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Delipidation of a Pestivirus: Viral Inactivation and Vaccine Development in Large Animals

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Abstract: Lipid Associated Viruses (LAVs) including HIV, hepatitis B, C and SARS are amongst the most malevolent viruses. These and a variety of other viruses have an outer lipid envelope which maintains inserted viral peptides in the "correct" functional conformation and orientation. Most solvents disrupt the lipid envelope and destroy infectivity but these solvents also result in a loss of antigenicity. Specific organic solvents consisting of butanol and Diisopropyl Ether (DIPE) delipidate the whole virion rendering it non-infective, but antigenic. In large animals such as cattle, delipidation of large amounts of the pestivirus Bovine Viral Diarrhea Virus (BVDV) result in the inactivation of the virus shown by *in vitro* and *in vivo* testing. The inactivated BVDV preparation when used as a vaccine results in anti-E2 antibody production in all vaccinated animals. Delipidation of lipid-associated enveloped viruses with specific organic solvents has potential as the basis for development of vaccines.

Key words: Delipidation, virus inactivation, vaccine development, BVDV, lipid-associated virus

INTRODUCTION

The incidence of viral diseases have been increasing over recent years. Viral diseases that had been confidently thought to be under control such as yellow fever have emerged again. Hepatitis C is now being recognized as a major problem in health care. A WHO report in 1998 stated that there were about 5 billion cases of disease and 6 million to 7 million deaths annually from childhood diarrhea, including those due to rotavirus infections and respiratory tract infections especially those caused by respiratory syncytial virus. Infections with HIV-1, tuberculosis and malaria together cause 7-8 million deaths annually, mainly in developing countries. Currently Human Herpes Virus are major problems. HIV-AIDS has emerged as an uncontrolled, worldwide public health emergency. Despite well-publicized advances in antiretroviral therapy, there is still no cure.

Our body is constantly being invaded by many organisms including viruses. Fortunately our body's defense mechanisms overcome a large number of viral infections. Our immune system is mainly responsible for the victory over these viruses. The current understanding is that the protein components of the virus are responsible for the body's immune response. When a "new" virus infects a body, the body is at a disadvantage and cannot respond rapidly to the insult. It takes some time before the body can get its multiple defense mechanisms established. If it is a very malevolent organism such as HIV the body may never establish an appropriate defense mechanism, especially as is the case with HIV, because this virus actually attacks the mechanisms that are responsible for the defense. In the majority of cases when the body is invaded by not so malevolent organisms the battle is long. However, the body is usually excellent in memorizing its enemy, the invading virus and if exposed to the same enemy again, rapidly establishes its defense system and usually rapidly overcomes its enemy.

Vaccines have come to the rescue of animals and humans on many occasions. Notwithstanding the vast improvements in the understanding of viral infections and their clinical sequelae these little viral critters have eluded their control by man through various means, such as, for example, mutations.

Vaccines are considered the first line of defense for reducing the excess morbidity and mortality that invariably accompany pandemics. An effective vaccine needs to be a close match to the actual infecting virus and this is the limitation that scientists face in the development of highly effective vaccines.

It is accepted that vaccine developments for various strains of viruses such as HIV, SARS and hepatitis have not been and will not be an easy process. It is also believed that the more that is understood of the virus and the disease the more complex the science.

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It must be understood that a vaccine does not prevent infection, it allows the animal to respond more rapidly after infection occurs. Disease and infection from viruses are two very different issues. On the one hand, there are many animals and humans that get infected with a virus but don't show disease. On the other hand, there are viruses that are very infective culminating in rapid onset of diseases. The latter occurrence is a major problem that animals and humans face. In order to overcome the morbidity and mortality of these viruses various categories of vaccine development have been studied. These categories are mainly composed of the following vaccines.

Live-attenuated vaccines: These vaccines have been used globally against many viral diseases, such as polio (Sabin vaccine) and measles. They consist of weakened (attenuated) live viruses that are too disabled to cause disease but can still infect cells and replicate within the body. After responding to the weakened viruses, the immune system is then prepared to protect against future infections by pathogenic strains.

