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Genetically engineered structure-based vaccine for bluetongue disease

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Summary

At present the only vaccines used against bluetongue (BT) disease are live-attenuated virus vaccines. Since viruses with RNA genomes such as BT virus (BTV) have a high frequency of mutations, live virus vaccines could have breakthroughs (vaccine failures) and mutate into virulent strains. In addition, multiple BTV serotypes exist in nature which could potentially cause additional problems with live virus vaccines. The BTV genome is made up of 10 segments and therefore potentially could exchange these segments (or genes) randomly between different viruses including vaccine strains, generating novel viruses with mixed genes. Hence it is necessary to develop BTV vaccines that pose no such threat. Ideally BTV vaccines should be completely devoid of harmful genes. Recent protein expression technology has provided novel approaches for the development of intrinsically safe vaccines. The technology involves the synthesis of immunogenic proteins and particles that elicit highly protective immune responses. We have generated such vaccines, termed virus-like particle (VLP) vaccines. These vaccines (which do not carry either BTV or foreign genes) give the immune system information about viral structures so that it can generate a complete defence against the real virus infection very efficiently. A series of vaccine trials were undertaken outdoors under natural UV light using 50-200 BTV-susceptible sheep per trial. Vaccination trials of sheep showed that the VLPs were highly immunogenic, and protected sheep when animals were challenged with virulent virus even 15 months after the first immunisation. Moreover, a cocktail of five VLPs afforded protection against not only each of the homologous BTV serotypes but also against certain heterologous serotypes that are genetically related to some of these vaccine strains. VLPs representing a number of serotypes are currently available and can be produced fairly quickly if there is such a need. Based on our sequence data it can be predicted that a mixture of seven or eight types of VLPs (that are already available) will provide protection against at least 10 or more serotypes depending on their phylogenetic relationships. BTV VLPs offer particular advantages as potential vaccines over other systems. Large quantities of VLPs can be produced and easily purified using a one-step protocol based on the physical proprieties of the particle. More importantly, these particles are devoid of any nucleic acid and thus pose no threat by re-assortment or the re-emergence of virulence that attenuated vaccines can cause.

Keywords

Bluetongue virus – Sheep – Vaccine – Virus – Virus-like particles.

Most of the current viral vaccines are prepared using attenuated or inactivated virus. This approach, although useful in many cases, has certain drawbacks including vaccine breakthroughs or disease caused by incompletely inactivated vaccine. Viruses with multiple serotypes, as well as segmented genomes, are especially challenging for development of safe vaccines as gene exchange between viruses may occur randomly. This may cause the generation of infectious virus with mixed genes. Production of live virus vaccines has to be undertaken in containment laboratories, with added costs both for the production and the safety and efficacy testing of vaccine lots.

Bluetongue (BT) virus (BTV) is the type member of the genus *Orbivirus*, in the family *Reoviridae*. This is one of the largest families of viruses and includes major human pathogens (e.g. rotavirus) as well as other vertebrate, plant and insect pathogens. Orbiviruses are distinct from other members of the *Reoviridae* in a number of ways; they multiply in

arthropod and vertebrate cells, often causing severe disease and high mortality. BTV is transmitted by *Culicoides* spp., causing disease in ruminants in many parts of the world. To date, some 24 different BTV serotypes have been identified (BTV-1, BTV-2, etc.) from different parts of the world (6). In sheep, the disease is acute and mortality may be high, whereas in cattle and goats the disease is usually milder (6). In a typical case in sheep, the onset of the disease is marked by high fever lasting about 5-7 days. By 7-10 days, distinctive lesions appear in the mouth and the tongue can be severely affected, turning blue. In contrast to sheep, infected cattle experience prolonged viraemia and infection during pregnancy can cause teratogenic defects in calves and abortion of the foetus.

