaccine into embryonated chick eggs. The machine can inject 20 000 to 30 000 eggs per hour. The success of these approaches will depend on whether the economic advantages claimed are realized. This is especially true where hygiene standards are high and day-old chicks do not face a massive challenge.

One different approach to embryonated egg inoculation is to inject an immunostimulant containing lymphokines. It is claimed that this has enhanced the efficiency of Marek's disease vaccination and, in addition, resulted in lower mortality, reduced feed conversion and lower production costs for commercial broilers (Miles *et al.*, 1993).

Genetic engineering

Initially, organisms were modified by egg passage (e.g. IB), by cell culture passage or by identifying a naturally occurring non-pathogenic strain (e.g. Rispen's vaccine against Marek's disease). The attenuation process can be enhanced by using chemical mutagens, and mutants can be selected using a range of techniques such as plaque purification, temperature sensitivity, colony size or an appropriate monoclonal antibody. Some proteins are only expressed when bacterial cells are grown *in vivo,* and it is suggested that this is the reason why *Pasteurella multocida* grown *in vivo* gives better vaccines. An attempt to clone the genes responsible for expressing these proteins is in progress and may give rise to better inactivated or attenuated vaccines (Jost *et al.*, 1993). It is now possible to modify the genome by chemical means, as in the Aujeszky's disease vaccine virus in which the thymidine kinase gene has been deleted.

It is possible to insert genes into vectors. These vectors can be organisms which do not replicate in the host, in which case an adjuvant is indicated. Thus, a recombinant baculovirus (expressing the haemagglutinin and neuraminidase protein of Newcastle disease virus), combined with an oil adjuvant, is effective in protecting

against Newcastle disease challenge (Nagy *et al.*, 1991).

The alternative approach is to incorporate one or more genes into a vector which does replicate in the host. Avian poxviruses, avian herpesviruses (serotype 2 and turkey herpesvirus) and avian adenoviruses have been investigated. An example is the incorporation of the gene coding for the haemagglutinin of avian influenza (H5N2) into a poxvirus (Beard, Schnitzlein and Tripathy, 1991). The gene coding for the fusion protein (F) of Newcastle disease virus has also been incorporated into poxvirus (Iritani *et al.*, 1991). This gives immunity but does not give rise to H1 antibodies, thus allowing a test to detect infection by the Newcastle disease virus. Antibody to Newcastle disease did not prevent the development of immunity, but antibody to fowl pox prevented the poxvirus growing. Thus, this vaccine could be used to vaccinate ducks with maternal antibody to Newcastle disease. Similarly, a fowl poxvirus expressing the turkey rhinotracheitis fusion glycoprotein gave partial protection to challenge by turkey rhinotracheitis virus (Quinzhong *et al.*, 1993).

A promising approach is to use a turkey herpesvirus (THV) as the vector. A recombinant THV expressing the Newcastle disease virus fusion protein gave good protection against both Marek's and Newcastle diseases (Morgan *et al.*, 1992). THV is used widely to vaccinate day-old chicks. It is universal and safe (unlike fowl pox which is absent in some areas and which may cause local reactions), it does not spread horizontally and, most important, it gives a persistent viraemia lasting several weeks. This approach may well overcome the problems of maternal immunity. Thus, if genes giving resistance to a number of diseases were incorporated, they could stimulate the immune response to each agent at the appropriate time as maternal antibody wanes. This would overcome the hurdle of varying levels of antibody to different viruses and also to the same virus, owing to the fact that the flock is made up of the progeny of a number of breeding flocks.

There is always the problem of the maternal antibody to the vector preventing the vector growing. In the case of THV this could be overcome by using the THV in alternate generations (it does not occur as a natural infection in chickens), as is already the practice.

While it is possible to express the genes encoding protein antigens *in vitro* for use as subunit vaccines, and also to synthesize peptide vaccines chemically, these solutions may present problems regarding the cost of production and administration of these vaccines, difficulties in obtaining the correct folding of the protein and the limited number of antigenic determinants, which could mean that strains of agents could arise which are not neutralized.

Although, in theory, anti-idiotype vaccines are possible, in practice they have been poorly immunogenic and show little immediate promise. A different approach is the use of cytokines as adjuvants. These can be incorporated in the injectible vaccine which may also include slow-release mechanisms and immunostimulating complexes (ISCOMs). Such an approach has been taken with a subunit vaccine against avian influenza in which nucleoprotein with residual haemagglutinin activity was incorporated into ISCOMs (Sivanandan *et al.*, 1993).

Liposome-adjuvanted egg drop syndrome 1976 vaccines appeared as efficacious as oil-emulsion vaccines without the side-effects of the oil (Jing-Sheng and Yi-Zhu, 1993).

Using trivalent avian influenza antigens, Fatunmbi *et al.* (1992) found that charged liposomal avridine adjuvant produced a better antibody response than other uncharged liposomal avridine or oil-

emulsion adjuvants. The positively charged liposomal avridine adjuvant was superior to negatively charged adjuvant.

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Poultry vaccines (2)

Special requirements for village chickens

R.P. Spradbrow

The poultry industry in developing countries, especially the village poultry industry, encounters different problems to those found in the developed countries and, consequently, the requirements for vaccine by this segment of the market are very different. Certainly, there are elements of the international commercial industry with poultry enterprises close to the larger cities in many developing countries, and these enterprises have access to international breeding stock and international expertise as well as the opportunity and funding to draw vaccines of high quality from the international market. The vaccines used on these poultry populations in a developing country will seldom have been produced in that country. Instead, they will have been produced to meet the highest standards – as discussed elsewhere in this manual – and the manufacturers will have ensured as far as possible the absolute safety and adequate efficacy of the vaccines.

Village chickens comprise the major part of the poultry industry in many developing countries. Village flocks are small, of mixed age and poorly housed or even unhoused. The chickens gain much of their nourishment by scavenging in the village environment and by consuming supplements of household scraps if these are made available. The village flocks are poorly productive, as the major call on energy is for reproduction and maintenance of the population. Extremely high mortality rates ensure that few surplus eggs and birds become available for sale, barter or consumption. Nearly all the eggs are required for brooding. Two elements responsible for these extreme mortalities have been cited. First is the high wastage that occurs during the brooding season, which is probably caused by a combination of starvation, predation and infectious disease but is indirectly the result of poor husbandry and neglect. The second cause of attrition is more directly caused by infectious disease. Outbreaks of disease causing extremely high rates of mortality in village chickens have been reported from many developing countries. Where these diseases have been identified, the major culprits are Newcastle disease and fowl cholera, which are preventable and readily controlled if effective vaccines can be applied. It is the lack of suitable vaccines and appropriate methods of application that allows Newcastle disease and fowl cholera to plunder the village flocks. If these diseases could be controlled, villagers would have the incentive to make the changes in husbandry that would control brooding losses.

Suitable vaccines are the key to a "new science" of village chicken keeping. An analysis of the special requirements of village chickens indicates that their needs are not being served by the conventional vaccines available on the international market. A start has been made on developing vaccines against Newcastle disease that are specifically suited for delivery to and use in villages, (Copland, 1987; Spradbrow, 1992 and 1993/94). However, it is important that regulatory authorities recognizse the special needs of village chickens and that well-intentioned regu-

lations do not impose further barriers to the provision of suitable vaccines to village chickens.

Village flocks require special vaccines. The transport of vaccines to villages and their storage within these villages must usually be accomplished without refrigeration. It will still not be feasible in the forseeable future to establish adequate cold chains; therefore, the vaccines themselves must be thermostable. As long as villagers think that their flocks will survive the frequent outbreaks of infectious disease to which they are exposed, adequate housing will remain a rarity. This means catching chickens for individual vaccination will often be impossible. There seems to be no alternative to the oral application of vaccine, usually on food but also in water, in areas where the sources of drinking water can be controlled. When possible, individual applications of vaccine to the eye, nose or mouth is a more reliable method of inducing immunity with attenuated vaccines. Vaccines for use in village chickens also need to be extremely cheap. It may be necessary to reconsider some of the elements that add value to conventional vaccines – formulation, packaging, substrate, testing. Nor is it necessary to demand an extremely high degree of efficacy of a vaccine for use in village chickens. Absolute protection may require unrealistically large amounts of vaccine. The cost-effective compromise may be a moderate level of protection achieved with an inexpensive vaccine. In few places have conventional vaccines contributed to the protection of village chickens.