Whole inactivated vaccines: This vaccine approach consists of inactivating the whole pathogen by chemical modification so that it is no longer capable of replicating in the host. The advantage to this type of vaccine is that all potential antibody-inducing determinants on the surface of the virus are present without danger of infection. There are several disadvantages to inactivated vaccines: Primarily, without being infectious, these viral particles predominantly evoke a lesser antibody response; they are less effective than attenuated vaccines at inducing cell-mediated or mucosal immunity. Repeated boosters are often required to maintain the immune status of the individual. Lastly, the risk associated with this type of vaccine is that without careful surveillance, the pathogen may not be properly killed. The Salk polio vaccine and Pertussis vaccine are two therapies currently produced by formaldehyde inactivation.

Recombinant subunit vaccines: These vaccines introduce a harmless sub-unit or portion of a viral protein into the body. This was the basis of AIDSVAX, the first vaccine tested unsuccessfully for effectiveness in humans and which contains a portion of HIV's outer surface (envelope) protein, called gp120. The hepatitis B vaccine successfully uses this approach to confer protective immunity.

Live recombinant vector vaccines: These are created by genetically engineering relatively harmless, replicating viruses or bacteria to produce viral proteins, theoretically providing some of the advantages of live-attenuated vaccines without many of the safety concerns.

DNA vaccines: These vaccines also known as "naked DNA" or "nucleic acid" vaccines, use the actual genes of the virus as a vaccine. Once introduced into skin or muscle, the genetic material is taken up by cells in the body, which then produce the viral proteins; the immune system can then mount responses against the viral proteins.

Virus-like particle and synthetic peptide vaccines: This approach is similar to that of the sub-unit vaccine, but introduce particles of different sizes into the body. Virus-like particles are significantly more complex than the single proteins used in the sub-unit vaccines, consisting of several viral proteins engineered to mimic a viral particle. In contrast, synthetic peptides consist of small portions of viral proteins chosen specifically to focus the anti-viral immune responses on what are thought to be the most important regions of the viral proteins.

Combination vaccines: Combination vaccines combine several of the above approaches, based on the premise that protection from viruses may require a broad spectrum of immune responses. Another combination vaccine strategy uses a DNA vaccine to prime the immune system and a vaccine to boost the immune system to produce robust anti-viral immune responses.

The above vaccine strategies all have in common that the body recognizes protein particles of the virus generated by different means. It appears that the generated proteins of the virus are either in denatured forms or subunit forms of proteins. In addition only limited types of viral proteins have been investigated.

LAVs are amongst the currently most malevolent viruses. Lipid present in the outer layer of viruses contribute to the stabilization of the viral particle. Certain proteins may protrude from the lipid layer, but these outer layer membranous proteins constitute only a small fraction of the virus' total protein content. Many other functional proteins are imbedded within the core of the viral particle. Some LAVs are HIV, Hepatitis (HBV) and C Virus (HCV), Severe Acute Respiratory Syndrome Coronavirus (SARS), West Nile virus and Influenza virus including Bird Flu virus.

The problems associated with making inactivated vaccines against viruses with lipid envelopes are mainly due to the conformational changes in the antigenic epitopes which occur when the envelope is disrupted

by the extraction processes. The altered epitopes are either not recognized by the host immune system or elicit inappropriate responses. Experimental vaccines now include combination of structural (receptor and core) as well as non-structural proteins (Ferrantelli *et al*., 2004). Polyvalent vaccines characteristically elicit both cellular and humoral responses and this collaboration is the key characteristic of efficacy (Cham *et al*., 2006).

In the past a major limiting step was the obtaining of structural and non-structural proteins derived from viruses. Previously when lipid was removed by various means from the viral particle the proteins were obtained in denatured forms. However, a delipidation procedure has been described that removes lipid from lipid-associated particles that contain proteins. After delipidation with specific organic solvents the delipidated proteins remain soluble in their native forms. This procedure was first applied to delipidate plasma while preserving the biological activity, including enzymatic functions. The solvent extraction system used for delipidation consisted on an ether such as DIPE, a halogenated form of DIPE, or a mixture of an ether and an alcohol such as DIPE and butanol (Cham and Knowles, 1976a, b, c; Cham *et al*., 1995; Cham *et al*., 2005; Cham, 2007; Groener *et al*., 1984, 1986; Hayase *et al*., 1980). The epitopes of the delipidated proteins obtained using the DIPE-butanol system are not affected by delipidation (Avogaro *et al*., 1978; Curry *et al*., 1977; 1980, 1981; Fex and Hansson, 1978; Jaukiainen *et al*., 1982; Koren *et al*., 1985 a, b; Klein and Zilversmit, 1984; Meunier *et al*., 1986; Ockner *et al*., 1982; Pattnaik *et al*., 1978; Rustow *et al*., 1979; Staprans and Felts, 1977; Slater and Robertson, 1979; Shakespeare and Postle, 1979; Slater *et al*., 1980) and indeed it has been shown that the epitopes as measured by antigen-antibody interaction increases in such lipid-protein complexes after delipidation (Kostner *et al*., 1979) suggesting that the delipidation process may lead to more efficient processing of viral antigens. In 2001, it was first observed that lipid-associated viruses could be effectively delipidated and its potential therapeutic application of viral inactivation with a novel approach to vaccine strategy was described (Cham, 2001 a,b). The proof-of-concept of delipidation of viruses was shown by delipidation of human HIV viruses and Kunjin viruses which resulted in the inactivation of the viral particles (Cham, 2001 a).