Other orbiviruses infect a variety of animals and also cause significant diseases, for example the recent outbreak of African horse sickness virus (AHSV) in Spain and Portugal. The spread of *Culicoides* insects from endemic to non-BTV and non-AHSV regions in the past highlights the concern that these viruses are a threat to areas that are presently free from viral infection. As a result of its economic significance, BTV has been the subject of extensive molecular, genetic and structural studies and now represents one of the most well characterised viruses (1). BTV virions are architecturally complex structures composed of seven discrete proteins (Fig. 1) in a specific but non-equimolar ratio that are organised into two shells, the inner core and outer capsid (1). The virion contains a genome of ten double-stranded (ds) RNA segments. The outer capsid is composed of two major structural viral proteins (VP2 and VP5) and is involved in cell attachment and entry of virus during the initial stages of infection (8). Shortly after infection, BTV is uncoated (VP2 and VP5 are removed) to yield a transcriptionally active core particle that is composed of two major proteins (VP3 and VP7), three minor proteins (VP1, VP4 and VP6) and the dsRNA genome (1). While the four major proteins (VP2, VP3, VP5 and VP7) form the bulk of the virus capsid, the three minor proteins, together with genomic RNA, form the virus replication complex. In addition to the structural proteins, non-structural (NS) proteins NS1, NS2, NS3 and NS3A are made in BTV-infected cells which are involved in virus replication and assembly (1).

Of the ten BTV proteins, only the two outer capsid proteins, VP2 and VP5 that are responsible for virus entry into susceptible host cells (8, 9), are variable from serotype to serotype (24 serotypes), although close phylogenetic relationships are easily detectable indicating that the genome mutations may have played a major role in generating multiple serotypes (17). In contrast to VP2 and VP5, all five core proteins and the three non-structural proteins are highly conserved (17). Both by *in vitro* (tissue culture) and *in vivo* (animal) studies it has been shown that BTV is highly capable of reassorting the RNA segments between different serotypes (1). Therefore, potentially, attenuated virus vaccines might play a significant role in the generation of endemic strains.

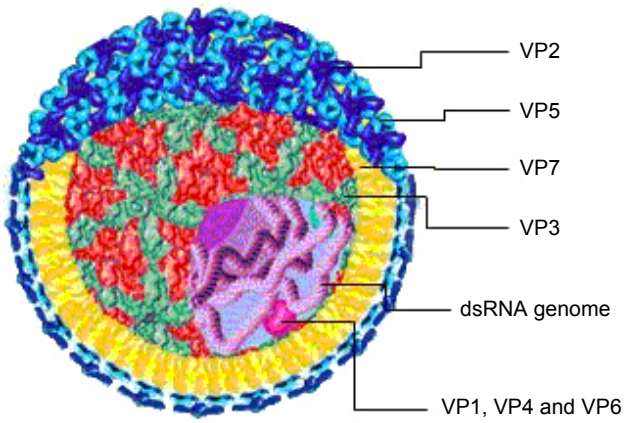


Figure 1
Bluetongue virus particle: a schematic of the virus particle showing the organisation of 7 BTV proteins and the RNA genome

In the past, live-attenuated orbiviruses have been employed as vaccines in those regions of the world where BTV or AHSV cause epidemics of disease in livestock. Although reasonably effective, since vaccine strains are replication competent and since orbiviruses have segmented genomes, there is some concern over the use of live-attenuated virus vaccines. Live virus vaccines may aid virus maintenance in nature, and facilitate genome segment reassortment and the generation of new viral genotypes. Moreover, since there are multiple serotypes of BTV and AHSV, it is likely that not all vaccine strains would have the required level of attenuation. In order to develop rationally designed BTV vaccines, over the past few years an understanding has been developed of the structural and functional relationships of the BTV genes and gene products and the assembly pathway for the formation of virions. Recent advances in gene manipulation have made it possible to express foreign genes in heterologous systems. The productivity and flexibility of insect baculovirus expression vectors and the ability of the baculovirus genome to incorporate (and express) large amounts of foreign DNA in *Spodoptera frugiperda* insect cells have permitted this system to be used for the expression of not only a single gene, but also for the simultaneous expression of dual and multiple genes. To accomplish this, several expression vectors have

been developed based on the resident promoters of the nuclear polyhedrosis virus of *Autographa californica* (AcNPV) (1, 2, 3). Using these various expression vectors, all ten genes of BTV have been expressed either individually or in various combinations using single, dual, triple and quadruple expression vectors and the structure-function of each gene and gene-product analysed (17). Information gained from these studies has served as platform for rationally designed safe vaccines for BTV and AHSV. Extensive clinical trials have been conducted using these proteins and protein structures, some of which are discussed below.