Many developing countries would prefer to produce their own veterinary vaccines for local use, especially the relatively simple avian vaccines that can be made in embryonated eggs. There are several reasons for this. One is the desire for national self-reliance and the belief that self-sufficiency in avian vaccines can eventually lead to independence in other areas of vaccine production. The overriding imperative is the need to conserve foreign exchange.

The purchase and transport of imported vaccines is a continuing drain on funds. On the negative side, a wasteful regional overcapacity for vaccine production can be reached, while the purchase of vaccine-producing equipment of any sophistication is also an expensive undertaking. Where vaccine is produced nationally, consideration should be given to establishing independent regional centres for testing.

Many of the standards developed for the testing of conventional avian vaccines are not appropriate for the testing of vaccines for village chickens. Tests for safety should be undertaken in local chickens and tests for efficacy should also be made locally, using indigenous chickens and challenge organisms of local origin. It should be recognized that safety and freedom from nominated adventitious agents are not synonomous. The first factor is essential, the second may be a luxury that can be postponed for vaccines for village use.

Several procedures that are used in the production of conventional vaccines might be questioned when vaccines for village chickens are being produced:

Is specific pathogen-free substrate required? Genuine specific pathogen-free flocks are not readily established in developing countries. The costs of initiation, maintenance and testing are enormous and the advantages for village-style vaccines are minimal. Some of the flocks claimed to be specific pathogen-free do not deserve that designation. Some receive live vaccines and the disease status of some is monitored only by the absence of clinical disease. Many are more realistically described as isolated flocks or minimal disease flocks. Vaccine seeds should be specific pathogen-

free and production of vaccine should use a seed lot system. This will allow an eventual increase in the standards of vaccine. However, rural poultry should not at this point be deprived of vaccines because of an absence of specific pathogen-free flocks.

Is freeze-drying necessary? Most conventional attenuated vaccines produced for use in commercial poultry are lyophilized. Compared with liquid vaccines, the lyophilized product has greater stability and is more convenient to store and transport. However, freeze-drying and suitable packaging of the dried product add to the cost of a vaccine. In some developing countries, avian vaccines are produced as "wet" vaccines. This may not be a disadvantage if vaccine is to be used soon after manufacture and if local production reduces the need to transport vaccines over long distances. Nevertheless, thermostability becomes an important character of the vaccine strain.

The sophisticated packaging of conventional vaccines is not necessary for products to be used in village chickens. There are frequent calls for the production of small-dose packs because village flocks are small and the supply of vaccine in packages that contain many doses is seen to be wasteful. The conventional 1 000-dose vial is admittedly an embarrassment for the owner of a flock of 20 chickens and there is an obvious need to share vaccine among village flocks. There is also a need to develop a philosophy of overdosing, rather than discarding unused vaccine. Lentogenic Newcastle disease vaccines are not harmful to chickens when given in doses larger than those recommended by the manufacturer. The problem in villages is not the 1 000-dose content of a single vial of vaccine, rather it is that of obtaining a single vial of vaccine in systems that are designed for bulk distribution to a commercial industry.

The logistics of delivering vaccine to villages is as important a problem as developing a suitable vaccine strain. The centralized preparation of food-based vaccines and their delivery to villages are not feasible in most countries. Each chicken requires 7 to 10 g of vaccine-coated food. In most areas, food and vaccine will need to be mixed in the villages, which will

TABLE 11

Comparison of poultry vaccine requirements for commercial and village use

	Commercial chickens	Village chickens
Place of production	Few large laboratories	Small local laboratories
Thermostability	Not essential	Very desirable
Market	International	Local
Seed material	Specific pathogen-free	Specific pathogen-free
Vaccine	Specific pathogen-free	Not necessarily specific pathogen-free
Lyophilization	Desirable	Not necessary
Efficacy	Extreme efficacy reqired	Moderate efficacy acceptable
Delivery	Individual vaccination or by aerosol, spray or drinking water	Delivery on food advantageous
Packaging units	Large dose (multidose vials)	Small-dose vials preferable

require the regular delivery of small quantities of vaccine. Recent experiments indicate that it may be possible to incorporate in a single pellet a quantity of Newcastle disease virus vaccine sufficient for a single chicken. This would help overcome the problems of central production and would ease the problem of transport.

Table 11 indicates some of the requirements that should be considered when comparing conventional vaccines and vaccines for village chickens.

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Fish vaccines

A. Adams, K.D. Thompson and R.J. Roberts

With fish being the primary source of animal protein in many countries, aquaculture is growing rapidly worldwide, and stress and diseases that accompany intensive fish culture have led to treatment with antibiotics and chemicals. However, as concern over pollution associated with chemical treatments and the emergence of multiple resistance to antibiotics make the control of infections more and more difficult, the emphasis should be on disease prevention by means of optimal husbandry and biological control methods, such as vaccination and immunostimulants.

FISH DISEASES

Fish in culture suffer a variety of diseases, including bacterial, viral, parasitic and fungal infections, some of which are shown in Table 12. The majority of infections described for fish in culture are bacterial. Vibriosis, associated with a number of marine vibrios, is the most widespread worldwide and not only causes huge economic losses to the marine fish culture industry but is the main disease affecting the farming of prawns in Southeast Asia and Japan. Furunculosis, hitherto thought of as a freshwater disease of wild salmonids, is the most serious disease affecting cultured salmonids, particularly in the seawater phase. Acute furunculosis is a common problem when Atlantic salmon smolts are transferred to the sea, at which time losses of up to 30 percent of the stock may occur. The disease is often complicated by simultaneous sea lice infection and pancreatic disease. Unfortunately, once furunculosis is introduced to a site it is very difficult to eradicate and is likely to recur intermittently among the ongrowing stock. Motile aeromonads appear to be the most important bacterial pathogens of freshwater fish in tropical countries. These micro-organisms have been reported to cause mass mortality in Indian major carp and can be detected during a variety of infections, including haemorrhagic septicaemia, asymptomatic septicaemia, epizootic ulcerative disease (EUS), tail rot and fin rot.

The exact aetiology of some of the newly emerging diseases, such as EUS, have not been unequivocally determined. In the case of EUS, bacterial, viral and fungal participants are all associated with the disease complex but the principal initiating factor has yet to be determined (Roberts, Willoughby and Chinabut, 1993).

As the aquaculture industry grows and new species of fish are intensively cultured, the range of diseases and affected species is similarly expanding. For example, pasteurellosis was only recently described in Europe and rickettsiosis in Chile, Ireland and Taiwan Province of China, but now both are recognized as highly significant pathogens.

COMMERCIAL VACCINES

Of the many infectious diseases affecting fish there are to date only five for which effective commercial vaccines have been produced. Three of these are bacterial