More recently it was reported that delipidation of a hepadnavirus, a Duck Hepatitis B Virus (DHBV), resulted in the inactivation of DHBV. In addition, the viral delipidated proteins retained epitopes that were capable of generating protective immunity and specific anti-DHBs antibody in recipients. It was reported that stabilization or the increase of the virus epitopes in a particle of differenct conformation to the native virion may have occurred and the core proteins of the inactive virus released from the viral particle by delipidation may have also played a key role in the vaccine efficacy (Cham *et al*., 2006). In mice a dose as little as 1μg of delipidated SIV virus elicited broad humoral as well as CD4+ and CD8+ responses (Kitabwalla *et al*., 2005).

With the DHBV studies the ducks were vaccinated with nanogram amounts delipidated DHBV (Cham *et al*., 2006), this is much lower than that used when purified surface antigen (25μg kg^{-1} body weight) was used to induce protective antibody levels in ducks. This suggests that the presentation of viral antigens are preserved or even enhanced by the DIPE-butanol delipidation process. This delipidation procedure has recently been applied to SARS and it was demonstrated that delipidation modified the SARS viral particles resulting in the enhancement of immunogenic responses in an animal model resulting in both neutralizing antibody and cell-mediated immune responses (www.lipidsciences.com).

The present study was undertaken to evaluate in large animals whether delipidation, with specific solvents, of the lipid-associated pestivirus BVDV inactivates the virus as determined by *in vitro* and *in vivo* techniques. Furthermore, it was of interest to establish whether the delipidation process might preserve viral antigens in authentic form for presentation to the immune system to allow an effective vaccine in large animals.

MATERIALS AND METHODS

A standard cattle pestivirus isolate (BVDV) was used in these experiments. The isolate, "Numerella" BVD virus, was isolated in 1987 from a diagnostic specimen submitted from a typical case of "Mucosal Disease" on a farm in the Bega district of New South Wales (NSW) Australia. This virus is non-cytopathogenic and reacts with all 12 of a panel of monoclonal antibodies raised at the Elizabeth Macarthur Agricultural Institute (EMAI), NSW, Australia, as typing reagents. Therefore, this virus represents a "standard strain" of Australian BVD viruses.

The Numerella virus was grown in Madin-Darby Bovine Kidney (MDBK) cells tested free of adventitious viral agents, including BVDV. The medium used for viral growth contained 10% Adult Bovine Serum (ABS) derived from EMAI cattle, all of which tested free of BVDV virus and BVDV antibodies. This serum supplement has been employed for over 30 years to exclude the possibility of adventitious BVDV contamination of test systems, a common failing in laboratories world wide that do not take precautions to ensure the test virus is the only one in the culture system. Using these tested culture systems

ensured high-level replication of the virus and a high yield of infectious virus. Titration of the final viral yield after 5 days growth in MDBK cells showed a titer of $10^{6.8}$ infectious viral particles per ml of clarified (centrifuged) culture medium.

Inactivation of infectious BVDV: One hundred millitre of tissue-culture supernatant, containing $10^{6.8}$ viral particles mL^{-1} was harvested from a 150cm^3 tissue-culture flask. The supernatant was clarified by centrifugation (cell debris pelleted at 3000 rpm, 10 min, 4°C) and 10 mL set aside as a positive control for animal inoculation (non-treated virus). The remaining 90 mL, containing $10^{7.75}$ infectious virus, was, treated using the procedure of Cham and Knowles (Cham and Knowles, 1976a, b, c). Briefly, 180 mL of a solvent mixture butanol:DIPE (40:60$^v/_v$) was added to the virus suspension in a 500 mL conical flask and mixed by swirling. The mixture was then shaken for 60 min at 30 rpm at room temperature on an orbital shaker. It was then centrifuged for 10 min at 400×g at 4°C, after which the organic solvent phase was removed and discarded. In subsequent steps, the bottom layer (aqueous phase) was removed from beneath the organic phase, improving yields considerably.