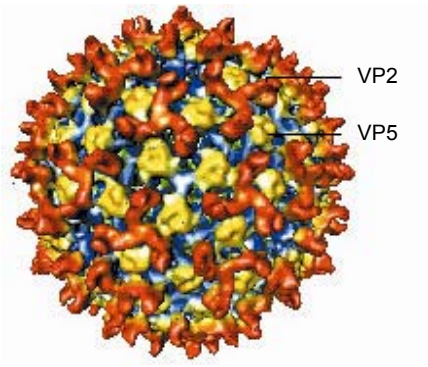
Three-dimensional structures of BTV virion and core

To design the virus-like particle (VLP) vaccine it is necessary to understand the structural organisation of the BTV capsid. Therefore, the three-dimensional structure of cores and virions were determined by cryoelectron microscopy (Cryo-EM) and computer image reconstruction methods which allow visualising the individual protein organisation in large particles such as viruses. The major advantage of Cryo-EM over conventional electron microscopic techniques is that the biological molecules are observed in a frozen hydrated state in amorphous ice, which closely resembles the native aqueous state. The problems associated with heavy metal stains, fixatives and dehydration are thus avoided.

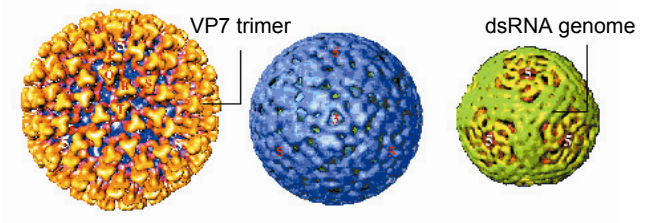
The surface of the core serves as a foundation for deposits of the two outer capsid proteins, VP2 and VP5. Cryoelectron micrographs analysis has revealed a well-ordered morphology of the virion outer capsid. This is in contrast to the morphology deduced by negative-staining methods, which indicated that the outer capsid of the complete BTV particle has a fuzzy appearance (1). The capsid has an icosahedral configuration and the two proteins of the capsid have distinctive shapes, one globular and almost spherical, the other sail-shaped (Fig. 2a) (10, 11). The globular proteins, 120 in number, sit neatly in the channels formed by each of the six-membered rings of VP7 trimers. The sail-shaped spikes, which project 4 nm beyond the globular proteins, are located above 180 of the VP7 trimers and form 60 triskelion-type motifs that cover nearly all the VP7 molecules. These spikes are trimers of the viral haemmagglutinating protein VP2, which also contains the virus-neutralising epitope, and the globular proteins are the trimers of VP5. The two proteins are attached to the surface of the VP7 layer and together they form a continuous layer (outer capsid) around the core except for holes on the fivefold axis. The structures of these two outer capsid proteins indicate that the formation of VP7 layer is essential for deposition of the outer capsid.

It was therefore necessary to examine the core structure. Cryo-EM analysis of BTV cores revealed that the core has a diameter of 69 nm and that the surface exhibits icosahedral symmetry with a triangulation number of 13 (Fig. 2b) (7). The core structure is divided into two concentric layers of protein enclosing the RNA and minor proteins. The surface layer of the core is made up of clusters of VP7 trimers, which bear prominent knob-like protrusions and which are organised into pentameric and hexameric units with channels between them (Fig. 2b, panel A). There are a total of 780 VP7 molecules per particle, 132 channels and 260 trimers or knobs at all the threefold axes. The smooth scaffold for the VP7 trimers is made up by 120 molecules of the second major protein VP3 (Fig. 2b, panel B) which is closely associated with the VP7 trimers. The remaining three minor enzymatic proteins, VP1, VP4, VP6 that are responsible for replication of the viral genome occupy the innermost component genomic RNA (Fig. 2b, panel C).