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TABLE 12
Fish diseases

Disease	Causative agent	Major fish species affected	Country / region
Bacterial disease			
Enteric red mouth	*Yersinia ruckeri*	• Salmonids, primarily rainbow trout	North America, Europe, South America
Vibriosis			
- Vibriosis	*Vibrio angulllarum, V. ordalii*	• Widespread in marine fish salmonids	Worldwide, Japan, North America
- Hitra disease	*V. salmonicida*	• Atlantic salmon	Norway, Faroe Islands
Furunculosis	*Aeromonas salmonicida*	• Salmonids	Europe
Bacterial kidney disease	*Renlbacterium salmoninarum*	• Salmonids	North America, Europe, Japan, Chile
Enteric septicaemia	*Edwardsiella ictaluri*	• Catfish	Southeastern United States, Canada
	E. tarda	• Eel, hirame	Japan
Motile aeromonad septicaemia	*Aeromonas hydrophila, A. caviae, A. sobria*	• Catfish, cyprinids, salmonids	Asia, Europe, United States
Pasteurellosis	*Pasteurella piscicida*	• Ayu, yellow tail, sea bream, sea bass, carp	United States, Japan, Europe, Taiwan Province of China
Bacterial cold-water disease	*Lyto psychrophilus*	• Salmonids	United States, Europe, Japan
Streptococcus infections	*Streptococcus* spp.	• Yellow tail, rainbow trout, ayu, tilapia, bass, bream	Japan, United States, Taiwan Province of China
Tuberculosis	*Mycobacterium marinum, M. fortuitum, M. chelonae*	• Snakehead, tropical aquarium fish, sea bass, wide variety of other species	Southeast Asia, Japan, Europe
Nocardiosis	*Nocardia asteroides, N. kampach*	• Tropical aquarium fish, yellow tail, rainbow trout and brook trout	Spain, Japan, Canada
Salmonid rickettsial septicaemia	*Piscirickettsia salmonis*	• Salmonids	Chile, Taiwan Province of China, Ireland
Epitheliocystis	*Chlamydia*-like organisms	• Wide variety of species	North America, Southeast Asia, Europe, South Africa
Clostridial infections	*Clostridium botulinum*	• Salmonids	Europe, United States
Columnaris disease	*Flexibacter columnaris, F. maritimus*	• All freshwater species, bream, bass, turbot, salmon	North America, Asia, Europe, Japan
Entrococcus infection	*Entrococcus serrolcida*	• Yellow tail	Japan
Bacterial gill disease	*Cytophaga* spp., *Flexibacter* spp. *Flavobacterium bronchiophilia*	• Wide variety of species	North America, Japan, Europe
Ulcerative septicaemia	*Pseudomonas* sp.	• Eels and others	Japan

(cont.)

TABLE 12 (continued)

Disease	Causative agent	Major fish species affected	Country / region
Viral diseases			
Infectious pancreatic necrosis	Birnavirus (ds RNA)	• Salmonids, sea bass, sea bream, turbot, Pacific cod	Europe
Viral haemorrhagic septicaemia	Rhabdovirus	• Salmonids	Japan, North America, Europe
Infectious haemorrhagic necrosis	Rhabdovirus	• Snakehead, carp, barbs	Japan, Taiwan Province of China, Canada, North America
Infectious salmon anaemia	Unknown (putatative virus)	• Atlantic salmon	Norway
Parasitic diseases			
Sea lice	*Lepeophtheirus salmonis*	• Marine-cultured salmonids	Northern circumpolar (Norway, Japan, Scotland, Ireland, Canada)
Proliferative kidney disease	Unidentified myxosporean extrasporgonic stage, PKX	• Freshwater salmonids	United Kingdom, Europe, United States
Costiasis	*Ichthyobodo necator*	• Freshwater, non-host-specific fingerling fish especially affected. Also a saltwater form	Worldwide, 2°-30°C
White spot	*Ichthyophthirius multifiliis*	• Freshwater, especially young fish; e.g. cyprinids, tilapiids, salmonids, ictalurids	Worldwide, 4°-25°C
Trichodinids	*Trichodina* sp., *Tripartiella* sp. and others	• Freshwater and marine non-specific salmonids flatfish (e.g. turbot) in culture	Worldwide
Myxosporeans	A range of pathogenic species, e.g. *Myxobolus* spp., *Sphaerospora* spp., *Kudoa* spp.	• Freshwater and marine. All cultured fish	Worldwide
Microsporeans	*Pleistophora* sp., *Glugea* sp. and others	• Freshwater and marine. One reported problem in flatfish	–
Fungal diseases			
Ichthyophoniasis	*Ichthyophonus* spp.	• Freshwater and marine species	Worldwide
Branchiomycosis	*Branchiomyces sanguinis, B. demigran*	• Cyprinids, eels, freshwater tench, stickleback	India, Japan, Eastern Europe
Saprolegniasis	*Saprolegnia parasitica* – diclina complex	• Cold, freshwater salmonids, catfish	Northern Europe, United States
Aspergillomycosis	*Aspergillus* spp.	• Tilapia	Worldwide
Epizootic ulcerative syndrome	Unknown (putative fungus)	• Freshwater and brackish species	Australia, Southeast Asia

diseases affecting salmonids – enteric red mouth (ERM), vibriosis and furunculosis – while the others are a bacterial disease in catfish – enteric septicaemia of catfish (ESC) and the viral infection, spring viraemia of carp (SVC), as shown in Table 13.

Fish vaccines first became available in 1976 when a commercial ERM vaccine was developed and registered in the United States (Tebbit, Erikson and Vande Water, 1981). By 1982, Busch stated that vaccination was the single most effective control for ERM. The disease is closely stress-related and the causative agent, *Yersinia ruckeri*, is an important primary pathogen for intensively cultured rainbow trout (Busch, 1982).

A commercial *Vibrio* vaccine was developed in 1980. This simple, killed whole-cell vibrio vaccine is highly effective against the marine pathogens *Vibrio anguillarum* and *Vibrio ordalii* in salmonids (Fryer, Rehovec and Garrison, 1978). The protective heat-stable lipopolysaccharide (LPS) antigens, derived from cell walls, which make the *Vibrio* vaccine so successful in salmonids have also proved effective for other farmed species, for example cod, eel, ayu (Kawai, Kusuda and Itami, 1981) with little or no modification to the vaccine's original design. More recently, with the upsurge in farming of sea bass and bream in the Mediterranean, the control of vibriosis has become important in these species too. Vaccines containing additional serotypes such as serotype III appear to be necessary for full protection (Vigneulle *et al.*, 1993). Generally, vibriosis in fish accompanies some other stress or physical trauma but some strains of *V. anguillarum* or *V. salmonicida* appear to be highly infectious primary pathogens. In Norway, where *V. salmonicida* causes Hitra disease or cold-water vibriosis, a combined vaccine is available because there is no cross-protection between the pathogens. As shown in Table 13, a triple vaccine is also available against furunculosis and the two *Vibrio* species.

Since the initial work of Duff (1942), many years of research have been dedicated to the development of a vaccine against furunculosis, the most economically important disease affecting salmonids. *Aeromonas salmonicida* is the causative agent and outbreaks of the disease are stress-associated, with high mortalities among salmon. Until recently, only limited success had been achieved with vaccination. In contrast to the early simple, killed whole-cell vaccines, the organisms for the new generation of furunculosis vaccines are cultured under iron-limited conditions which closely mimic the situation found in fish. This allows expression of the essential protective antigens, which include LPS and IROMPS (iron regulatory proteins) (Hirst and Ellis, 1994). The use of adjuvants, which enhance the immune response to the vaccine, also play an important role in the success of these vaccines while adjuvants do not appear to be necessary for the LPS-based ERM and *Vibrio* vaccines.

ESC is the major cause of mortality in farmed catfish in the southern United States and Asia. It was first identified in 1976 and is caused by the bacterium *Edwardsiella ictaluri*. The commercial vaccine available for ESC is based on a bacterin preparation and involves a two-step vaccination programme. Catfish fry (eight to ten days old) are immersed in the vaccine prior to ponding. They are then given an oral boost of vaccine 30 days before the start of the ESC season. The vaccination of fish by this method has proved effective in controlling ESC outbreaks.