The aqueous phase, after the butanol: DIPE treatment, was washed four times with an equal volume of fresh peroxide free Diethyl Ether (DEE) to remove all contaminating traces of butanol. After each washing, the contents of the flask was swirled to ensure even mixing of both aqueous and solvent phases before centrifugation as above (400×g, 10 min, 4°C). After 4 washes, the aqueous phase was placed in a sterile beaker covered with a sterile tissue fixed to the top of the beaker with a rubber band to prevent contamination and placed in a fume hood running continuously overnight (16 h) to remove all remaining volatile ether residue from the inactivated viral preparation. Subsequent culture of the treated material demonstrated no contamination. The treated viral preparation was then stored at 4°C under sterile conditions until inoculation into tissue culture or animals to test for any remaining infectious virus.

Testing of treated BVDV preparation by tissue-culture inoculation: Two milliliters of the solvent-treated virus preparation ($10^{7.1}$ viral equivalents) was mixed with 8 mL tissue-culture medium Minimal Eagles Medium (MEM) containing 10% tested-free adult bovine serum and adsorbed for 60 min onto a monolayer of MDBK cells in a 25 cm^3 tissue-culture flask. As a positive control, 2 mL of non-treated lipid-containing infectious virus (also containing $10^{7.1}$ live infectious viral equivalents) was similarly adsorbed on MDBK cells in a 25 cm^3 tissue-culture flask. After 60 min, the supernatant was removed from both flasks and replaced with normal growth medium (+10% ABS). The cells were then grown for 5 days under standard conditions before the MDBK cells were fixed and stained using a standard immunoperoxidase protocol with a mixture of 6 BVDV-specific monoclonal antibodies (EMAI panel, reactive with 2 different BVD viral proteins).

Testing of treated BVDV preparation by animal inoculation: An even more sensitive *in vivo* test is to inoculate naïve (antibody-negative) cattle with the inactivated-virus preparation. As little as one infectious viral particle injected subcutaneously in such animals is considered to be an infectious-cow dose, given that entry into cells and replication of the virus is extremely efficient for BVDV.

A group of 10 antibody-negative steers (10-12 months of age) were randomly allocated to 3 groups. The first group of 6 steers was used as the "vaccine" group, to test whether the BVD virus had been fully inactivated and also whether 2 doses of the inactivated virus preparation would elicit strong neutralizing antibody levels. Two steers were inoculated with non-inactiveated vaccine to act as a positive-control for the vaccine group, while the 2 remaining steers acted as negative "sentinel" animals to ensure there was no natural pestivirus transmission occurring naturally, within the vaccinated group of animals. The positive control animals (inoculated with live, infectious virus) were run under separate, quarantined, conditions to stop them infecting any other animals when they developed a transient viraemia after infection (normally at 2-17 days after receiving live BVDV virus) (Baule *et al.*, 2001). Antibody levels were measured in all 10 animals using a validated, competitive ELISA developed at EMAI. This test has been independently validated by CSL Ltd and is marketed by IDEXX Scandinavia in Europe (Kampa *et al.*, 2007).

Total 6 animals in the vaccine group each received a subcutaneous injection of 4.5 mL of the inactivated BVDV preparation, incorporated in a commercial adjuvant, on each of 2 occasions, 4 weeks apart. Since each ml of the test "vaccine" contained $10^{6.8}$ viral equivalents, the total viral load before inactivation was $10^{7.4}$ $TCID_{50}$ (50% tissue cell infectious doses). The positive-control animals received 5 mL each of the non-inactivated preparation, that is, $10^{7.5}$ $TCID_{50}$ injected subcutaneously in the same way as the vaccine. The remaining 2 "sentinel" animals were not given any viral antigens, being grazed with the vaccinated animals throughout the trial to ensure there was no natural pestivirus activity occurring in the group while the trial took place.

RESULTS AND DISCUSSION

Tissue culture *in vitro* inoculation studies: There were no infected cells in the monolayer of MDBK cells that was inoculated with the organic solvent treated virus. In contrast, approximately 90% of the cells in the control flask (that was inoculated with non-inactivated BVDV) were positive for virus as shown by heavy, specific, immunoperoxidase staining. These results showed that, under *in vitro* testing conditions, no infectious virus remained in the inactivated BVDV preparation.