A An icosahedral complex whole virus particle viewed along a two-fold axis, showing the topography of the two outercapsid proteins, one globular-shaped (VP5) and the other sail-shaped (VP2) protruding 4 nm above the surface of the particles



B BTV core viewed along the icosahedral three-fold axis



Showing the protrusions of 260 VP7 trimers in the outer layer

Showing the smooth inner VP3 layer formed by 120 molecules

RNA genome and replication enzyme complex formed by VP1, VP4 and VP6 (7)

Figure 2
Surface representation of a cryoelectron micrograph of bluetongue virus

Assembly of bluetongue core-like and virus-like particles by baculovirus expression systems

Since VP2 and VP5 together induced a protective immune response in sheep, it is likely that the immunity would be enhanced if these proteins could be presented in a similar manner as in native virion particles. It would be rewarding if virus capsid structures without the genetic materials could be synthesised. The flexibility of baculovirus expression vectors and the capacity of the baculovirus genome to accommodate large amounts of foreign DNA enabled exploitation of the system for the simultaneous expression of multiple BTV genes in a single insect cell. Since 3D studies indicated that it might be possible to obtain a stable scaffolding core structure consisting of only the VP3 and VP7 which may allow eventually assembling VP2 and VP5 on the surface, dual and multigene baculovirus vector systems were prepared.

To assemble the VP3 and VP7, a dual baculovirus expression vector was utilised to express the coding sequences of the L3 (VP3) and S7 (VP7) genes of BTV (5). Recombinant baculoviruses synthesising both proteins were isolated and indeed core-like particles (CLPs) were produced and distributed throughout the infected insect *Spodoptera* cells. Gradient-purified CLPs were similar in size and appearance to cores prepared from BTV (Fig. 3). VP3 and VP7 were the only protein components identified in the expressed particles and the molar ratios of these two proteins were similar to those of VP3 and VP7 derived from infectious BTV. The CLPs appeared to lack nucleic acids when analysed by phenol-chloroform extraction and alcohol precipitation.

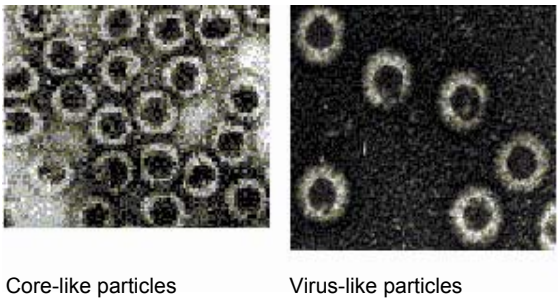


Figure 3
Electron micrographs of negatively stained, baculovirus-expressed core-like particles consisting of VP3 and VP7 and virus-like particles consisting of VP2, VP3, VP5 and VP7

Subsequently baculovirus multigene vectors were constructed to co-synthesise up to four BTV proteins in the same cell (2). Two different expression cassettes were generated; one that expressed VP2 and VP5 simultaneously and the other that expressed VP2, VP3, VP4 and VP7 proteins in a single cell (1, 4). The expressed proteins from the quadruple vector assembled into virtually homogenous double-capsid particles (Fig. 3). Co-infections with two dual expression vectors (namely VP3/VP7 and VP2/VP5) gave VLPs that contained different amounts of the outer capsid proteins, depending on the experiment (4). The formation of complete VLPs in the absence of non-structural proteins or the internal minor proteins implies that these proteins are not necessary for the assembly of these double-capsid particles or for CLPs. VLPs express high levels of hemagglutination activity, similar to that of BTV virions. Furthermore, antibodies raised to the expressed particles gave high titres of neutralising activity against the homologous BTV serotype (4). When the 3D structure of CLPs and VLPs were analysed by Cryo-EM, both types of particles were clearly comparable to that of authentic cores and virions and exhibited essentially the same basic features and full complement of the two or four proteins (11, 14). VLPs synthesised by recombinant baculoviruses were also characterised further at the biological and immunological levels and compared to those of the native virion. VLPs exhibited high levels of haemagglutination activity similar to those of authentic BTV. Further antibodies raised to the expressed particles contained high titres of neutralising activity against the homologous BTV serotype emphasising their authenticity at a functional level (4).