The one commercial viral vaccine for SVC has been available in the Czech Republic and Slovenia since 1981 and also includes an oil-based adjuvant for administration by injection. SVC is caused

TABLE 13
Commercially available fish vaccines

Disease	Manufacturer	Country of origin	Product name/method of administration
Furunculosis	Aqua health	United Kingdom	Furogen/injection
Furunculosis	Aqua health	United Kingdom	Furogen B/immersion
Vibriosis	Aquaculture Vaccines Ltd	United Kingdom	AquaVac Vibrio/immersion
Vibriosis	Biomed Inc.	United States	Biojec 1300 injection or immersion (non-adjuvanted)
Furunculosis	Aquaculture Vaccines Ltd	United Kingdom	AquaVac Furovac-immersion/immersion
Furunculosis	Aquaculture Vaccines Ltd	United Kingdom	AquaVac Furovac-5/injection or immersion (non adjuvanted)
Furunculosis	Aquaculture Vaccines Ltd	United Kingdom	AquaVac Furovac-5/injection (oil-based adjuvant)
Furunculosis	Biomed Inc.	United States	Biojec 1500/injection
Furunculosis	Apothekernes Laboratorium	Norway	Apoject 1-Fural/injection (oil-based)
Furunculosis and cold-water vibriosis	Apothekernes Laboratorium	Norway	Apoject 2-Fural/injection (oil-based)
Furunculosis, cold-water vibriosis and vibriosis	Apothekernes Laboratorium	Norway	Apoject 3-Fural/injection (oil-based)
Cold-water vibriosis and vibriosis	Apothekernes Laboratorium	Norway	Apovax/immersion (water-based)
Furunculosis	Ewos Aqua A.S.	Norway	Lipogen Mono/injection (oil-based)
Furunculosis, cold-water vibriosis and vibriosis	Ewos Aqua A.S.	Norway	Lipogen Triple/injection (oil-based adjuvant)
Furunculosis	Ewos Aqua A.S.	Norway	Furogen/immersion (water-based)
Furunculosis	Ewos Aqua A.S.	Norway	Furogen/immersion (water-based)
Furunculosis, cold-water vibriosis and vibriosis	Ewos Aqua A.S.	Norway	Furogen B/dip (water-based)
Furunculosis	Ewos Aqua A.S.	Norway	Oravacc F Vet/Oral (water-based)
Furunculosis, cold-water vibriosis and vibriosis	Intervet Norbio A.S.	Norway	Norvax triple/injection (oil-based)
Furunculosis, cold-water vibriosis, vibriosis + infectious pancreatic necrosis	Intervet Norbio A.S.	Norway	Norvax triple + IPN/injection (oil-based)
Cold-water vibriosis and vibriosis	Intervet Norbio A.S.	Norway	Norvax vibriosis/dip (water-based)
Furunculosis, cold-water vibriosis and vibriosis	Biomed Inc.	United States	Bioject 1900/injection (oil-based adjuvant)
Enteric septicaemia of catfish	Biomed Inc.	United States	Biomed ESC/immersion (water-based)
Enteric red mouth	Biomed Inc.	United States	Bioject 1100 immersion (water-based)
Enteric red mouth	Aquaculture Vaccines Ltd	United Kingdom	Aqua Vac ERM/immersion
Enteric red mouth	Aqua health	United Kingdom	Ermogen/immersion
Spring viraemia of carp	Bioveta	Czech Republic	Injection

by *Rhabdovirus carpio* (two serotypes) and the disease is widespread in areas of carp culture.

Adjuvants

Most water-based vaccines for salmonids contain either glucan- or aluminium-based adjuvants which are partially effective and have few side-effects. The latest trend in salmonid vaccines is towards oil-based adjuvants which have been found to be more effective. These do, however, need to be injected into the fish and have many side-effects, for example suspected reduced growth rate, reduction of fertility in broodstock, reduced carcass quality owing to pigmentation at the site of injection, difficulty in administration owing to high viscosity and an increased hazard to human operators if accidentally self-injected. The use of mineral oil adjuvants has been accepted in Norway, but a licence for their use has not yet been granted in the United Kingdom.

Index of efficacy

The effectiveness of fish vaccines is calculated in terms of relative percent survival (RPS) using the following formula:

$$\text{RPS} = 1 - \frac{\text{percent vaccinate mortality}}{\text{percent control mortality}} \times 100$$

Amend and Fender (1976) describe the additional considerations which need to be met.

During the development of these commercial vaccines many parameters important to the success of vaccination have been determined, for example the route of administration, the length of protection obtained, the size of the fish and the temperature dependence of the immune response (Johnson and Amend, 1983a and 1983b; Johnson, Flynn and Amend, 1982a and 1982b; Tatner and Horne, 1985).

In general, intraperitoneal injection appears to be the most effective route of vaccination, although not the most practical. Much effort is therefore being put into the development of immersion and oral vaccines.

DEVELOPMENT OF VACCINES

The primary considerations for any successful vaccine for aquaculture are cost-effectiveness and safety. To accomplish this the vaccine must provide long-term protection against the disease under the intensive rearing conditions found on commercial fish farms. Consideration must be given to all the serotypic variants of the disease agent, the time and age at which the animal is most susceptible to disease, the route of administration and the method of vaccine preparation (i.e. killed, attenuated, subunit, recombinant).

All the commercial vaccines currently available comprise *inactivated* (killed) disease agents. When that approach failed in the development of vaccines, particularly viral vaccines, live attenuated vaccines were developed.

Whenever a *live* vaccine is used there is always concern that the attenuated strain (usually the result of a gene deletion) may back-mutate and revert to the virulent wild type. Many of the successful vaccines against viral diseases of humans (e.g. rubella, measles, poliomyelitis) and in domestic animals (e.g. rabies, distemper) are attenuated organisms. The licensing of such vaccines may, however, prove to be very difficult in aquaculture. An alternative approach has been to prepare subunit vaccines using recombinant technology, where the specific components of the disease-causing agents are isolated and, following amplification, used in vaccines. To increase the amount of antigen available, amplification is acheived by cloning the genes coding for specific antigens and incorporating them into

bacterial DNA, where they are expressed. Using fermentation technology for the growth of bacterial cells, expressed "foreign" proteins (antigens) can be produced in bulk.

Bacterial vaccines

Numerous fish vaccines are currently being developed, although some of them with only limited success. For example, vaccination would be an ideal approach for controlling bacterial kidney disease (BKD) because it is widespread and its control by means of chemotherapy is unsatisfactory. Experimental vaccination of coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*Oncorhynchus nerka*) indicated that agglutinating antibodies were produced but the response was slow to develop and these antibodies were not protective (Evelyn, 1971; Evelyn, Ketcheson and Prosperi-Porta, 1984; Baudin-Laurencin, Vigneulle and Mevel, 1977). Kaattari *et al.* (1987 and 1988) investigated potential BKD vaccines containing the highly immunogenic *Vibrio* vaccine components as an adjuvant; however, the results were inconsistent. Paterson, Desautels and Weber (1981) and McCarthy, Croy and Amend (1984) achieved the most promising results in Atlantic salmon and rainbow trout, where protection was conferred against a natural challenge and experimental challenge, respectively. One vaccine was adjuvanted with Freund's complete adjuvant while the other was pH-lyzed and non-adjuvanted. Other researchers (Bruno and Munro, 1984; Sakai, Atsuta and Kobayashi, 1993) have been unable to reproduce these results, and so the search for a BKD vaccine continues. The preparations tested so far appear to be inadequate. This may be because they contain inappropriate antigens and, whichever protective antigens are present, are only weakly immunogenic or responses to them are suppressed by other antigens.

It has been reported that certain components of the extracellular proteins (ECP) from *Renibacterium salmoninarum*, for example the 57 kDa protein, are immunosuppressive (Turaga, Wiens and Kaattari, 1987). These antigens must therefore be omitted (or modified) from any successful vaccine preparation. Hastings and Ellis (1988) showed that rainbow trout only responded to five out of 30 ECP components from *A. salmonicida*, while rabbits responded to 15. Thus, it is important to establish which antigens the fish respond to and whether these are protective. Current research in the United Kingdom and Canada is based on the purification of antigens from *R. salmoninarum* and its extracellular products, and includes investigation into their effects on the immune system prior to their incorporation into a recombinant vaccine.

The other vaccines currently being developed against bacterial fish pathogens include atypical *A. salmonicida*, *A. hydrophila*, *Edwardsiella ictaluri*, *Flexibacter columnaris*, *Streptococcus* sp. and *Pasteurella piscicida*.

Atypical furunculosis (caused by atypical non-pigmented *A. salmonicida*) is the main bacterial disease affecting Icelandic fish farming. An experimental vaccine given by injection has been developed (B. Gudmundsdottir, personal communication). The protective antigen was identified by passive immunization of Atlantic salmon with antisera against purified antigen raised in rainbow trout and rabbits. A method to culture the bacterium with the maximal expression of protective antigen has also been developed. The vaccine results in 70 to 100 percent RPS when administered with mineral oil as an adjuvant to Atlantic salmon parr. Protection appears to last for 12 months and there is a good correlation between antibody production and protection. Field trials have not yet been completed.