Whole animal *in vivo* inoculation studies: There was no antibody development in any of the vaccinated steers receiving the delipidated BVD virus preparation until a second dose of vaccine was given. Thus, at 2 and 4 weeks after a single dose, none of the 6 steers seroconverted showing that there was no infectious virus left in a total volume of 27 mL of the delipidated virus preparation. This is the equivalent of a total inactivation of $10^{8.2}$ $TCID_{50}$. In contrast, there were high levels of both anti-E2 antibodies (neutralizing antibodies) and anti-NS3 antibodies (not shown) at both 2 and 4 weeks after inoculation in the two steers receiving 5 mL each of the viral preparation prior to delipidation. This confirmed the infectious nature of the virus prior to delipidation. These *in vivo* results confirm the findings of the *in vitro* tissue-culture test. The two "sentinel" animals remained seronegative throughout, showing the herd remained free of natural pestivirus infections (Table 1).

Table 1: Anti-E2 Antibody results at 0, 2 and 4 weeks after the inoculation of a single dose of delipidated virus, live virus and non inoculated controls in steers

	E2 CTB-ELISA % inhibition *		
Group and animal no.	Before inoculation	T 2 weeks	T 4 weeks
Inoculated with delipidated virus			
U118	0.4	2.8	2.6
U172	1.9	1.6	5.4
U181	1.8	4.0	0.7
U224	1.7	1.6	3.0
U253	1.4	1.9	1.0
U298	1.0	0.6	4.3
Inoculated with live virus			
U104	1.9	35.4	74.8
U228	0.3	29.5	84.8
Non-inoculated sentinels			
U203	1.5	0.1	1.3
U231	0.3	0.4	4.0

Table 2: Anti-E2 Antibody results of animals that received an inoculation of delipidated virus, live virus and non inoculated controls in steers 4 weeks after the first primary dose

	E2 CTB-ELISA % inhibition *	
Group and animal no.	Priming dose dose	4 week after priming does
Inoculated with delipidated virus		
U118	2.8	86.5
U172	1.6	31.5
U181	4.0	26.9
U224	1.6	79.5
U253	1.9	74.3
U298	0.6	23.3
Inoculated with live virus		
U104	35.4	76.3
U228	29.5	85.4
Non-inoculated sentinels		
U203	0.1	1.4
U231	0.4	0.4

Weeks after Priming

Delipidated BVDV preparation as a vaccine in steers: All 6 steers that had received an initial dose of 4.5 mL of the delipidated BVDV preparation were again injected subcutaneously with a similar dose at 4 weeks after the first priming dose. At this time there were no antibody responses after the initial dose. It is normal for an animal to react after the second dose. Strong secondary immune responses for anti-E2 antibody levels (equivalent to serum neutralizing antibodies SNT) were observed in 3 of the 6 steers at 2 weeks after the second dose of the delipidated virus. This response was more than 70% inhibition in a competitive ELISA. The remaining 3 animals showed weaker antibody responses (23-31% inhibition) (Table 2).

In contrast to the anti-E2 antibody responses, only one animal developed a strong anti-NS3 antibody response (93% inhibition) at 2 weeks after the second dose of delipidated BVDV. A second animal had a weak anti-NS3 response (29% inhibition) and four animals showed no antibody following administration of 2 doses (not shown). This was not unexpected since similar responses following administration of inactivated BVDV vaccines by other means have been observed previously. The reason is that NS3 antigen is normally cell-associated only and there were no cells incorporated in the delipidated inactivated preparation used in these experiments. NS3 protein has major regulatory roles in viral replication and is not present within the infectious virus (Baker, 1987; Donofrio *et al*., 2006). The antibody levels in steers following 2 doses of the delipidated BVDV preparation demonstrate its potential as a vaccine since antiE2 antibody levels were measurable in all 6 vaccinated steers at 2 weeks after the second dose.

CONCLUSION

Inactivation of large amounts of infectious BVDV using the delipidation procedure with the butanol-DIPE system was shown to be complete, as assessed by both tissue-culture inoculation and the inoculation of relatively large doses of the inactivated preparation into all antibody-negative bovine animals.

Antibody levels measured following 2 doses of the inactivated BVDV preparation have shown that it has potential as a "vaccine", with anti-E2 antibody levels measurable in all vaccinated bovine animals at 2 weeks after the second dose.

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