Virus-like particles as vaccine

Since recombinant baculovirus-derived VLPs elicited strong neutralising antibodies in guinea-pigs, it can be anticipated that VLPs should elicit protective responses in sheep against BTV infection. Consequently, a number of experiments were performed to examine the protective efficacy of VLPs in sheep. In each experiment, BTV-susceptible, one-year old Merino sheep (BTV-free) were divided into groups, and each group was immunised subcutaneously with purified VLPs in saline containing various amounts of protein suspended in 50% Montanide Incomplete Seppic Adjuvant (ISA-50, Seppic, Paris). Each animal received 2 ml of the mixture. For each concentration of protein, a minimum of two sheep was used. For control experiments, one group of sheep received only saline. All vaccinated animals were boosted with the same amounts of protein on day 21. From the day of challenge to day 21, serum was collected from each animal at intervals and virus neutralisation tests were performed by plaque reduction neutralisation assay. Sheep that received VLPs developed demonstrable neutralising antibodies, albeit to different levels (15, 16). The levels of neutralising

antibodies depended on the amount of VLPs administered (Fig. 4). Significant levels of neutralising antibodies were elicited with all concentrations of VLPs and persisted throughout the study. The control sheep inoculated with saline remained seronegative. All sheep were challenged by subcutaneous inoculation of 1 ml of infective sheep blood containing virulent BTV-10 (South African strain) at day 117 (Fig. 4). The clinical reaction index (CRI) of the animals and viraemia were monitored from 3 to 14 days post-challenge (13). The challenged sheep neither developed clinical signs nor viraemias, indicating suppressed replication of BTV. The post-challenge blood samples of the sheep that only received saline were viraemic and these sheep developed high neutralising antibody titres indicative of a primary infection. In summary, protective immunity to BTV disease was obtained by vaccinating sheep with doses of 10 µg or more of BTV VLPs. The duration of protection obtained with only 10 µg VLPs was much higher than was obtained with the high doses (100 µg or more) of single (e.g. VP2) or dual antigens (e.g. VP2 and VP5) (data not shown) (17).

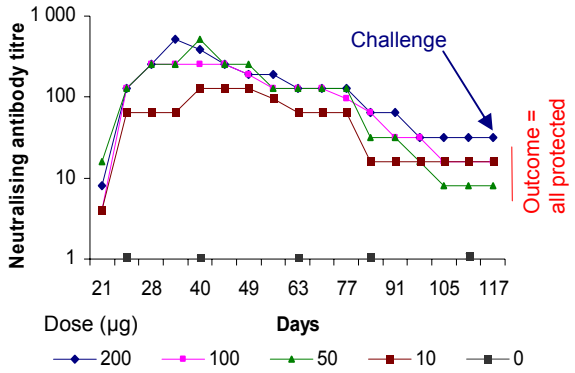


Figure 4
Vaccination trials of sheep with bluetongue virus serotype 10 virus-like particles
2-4 sheep were vaccinated with various doses of VLPs (1 ml) in the presence of 50% ISA-50 (1 ml)
Neutralising antibodies of sheep at various intervals after vaccination with bluetongue are indicated
VLPs and their protective responses following challenge (after 4 months) with homologous virulent bluetongue viruses

To analyse further the protective effects and duration of VLP vaccination, a similar protocol was employed for VLPs (10 µg or 50 µg per sheep) representing BTV-10 and BTV-17 (16). The neutralising antibody titres of the vaccinated sheep were determined at weekly intervals and over a sixty-week period after the booster. Both types of VLP elicited (to various levels) antibodies that neutralised the homologous virus. In almost all cases these neutralising titres remained high throughout the sixty-week period. The neutralising antibody titres for the animals that received 50 µg doses of VLPs were not significantly higher than those that received the 10 µg doses (Fig. 5). Sheep vaccinated with the mixture of the two types of VLPs induced antibodies that neutralised both types of virus as well as some related heterologous viruses (e.g. BTV-4) when tested by plaque reduction assays. As expected, the control sheep that were inoculated with saline remained seronegative. All the sheep were challenged 14 months after the booster vaccination by the subcutaneous injection of virulent BTV. The animals that were challenged with the homologous viruses (BTV-10, BTV-17) were completely protected and showed no clinical reactions, even those that received 10 µg doses of VLP (Fig. 5). Also, no viraemias were detected in these animals after challenge. In addition, some animals inoculated with 50 µg VLPs were also partially protected when