The antigenic diversity of *A. hydrophila* is the major limitation in the development of an effective vaccine and it seems likely that a polyvalent preparation will be necessary. Although there is some doubt as to whether these bacteria ever act as a primary pathogen, they do make a significant contribution to the disease process in the fish they invade. As part of the development of a vaccine, research groups in Southeast Asia are currently investigating the immune response of carp and catfish to *A. hydrophila* antigens.

Moore, Eimers and Cardell (1990) demonstrated the feasibility of immunizing channel catfish against columnaris disease by immersion vaccination with formalin-inactivated *Flexibacter columnaris*. However, a commercial vaccine has not yet been developed.

Streptococcal and *Pasteurella* spp. infections, hitherto reported principally in Japanese marine cultured fish populations, have recently become a problem in Mediterranean countries. The Japanese researchers Iida, Wakabayashi and Egusa (1982) and Sakai *et al.* (1987) reported that experimental vaccination provided protection from streptococcal infections, but a commercial vaccine is not yet available. There appear to be two serotypes. A cocktail immersion vaccine to prevent *Streptococcus* sp. and *V. anguillarum* is currently being tested in Japan and field trials are also being carried out in Italy (Ghittino, personal communication) on a potential *Streptococcus* vaccine.

Pasteurellosis has been of huge economic significance to yellow tail culture in Japan and, in 1991, was almost simultaneously reported as affecting sea bream in Italy, France and Greece. There appears to be homogeneity within this species of bacterium, although little has been published on its virulence factors and protective antigens. A *Pasteurella* vaccine is currently being field tested and researchers in Japan, the United Kingdom and Italy are continuing with development work to determine an immune response to the bacterium and investigate the pathogenesis of the disease.

Viral vaccines

The development of a successful vaccine by culturing the causative agent under defined conditions and subsequently inactivating it is not always feasible. It may be necessary to select protective antigens and engineer a recombinant vaccine genetically. This appears to be the case for most fish viral vaccines under development and requires the molecular cloning and expression of the viral genes in the bacterium *Escherichia coli* or the yeast, *Saccharomyces cerevisae*.

Viral haemorrhagic septicaemia (VHS) is a fish rhabdovirus responsible for severe losses in many continental European trout farms. It is an economically devastating disease for the aquaculture industry, since it may affect all age groups. In the past, inactivated vaccines have been developed; however, these were immunogenic only when given by injection (de Kinkelin, 1988). Following the failure of inactivated vaccines, several live vaccines were developed and appeared to provide protection against some of the serotypes. The vaccine strains were attenuated by successive passage in cell lines, but significant mortality associated with the virus was still observed and 2 to 13 percent of the fish died as a result of the vaccination alone (de Kinkelin and Bearzotti-Le Berre, 1981; Bernard, de Kinkelin and Bearzotti-Le Berre, 1983).

More recently, a subunit vaccine for VHS has been developed using recombinant DNA technology. The protective epitopes of VHS appear to be located on the surface glycoprotein of the virus, and antibody directed against the viral glycoprotein-neutralized viral infectivity (de Kinkelin,

Bernard and Hattenberger-Baudovy, 1984; Lorenzen, Oleson and Vestergaard-Jørgensen, 1990). The gene coding for the VHS glycoprotein was cloned and expressed in bacteria (Thiery *et al.*, 1990) and yeast (Lorenzen, 1991) to produce the glycoprotein inexpensively and in large quantities. Preliminary results indicate that this material induced protection following injection but no studies on immersion vaccination have been reported (Jørgensen, 1992). Thus, for VHS, a recombinant subunit vaccine may be a promising way of producing an inexpensive safe vaccine in fish.

Recent results obtained by Lecoq-Xhonneux *et al.* (1993), however, suggest that further research is needed prior to commercial exploitation, since the yield of antigens expressed was low and there was a problem with the route of delivery of the vaccine (i.e. it is only effective when introduced intraperitoneally, not orally).

Several research groups are actively developing vaccines for infectious pancreatic necrosis virus (IPN). This is a very important pathogen of farm-reared salmonids. Mortality is highest in young fish and survivors become life-long carriers, thereby maintaining the virus in the population by the continual shedding and transmission of the disease. The virus is serotypically heterogenous and has two major serotypes, the first of which comprises nine subtypes which are all pathogenic for salmonid fish.

Several inactivated IPN vaccines have been tested but only vaccine administered by injection-induced protection. The preferred route of immersion (since infected fish are usually small) was ineffective (Dorson, 1977). This was also found when individual virion polypeptides were tested. The virion proteins appeared to have lost their antigenicity following disruption with sodium dodecyl sulphate, urea and acetic acid (Hill, Dorson and Dixon, 1980).

Studies by Bootland, Dobos and Stevenson (1990) suggest that the age and size of the fish at the time of immunization are important factors in the development of protective immunity. They reported that only fry immunized at two to three weeks after hatching were protected. It appears that very young fish are capable of responding to vaccination but that growth rates must also be considered. These fish responded while in a slow weight-gaining phase.

Two attenuated IPN vaccines have also been tested and these appear to have provided protection when applied by immersion (Dorson, Castric and Torchy, 1978; McAllister, 1984). Further development was not pursued. Instead, as with other viral vaccines, many researchers are following the route of recombinant subunit vaccines for IPN (Havarstein *et al.*, 1990; Lawrence *et al.*, 1989; Hah, Park and Jeong, 1992). In these studies, the major capsid protein VP2 has been identified as the virion protein responsible for inducing protective immunity in fish.

The drawbacks of killed and attenuated vaccines (Leong, Fryer and Winton, 1988) have led research efforts to develop an effective subunit vaccine for infectious haematopoietic necrosis (IHN) virus (Leong *et al.*, 1992). During these studies, monoclonal antibodies to the virion proteins were produced, enabling characterization of the fish immune response to viral antigens and the development of *in situ* hybridization probes to detect the virus in vaccines. IHN is caused by a rhabdovirus and produces a severe disease among fry and juveniles of susceptible species of salmonid fish. Engelking and Leong (1989) demonstrated that the viral glycoprotein purified from one isolate could induce protective immunity in fish to a wide variety of IHN virus isolates from different geographical locations and different fish species. In consequence, the viral glycoprotein gene

from one strain was cloned and a recombinant vaccine prepared. Further studies have identified a specific immunodominant region in the middle of the gene (Xu *et al.*, 1991; Mourich and Leong, 1991). A field trial (immersion immunization) of the IHN subunit vaccine was undertaken in Idaho, United States, and showed it to be very effective in inducing protective immunity. A commercial vaccine, however is not currently available.

Two other viral vaccines currently under development for non-salmonid species are those for SVC and channel catfish virus (CCV). Despite a commercial inactivated SVC vaccine being available in the Czech Republic and Slovenia, attenuated vaccines have also been developed (Fijan *et al.*, 1977) and appear to offer protection. However, although the live vaccine in particular provided excellent protection, the vaccinated fish appeared to become asymptomatic carriers of the virus. When these fish were reared beside unvaccinated stock, an SVC outbreak occurred. A subunit vaccine is therefore an attractive alternative. A reliable challenge model for the disease also needs to be developed so that vaccine potency testing can be performed under standard conditions.

CCV, caused by *Herpesvirus ictaluri*, is an acute, highly infectious disease of juvenile channel catfish (*Ictalurus punctatus*). The onset of the disease is sudden and 100 percent mortality can occur within ten days. Once fish are infected with the virus, survivors become carriers for their entire lives. At present, CCV outbreaks are prevented by management strategies such as the use of resistant strains of channel catfish and virus-free broodstock for the stocking of fish farms. Vaccination would seem to be the ideal strategy in areas where CCV is endemic. An attenuated live vaccine was developed by serial passage in a tissue culture cell line derived from the walking catfish (Noga and Hartman, 1981). This was protective both by injection and immersion. Booster vaccination appeared to increase the efficacy of the vaccine greatly (Walczak, Noga and Hartman, 1981). This vaccine is not available commercially and has not been licensed, probably because of concerns regarding the risk of reversion to virulence and the possible establishment of carriers among vaccinated fish. If carrier fish are generated as a result of vaccination, these fish can no longer be certified as virus-free broodstock. Attempts to develop a killed CCV vaccine have been hindered by its poor immunogenicity (Plumb, 1973). A subunit vaccine for CCV was recently developed (Awad, Nusbaum and Brady, 1989). The envelope components of the virus appear to be capable of inducing protective immunity and, once these have been determined more specifically, an effective recombinant vaccine may be possible.

Parasitic vaccines

Monoclonal antibody (MAb) probes are proving to be useful tools in the development of vaccines against sea lice (*Lepeophtheirus salmonis*) and the protozoan infection proliferative kidney disease (PKD) in salmonids. A vaccine against sea lice remains a high priority for the aquaculture industry. Monoclonal antibodies were used to produce extracts from lice and to select individual antigens from a sea louse recombinant DNA library (Andrade-Salas *et al.*, 1993). These clones form the basis of experimental vaccines which are currently under investigation in the United Kingdom.

PKD is the most economically damaging disease affecting the trout industry in Europe. MAb probes have been produced against PKX, the causative agent, in an effort to map out the antigens on the parasite's surface (Adams, Richards and Marin de Mateo, 1992) and have shown that PKX from different species of fish and

different geographical locations share common antigens (Marin de Mateo *et al.*, 1993). At present, studies are under way to culture the parasite and investigate protective antigens.

A successful parasite vaccine has been described by Woo and Li (1990) working in Canada. Live attenuated *Cryptobia salmositica*, a pathogenic haemoflagellate causing cryptobiosis, was injected intraperitoneally into rainbow trout but the strategy for the delivery of the vaccine has still to be resolved.

Numerous other parasites cause significant losses to the aquaculture industry, for example *Icthyobodo necatrix*, *Ichthyopthirius multifiliis* and *Trichodina* sp., but their control by vaccination has not yet been attempted.

Future prospects

Fish vaccines have become much more sophisticated in recent years, with the trend being for the development of subunit recombinant vaccines in preference to the original killed whole-cell preparations. This has been necessary because the simpler approach did not succeed for many of the important diseases and attempts at attenuated vaccines in general have not been encouraging from a safety point of view. The cost of producing such "high-tech" vaccines must however be considered and the costs and benefits weighed up prior to commercialization.

Direct DNA vaccination has recently been successfully performed in cattle against infectious bovine rhinotracheitis virus (Cox, Zamb and Basiuk, 1993). Muscles can apparently take in naked DNA and then express it for long periods (Wolff *et al.*, 1992). DNA vaccination may prove to be a more cost-effective method of vaccinating fish against viral infections in the future.

As the range of cultured species increases, there is also a need for basic research on the immune responses of new species as well as for studies on genetically resistant species in parallel with vaccine development.

Immunostimulants. An approach recently taken by the aquaculture industry is the use of immunostimulants, either by themselves to counteract stress-induced immunosuppression in fish, or in vaccines as adjuvants. They may serve to boost the immune system in the short term and therefore have great potential. One group receiving a great deal of attention at present is the β-glucans. They apparently function as immunostimulants of the non-specific defence mechanism of fish (Robertsen, Engstad and Jørgensen, 1994).

Glucans are major structural polysaccharides from fungal and yeast cell walls, composed of glucose units which are held together through β-1,3 and β-1,6 bonds (Rosenberg, 1976; Duffus, Levi and Manners, 1982). Both soluble β-glucans (e.g. scleroglucan, schizophyllan and lentinan) and microparticulated β-glucans from yeast (M-Glucan, Macroguard®) have been shown to function as immunostimulators in fish (Robertsen, Engstad and Jørgensen, 1994). Intraperitoneal injections of glucans have resulted in enhanced disease protection of carp to *Edwardsiella tarda* infection (Yano, Mangindaan and Matsuyama, 1989; Yano, Matsuyama and Mangindaan, 1991) and yellow tail to *Streptococcus* spp. (Matsuyama, Mangindaan and Yano, 1992). Yeast glucan has been found to increase disease resistance in Atlantic salmon to *Vibrio anguillarum*, *V. salmonicida*, *Yersinia ruckeri* and *Aeromonas salmonicida* (Robertsen *et al.*, 1990; Robertsen, Engstad and Jørgensen, 1994) and in channel catfish to *Edwardsiella ictaluri* (Chen and Ainsworth, 1992).

The exact mode of action of glucans remains unclear, but enhanced protection against microbial pathogens, observed

after administering glucans to fish, correlates with increased blood lysozyme and complement activities and enhanced phagocytosis and killing of bacteria by headkidney macrophages (Yano, Mangindaan and Matsuyama, 1989; Engstad, Robertsen and Frivold, 1992; Chen and Ainsworth, 1992; Matsuyama, Mangindaan and Yano, 1992; Jørgensen *et al.*, 1993). It is believed that such macrophages may have an essential role to play in immunostimulation, since it has been shown that Atlantic salmon macrophages possess receptors for β-glucan (Robertsen, Engstad and Jørgensen, 1994).

Robertsen, Engstad and Jørgensen (1994) suggested that non-specific defence may be more effective against opportunistic pathogens than specific bacteria. Glucans, therefore, probably benefit most during stress-related conditions, such as handling, transportation or smoltification, when an increased susceptibility to disease occurs. Glucan-supplemented diets and furunculosis vaccines are now commercially available. However, the dose, the type of glucan and the route of administration must all be considered when examining the effects of glucans on disease resistance (Ainsworth, Mao and Boyle, 1994). Further studies are required to standardize these parameters.

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Role of the Office international des Epizooties and international organizations in vaccine quality standardization

M. Truszczynski and J. Blancou

Harmonization and standardization of requirements for veterinary vaccines have two important goals:

- to keep the quality of vaccines at a high level;
- to prevent or reduce potential trade barriers.

Specialized groups established by governments or groups of governments, such as the European Union (EU), formulate legislation and requirements on the basis of the latest technology to ensure the high quality and safety of these products.

Every new vaccine developed in a country or imported from another has to be registered according to the legislation and requirements in force in the country where the vaccine will be used.

The registration file must report on the results of experiments directly related to the product itself (Pensaert, 1992) and include:

- an analytical section, characterizing the different components and the substrates on which the antigens are produced;
- a toxico-pharmacological note, indicating the absence of remaining pathogenicity or abnormal toxicity;
- a section on the product's efficacy, describing clinically oriented experiments with relation to immunity and protection (degree, duration, etc.).

The analytical section should include the controls which have been carried out both on the product during manufacturing and on the final product. These controls are performed in the producers' laboratory or in a laboratory of their choice.

The registration files are examined by experts appointed by the ministry of health and, particularly, the national commission for veterinary drugs. The expert who examines the analytical section may require additional controls if those described in the original file are incomplete. These additional controls may have to be carried out in an officially accepted laboratory, for instance if the vaccine originates from a country for which there is little information on the quality of the local control system.

When the registration data are adequate, the commission for veterinary drugs gives a favourable assessment to the minister of health and the product is registered for marketing.

After registration, every production batch of the registered vaccine must be controlled by the producer, both during manufacturing and at the final product stage, and these controls are the responsibility of an industrial specialist. Further official controls are not required for the majority of bacterial vaccines although, in many cases, the bacterial strain used in the vaccine is controlled annually by the appropriate government institute. However, all viral vaccines and also some bacterial vaccines (e.g. for brucellosis) must undergo an official batch control by the national veterinary institute or an

equivalent institution before release. Every imported vaccine must undergo an official batch control in an officially accepted laboratory chosen freely by the importer. Additionally, a control is prescribed by the national veterinary institute.

For batch controls, representative samples are collected under the responsibility of the laboratory and according to information supplied by the producer with regard to the number of doses per batch, the homogeneity of the batch, conditions of storage, etc.

In the laboratory, the samples are examined to identify the active component(s), to ascertain the absence of contaminating agents and to ensure the product's sterility, safety and potency. For these controls, the relevant monograph of the European Pharmacopoeia (see p. 149) is followed. If there is no monograph for a particular vaccine, the tests described in the original registration file must be applied. For inactivated vaccines, the potency test may be performed on the target species or on a laboratory animal species but, in the latter case, the relation between the potency in the laboratory and that in the target species must be demonstrated by the producer. For live vaccines, potency is generally limited to quantitation of the organism or of the virus, using methods described in the original registration file. If the results are satisfactory, the batch will be given an official batch number and be released on to the market.