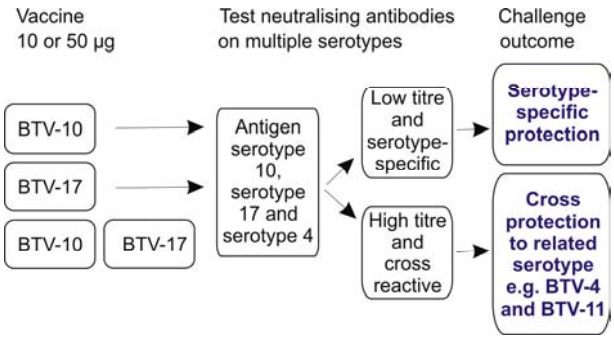


Figure 5
Virus-like particle vaccination trials of sheep with homologous and heterologous virulent virus challenge
A group of sheep received two doses of mixtures BTV-10 (10 µg) and BTV-17 (10 µg) or two doses of mixtures of BTV-10 (50 µg) and BTV-17 VLP (50 µg)
Neutralising antibodies of sheep at various intervals after vaccination were determined and protective responses following challenge (after 14 months) with homologous or heterologous virulent viruses were assessed as described in Figures 2 and 4

challenged with heterologous virus. By comparison, the control animals developed high or moderate signs of disease (BTV-10, CRI: 7.1-8.0; BTV-17, CRI: 1.6-2.7) and produced viraemias. Similar vaccination trials with a cocktail of five different BTV VLPs (Fig. 6) representing five different serotypes were also undertaken. When these vaccinated animals were challenged with heterologous types that were not included in the cocktail, very encouraging data was obtained. There were clear indications of cross protection and such protection was dependent on both the amount of VLP in the vaccine dose as well as the sequence variation of the outer capsid proteins.

In summary, the data showed that long-lasting protection against homologous BTV challenge was

provided by vaccination with VLPs. Some preliminary evidence was obtained for cross-protection, depending on the challenge virus and the amounts of antigen used for vaccination (16).

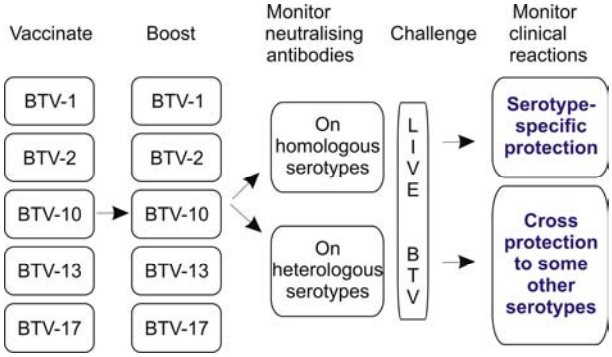


Figure 6
Virus-like particle vaccination trials with a cocktail of bluetongue virus serotypes 1, 2, 10, 13 and 17 VLPs (10 µg each) in 50% ISA-50 given in each step, boosted after 21 days
The protection against various virulent virus challenges were determined as in Figures 2 and 4

Protection afforded by cores

BTV cores are conserved across the 24 serotypes. Therefore, it would be highly beneficial if synthetic CLPs could afford any protection against BTV infection. The question of whether CLPs containing the two conserved proteins, VP3 and VP7, would provide a measure of homologous and heterologous BTV protection by cell-mediated mechanisms was therefore investigated. For initial studies, two groups of five sheep each were used. One group of five sheep inoculated with 100 µg BTV-10 CLP in ISA-50 boosted on day 21 was challenged with BTV-10 two weeks later. All sheep developed viraemias and neutralising antibodies after challenge (18). However, with the exception of fever, the vaccinated sheep developed only slight clinical reactions whereas controls showed characteristic mouth and feet lesions in addition to fever. The average CRI of the vaccinated sheep was 3.5 whereas that of the control sheep was 9.0 (Fig. 7). In summary, partial protection against BTV challenge was afforded by CLP vaccination.

Discussion

BT has been known to be associated with disease and mortality in sheep and cattle for decades. Despite the fact that this can have serious economic impacts, not only in terms of animal health but for some countries import and export regulations for sheep and cattle, only live-attenuated vaccines have been developed in South Africa and in the United States. In South Africa, sheep are vaccinated with three different pentavalent live-attenuated virus vaccines at three-week intervals. Conventional live-attenuated virus vaccines have certain inherent disadvantages. In the case of BT, such virus vaccines can cause infection in the foetus with teratological consequences. When used as a polyvalent vaccine, interference occurs between the component BTV serotypes, resulting in the development of incomplete immunity. Moreover, live-attenuated vaccine strains may be neutralised passively by the antibody in maternal colostrum.

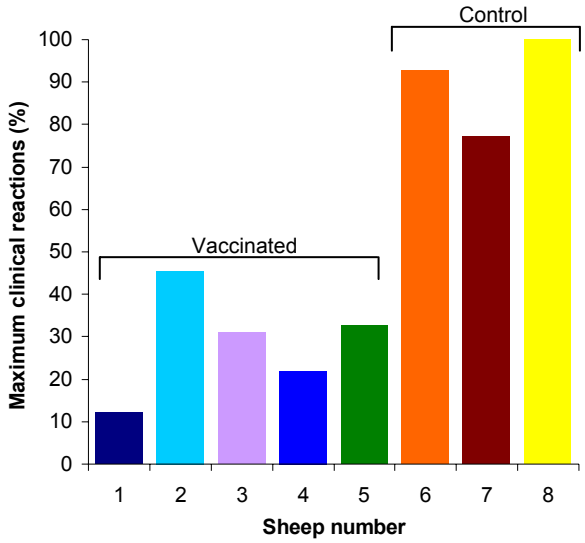


Figure 7
Immunisation of sheep with core-like particles showing partial protection against heterologous virus challenge

Recent developments in biotechnology have made it possible to synthesise double-shelled BTV-like particles, mimicking authentic virions but lacking the harmful genetic material and viral replicating machinery. Therefore, these particles are as safe as subunit vaccines, and potentially as effective as 'whole' virus vaccines. A number of vaccination trials in BTV-susceptible sheep were undertaken. The results clearly demonstrated that VLP vaccines are highly efficacious. A very small amount (10 µg) of VLPs (10-20% of the VLP mass is VP2, i.e. 1-2 µg of VP2 per dose), in the presence of appropriate adjuvant, protected the sheep against the disease. There are several possible explanations. Firstly, the conformational presentations of the relevant epitopes on VP2 probably mimic those on the authentic virus. Secondly, both VP2 and VP5 are present. Thirdly, VP3 and VP7 may provide a necessary scaffold for VP2 and VP5 antigen presentation. Fourthly, any of the four BTV proteins might have a direct role in eliciting cell-mediated immunity induced by the BTV VLPs. It can also be anticipated from the results obtained that this technology has much to offer for development of vaccines for both veterinary and human diseases.

VLPs offer particular advantages as potential vaccines over other systems. An additional advantage is that large quantities of these particles (BTV proteins) can be produced due to the high expression capabilities of baculovirus vectors (produced in serum-free medium), and purified using a one-step generic protocol based on the physical proprieties of the particle.

Complete sequence analysis of cDNA clones of viral RNA species, have demonstrated that both outer capsid proteins VP2 and VP5 are among the most variable proteins of different BTV serotypes. Depending on the serotype, they exhibit sequence relationships to other BTV serotypes (17). Data indicating that antigens of one BTV serotype (12) could neutralise other BTV serotypes and a mixture of two or five VLPs demonstrated no interference but protected against both homologous and heterologous virulent virus challenge. There is every reason to believe that VLPs representing only a few serotypes would afford protection against other serotypes in addition to the vaccine types and that not all 24 types would be needed to be present in the vaccine cocktail. This is an exciting prospect for the future.

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