Depending on the country or group of countries, there are varying degrees of difference in their legislation. With this in mind and in the spirit of international harmonization aimed at reducing or preventing potential trade barriers, the United States Animal Health Institute (AHI) has proposed the formation of an international working group consisting of itself, the European Federation for Animal Health (FEDESA), the EU's Committee for Veterinary Medicinal Products, the United States Department of Agriculture and international industry and government officials (Draayer, Hilsabeck and Miller, 1992; Folkers, 1992; Watson, 1992). It is suggested that annual meetings be held in conjunction with either FEDESA or AHI meetings. Issues to be dealt with include the standardization of test requirements, time-frames for implementation and other issues that could reduce or prevent trade barriers without adversely affecting product quality.

The main purpose of the working group would be to harmonize European and United States legislation as a necessary step towards international standardization and the prevention or reducion of potential trade barriers.

There are, in addition, international organizations acting in parallel – also with the goal of international harmonization and standardization of vaccines – but independently from governments and in more general terms. These organizations include the Office international des Epizooties (OIE), the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) as well as the Pharmacopoeias (e.g. the European, United States and Japanese Pharmacopoeias).

OFFICE INTERNATIONAL DES EPIZOOTIES

The OIE, whose purpose is to harmonize and coordinate animal health activities at the international level, also contributes to the standardization of vaccine quality. The three principal aims of the OIE (Truszczynski and Blancou, 1992) are:

- The provision of information on animal health worldwide.
- International coordination of research into and control of certain animal diseases.
- The harmonization of import and export regulations for animals and

animal products at the international level.

A measure of the development and growing international recognition of the OIE is that it was established by 28 countries in 1924 while it now has 136 member countries. Seventeen are from the Americas, 42 from Africa, 43 from Europe and 34 from Asia. The OIE operates under the authority of its International Committee, formed by the delegates of the member countries, under the leadership of an elected president.

The Central Bureau, located at OIE's headquarters in Paris (12 rue de Prony), is headed by the Director-General, at present Dr J. Blancou. The Central Bureau implements decisions of the International Committee and the different Commissions.

There are five *Regional Commissions*, covering Africa, the Americas, Asia, the Far East and Oceania, Europe and the Middle East.

There are also *Specialist Commissions* for The International Animal Health Code; Standards; Foot-and-Mouth Disease and other Epizootics; and Fish Diseases, including those of crustaceans and molluscs. In addition, there are three working groups: Animal Health Information Systems, Veterinary Drug Registration and Biotechnology.

The International Animal Health Code Commission draws up animal health recommendations for the import and export of animals and animal products and contributes through the *Animal Health Code*, an important OIE publication, to international harmonization in this area. *The Standards Commission* establishes standards for diagnostic methods, including diagnostic biologicals, and for vaccines. *The Foot and Mouth Disease and other Epizootics Commission* contributes to the development and standardization of vaccines against foot-and-mouth disease and strategies for the eradication or control of this as well as other diseases. *The Fish Diseases Commission* establishes standards for diagnostic methods and vaccines for fish diseases.

From this short description it can be concluded that all the Specialist Commissions are interested in the international harmonization and standardization not only of vaccines but also of diagnostic methods, including diagnostic biologicals. These activities relate to those important diseases included in the Lists A and B prepared by the OIE (Truszczynski and Blancou, 1992).

List A includes contagious diseases which spread rapidly and the scope of which extends beyond national borders. These diseases have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products.

List B includes contagious diseases which are considered of socio-economic and/or public health importance within countries and which, naturally, are also of significance to the international trade of animals and animal products.

Among these Specialist Commissions, the one most closely connected with the standardization of diagnostic methods and vaccine quality is the Standards Commission, whose role includes participation in the standardization of biologicals, including vaccines used for prophylactic purposes for List A and B diseases.

To accomplish this goal, the Standards Commission undertakes the following activities:

- regular updating of the *OIE manual of standards for diagnostics tests and vaccines;*
- the organization of reference laboratories for several diseases in Lists A and B;
- the establishment of international standards for diagnostic tests and vaccines.

OIE manual of standards for diagnostic tests and vaccines

The purpose of the OIE manual (OIE, 1992) is to provide a uniform approach to the diagnosis of important animal diseases and to the production and control of biological products, mainly vaccines used in the control of List A and B diseases. This is implemented by the presentation in the manual of standard methods for laboratory diagnosis of diseases and for the production and control of vaccines and other biological products for veterinary use in laboratories all over the world.

The first edition of the manual was published in three volumes in 1989-1991, each covering about 30 List A or B diseases. After revision and additional editorial work, a single combined volume was published as the second edition of the manual in 1992. It contains information on 15 List A diseases and 75 List B diseases. In future, the Standards Commission plans to produce a new updated edition every four years. The 1992 edition of the manual has been distributed worldwide, recommending:

- "prescribed tests" which should be used for diagnosis; and
- requirements for vaccines, in relation to List A and B diseases.

This will undoubtedly help lead to an internationally unified approach to the diagnosis and quality control of vaccines.

Organization of OIE reference laboratories for List A and B diseases

The standardization of "prescribed tests" and the production and quality control of veterinary vaccines can only be achieved when the necessary standards are made available. The goal of standardization and availability of standards is intended to be achieved through the OIE-organized reference laboratories.

These laboratories must fulfil a specific function or range of functions at an internationally recognized level related to the standardization of diagnostic methods and vaccines applied in the prophylaxis and control of List A and B diseases, especially those of greatest importance and those causing high economic losses. The designation of these laboratories is proposed by the OIE Standards Commission and ratified by the OIE International Committee. Designation does not imply that financial support will be given by the OIE.

The functions and responsibilities of the OIE reference laboratories are as follows:

- the provision of a centre of excellence in a designated activity;
- the standardization of methodology;
- the storage and distribution of standard antisera, antigens and other important reagents;
- the development of new methods;
- the provision of consultant assistance to the OIE;
- training in a designated activity;
- the organization of scientific meetings on behalf of the OIE;
- the coordination of collaborative studies;
- the provision of assistance to the OIE in collecting and disseminating necessary information.

The Director-General of the OIE sent letters to selected laboratories around the world inviting them to become OIE reference laboratories for the more important diseases of Lists A and B. Because of the necessity of some regionalization, in several cases a number of reference laboratories for the same disease were organized in different countries or regions. At the beginning of 1994, 101 OIE reference laboratories were designated for 37 List A and B diseases or groups of diseases. The majority of these laboratories developed significant activities in accordance with their responsibilities, and this is reflected in annual reports which they submit to the OIE.

OIE requirements for vaccines

As mentioned above, these requirements are part of the OIE manual's chapters on the List A and B diseases for which vaccines are available. The chapters contain general information, indicating recommended vaccines, data on seed management (including characteristics of the vaccinal strains, culture, validation as a vaccine), manufacture, in-process control and batch control (including sterility, safety and potency tests).

The Standards Commission is aware that the manual's coverage of standards for vaccines is less comprehensive than it is of diagnostic methods. Nevertheless, some chapters are exemplary and provide a good model for others to follow in the preparation of the next edition.

The Commission will, however, avoid any involvement in product licensing procedures. It will be essential to ensure that the OIE manual keeps up to date with developments in vaccine technology through current advances in microbiology, immunology and biotechnology. Following from this, the importance of consistency between the OIE manual, EU regulations and the European Pharmacopoeia must also be emphasized.

In future, the OIE reference laboratories will prepare for distribution the standards that are essential in vaccine production and control. The present role of the OIE is, however, restricted to the distribution of information contained in the manual and which is undoubtedly contributing to international harmonization in the area of vaccine production.

OIE COOPERATION WITH OTHER INTERNATIONAL ORGANIZATIONS

FAO

FAO plays an important role in improving the quality standards of vaccines. Its main interest is in developing countries and activities are concentrated on rinderpest eradication campaigns (Pan-African Rinderpest Campaign [PARC], Western Asia Rinderpest Eradication Campaign [WAREC] and South Asia Rinderpest Eradication Campaign [SAREC]) in 53 countries on three continents; foot-and-mouth disease control in Asia, Africa and South America; and Newcastle disease of poultry in the same areas.

During the last decade, through national and regional projects, FAO has provided expertise to individual laboratories and assistance in the setting up of networks. FAO has also cooperated with international organizations (WHO, OIE) in the standardization of particular products and the holding of regional training courses on vaccines and their associated technologies.

The FAO Expert Consultation on Quality Control of Veterinary Vaccines in Developing Countries, held in Rome, Italy, in December 1991 (FAO, 1991; Rweyemamu, Sylla and Palya in FAO, 1993), recommended that closer cooperation be established with other international organizations to develop a more coherent approach on guidelines for vaccine quality control. FAO, WHO and OIE should work jointly with developing countries to agree on priorities and carry forward regional participation in standardization, including the development of appropriate laboratory facilities and post-licensing surveillance.

This should include participation at the national level as well as within regions and would require those countries with no procedures to begin standardization and regulation, unless a decision has been taken to accept standards agreed by other countries or regions. Regional repositories of master seed stocks of vaccine strains, cell lines and challenge strains should be established to aid in attaining uniform quality of vaccines.

The consultation recognized the value of the activities of the Pan African Veterinary Vaccine Centre (PANVAC) and

recommended that FAO, together with the Organization of African Unity (OAU) and the Interafrican Bureau for Animal Resources (IBAR), should solicit appropriate regional and international support for the conversion of PANVAC to the status of a long-term programme institute, with appropriate facilities and support.

It was also stated that tests required by national authorities to license or release vaccines should be identical with those recommended by the appropriate international organizations. The meeting recommended the preparation of a manual on veterinary vaccines, providing guidelines for the production of veterinary vaccines in developing countries, as a project to be implemented by FAO and the other international organizations concerned.

World Health Organization

WHO, located in Geneva, Switzerland, is a specialized United Nations agency whose primary responsibility is in international and public health matters. However, it also plays an essential role in the standardization of vaccine quality.

The WHO Expert Committee on Biological Standardization is mainly responsible for human pharmaceutical products (vaccines) but also for some veterinary biologicals and products of interest to animals as well as humans. The committee has published guidelines for the preparation and establishment of reference materials and reference reagents for biological substances (WHO, 1978) and definitions of these materials and reagents have been formulated. According to these definitions, an international biological standard is a biological substance to which WHO has assigned an international unit on the basis of data obtained in a worldwide study.

The prime function of an international biological standard is to enable the activity of a sample of unknown potency to be measured in a biological system and expressed conveniently in international units. The international biological reference preparation is a biological substance which may be used for a purpose similar to that of a standard but which has been established without a full collaborative study, or after such a study has shown that it is not appropriate to establish it as an international standard.

Besides this activity, the WHO Expert Committee on Biological Standardization establishes more general requirements for biological products, among them vaccines for veterinary use. A good example is in the manufacturing and control requirements for rabies vaccines for veterinary use (WHO, 1981).

With regard to products for veterinary use or of animal origin, the committee has established requirements for tuberculins (human and bovine), anthrax spore vaccine (live vaccine for veterinary use), immune sera of animal origin, rinderpest cell culture vaccine (live) and rinderpest vaccine (live), *Brucella abortus* Strain 19 vaccine and *Brucella melitensis* Strain Rev.1 vaccine (live for veterinary use).

Requirements are revised regularly for most products as major advances in technology for manufacturing or quality control are reported and accepted by the international scientific community.

In addition to specific products, the Expert Committee on Biological Standardization also issues general recommendations on biological substances and test systems, for example on the national control of vaccines and sera and requirements for immunoassay kits. It also regularly reviews and updates the list of international biological reference preparations and reagents.

Technical units such as the Veterinary Public Health (VPH) Unit of WHO are associated with the committee's work each

time a substance or a subject falling within a specific technical unit's domain of expertise comes under discussion.

European Pharmacopoeia

One effective approach to vaccine quality standardization is based on the European Pharmacopoeia (Artiges, 1992). The 19 signatory countries – the 12 (in 1992) EU Member States, countries of the European Free Trade Association (EFTA) and Cyprus – are committed to setting up common monographs and making them official in their territories. The European Pharmacopoeia has its own secretariat, administratively attached to the Council of the EU, and a laboratory, both of which are located in Strasbourg.

Monographs and general analytical methods are prepared by specialized expert groups made up of scientists from universities and national control laboratories, etc. Before their final adoption, the texts, which in most cases have been the object of interlaboratory cooperative tests, are published in public inquiry form in the European Pharmacopoeia's *Pharmeuropa*, which appears four times a year in English and French.

The close cooperation between the European Pharmacopoeia and those countries retaining their own national pharmacopoeias should be mentioned, i.e. Germany, Austria, Belgium, the United Kingdom, France, Italy, Switzerland and the Nordic countries. The latter have brought all their general methods in line with those of the European Pharmacopoeia.

To date, all general analytical methods are harmonized: there are 17 general monographs defining the main pharmaceutical forms and about 800 monographs covering starting materials and certain biological preparations such as immunosera and vaccines, both human and veterinary.

The second edition of the European Pharmacopoeia has been published since 1980 in the form of fascicles. Publication is annual (between June and September) and implementation of the standards in all member countries is effective on 1 January of the following year.

Operationally, the roles of the pharmacopoeias and the regulatory governmental agencies (mentioned earlier) are complementary:

- the regulatory agencies approve new drugs and vaccines on the basis of proven safety and efficacy and approved specifications, tests and methods of analysis for each application;
- the Pharmacopoeia establishes public standards that apply to any manufacturer of a particular drug substance or pharmaceutical product.

In Europe, there is a close legal connection between licensing systems and pharmacopoeias regarding their legal (Directive EEC/75/81/852) as well as practical aspects (shared experts and a close relationship between secretariats).

The European Pharmacopoeia provides detailed quality standards for vaccines through a series of monographs. These are assembled by an expert group and, once published, are mandatory for the member states. There are currently 33 monographs on veterinary vaccines and other biologicals. Among them are: anthrax spore live vaccine for veterinary use, avian infectious bronchitis live vaccine (freeze-dried), canine distemper live vaccine (freeze-dried), *Clostridium botulinum* vaccine for veterinary use, *C. chauvoei* vaccine for veterinary use, *C. perfringens* vaccine for veterinary use, equine influenza vaccine (inactivated), foot-and-mouth disease vaccine (inactivated), swine erysipelas vaccine (inactivated) and swine fever live vaccine (freeze-dried).

The monographs on veterinary vaccines

provide information on identification tests, testing for contaminants and storage, etc. The European Pharmacopoeia also provides a limited number of reference preparations for use as working standards – among these preparations a rabies vaccine and a bovine tuberculosis purified protein derivative (PPD). A *B. melitensis*, Rev.1 vaccine is also currently under study.

Except in the case of biotechnology products, the European Pharmacopoeia maintains only limited liaison with other major pharmacopoeias such as those of the United States and Japan. For veterinary vaccines the differences between these pharmacopoeias are of considerable significance.

In general, the European Pharmacopoeia monographs are more detailed than the OIE manual's requirements for biological products (OIE, 1992), but they only deal with vaccines of significance to Europe. Although several of these vaccines are also important to other continents, the European Pharmacopoeia may be less accessible, particularly in developing countries, than the OIE manual.

In the next edition of the OIE manual, the sections on vaccines will be revised in the full light of standards already established in the European and other major pharmacopoeias.

Pan American Health Organization

The Pan American Health Organization (PAHO) contributes towards the standardization and quality control of foot-and-mouth disease (FMD) and some other vaccines in South America (FAO, 1991). Through the work of PAHO, standards of vaccine production and quality control in particular have been significantly improved in the last decade, and vaccination campaigns against FMD are having a significant effect on the incidence of the disease.

European Union

Cooperation between OIE and the EU (DGXII – Science, Research and Development) in the control of veterinary reagents and vaccines has been initiated (Lee Aileen, 1992).

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