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ScienceDirect
Journal of Virological Methods 137 (2006) 160–163
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Short communication

Delipidation of a hepadnavirus: Viral inactivation and vaccine development

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Received 20 December 2005; received in revised form 21 May 2006; accepted 25 May 2006
Available online 30 June 2006

Abstract

Many viruses including HIV, hepatitis C and hepatitis B, have an outer lipid envelope which maintains inserted viral peptides in the "correct" functional conformation and orientation. Disruption of the lipid envelope by most solvents destroys infectivity and often results in a loss of antigenicity. This communication outlines a novel approach to viral inactivation by specific solvent delipidation which modifies the whole virion rendering it non-infective, but antigenic.

Duck hepatitis B virus (DHBV) was delipidated using a diisopropylether (DIPE) and butanol mixture and residual infectivity tested by inoculation into day-old ducks. Delipidation completely inactivated the DHBV ($p < 0.001$).

Delipidated DHBV was then used to vaccinate ducks. Three doses of delipidated DHBV induced anti-DHBs antibody production and prevented high dose challenge infection in five out of six ducks. In comparison, five of six ducks vaccinated with undelipidated DHBV and four of four ducks vaccinated with glutaraldehyde inactivated DHBV were unprotected ($p < 0.05$).

Although this solvent system completely inactivated DHBV, viral antigens were retained in an appropriate form to induce immunity. Delipidation of enveloped viruses with specific organic solvents has potential as the basis for development of vaccines.

Keywords: Delipidation; Vaccine development; DHBV; Virus inactivation

The major blood borne viruses HIV, hepatitis C and hepatitis B, together infect 500 million people world wide, causing premature death in many and increasing morbidity in others. Many strategies to control, prevent and treat these viruses have placed a large strain on the health care dollar.

Failure to develop vaccines against major virus diseases such as HIV and HCV, despite a massive investment in research, highlights gaps in understanding of the host response on one hand and on the other limitations in the technology of producing and presenting virus antigens in an appropriate form. The difficulty in making inactivated vaccines against viruses with lipid envelopes is mainly due to the conformational changes in the antigenic epitopes which occur when the envelope is disrupted by the extraction processes. These altered epitopes are either not recognised by the host immune system or elicit inappropriate responses.

In recent years the importance of polyvalent responses in determining outcome of infection has been well recognised in hepatitis B. More recently heterologous cross protection afforded by infection and immunisation with influenza strains has extended this concept to preventive vaccination, and experimental vaccines for HIV now include combinations of structural (receptor and core) as well as non-structural proteins (reviewed in Ferrantelli et al., 2004). Polyvalent vaccines characteristically elicit both cellular and humoral responses and it is thought that collaboration between them is the key characteristic of efficacy.

Experimental production of polyvalent vaccines by expression of viral genes in yeast, baculovirus or sometimes mammalian cell systems makes many assumptions about the critical epitopes, but attempts to use inactivated cell culture supernates as a source of authentic immunogens has failed for most enveloped viruses. A similar technical problem was addressed in blood product research, where the aim of lowering the lipid concentration of plasma while preserving the biological activity, including enzymatic functions, of plasma proteins was met by use of a solvent extraction system of either an ether such as diisopropylether (DIPE) or a mixture of an ether and an

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doi:10.1016/j.jviromet.2006.05.027

alcohol such as DIPE and butanol (Cham et al., 1995; Cham and Knowles, 1976a,b; Kostner et al., 1997). Delipidation of serum with DIPE or DIPE–butanol mixtures causes dissociation of lipids (which are transferred into the organic solvent) and apolipoproteins which remain soluble in the serum. Removal of lipid from such proteins does not affect their physiological role, and does not affect lipid-related enzyme activities (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 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.

Because of the ongoing issue of viral contamination in blood products it was of interest to determine whether this process inactivates enveloped viruses, and additionally whether treatment might preserve viral antigens in authentic form for presentation to the immune system. The duck/duck hepatitis B (Hepadnaviridae) model was chosen because it has been used for many years as a surrogate for hepatitis B virus in anti-viral testing, due to its similar virology and inactivation kinetics. In addition, it is known that hepatitis B vaccine efficacy depends on presentation of the viral surface antigen in particulate form, and the parameters of infection and immunity are readily measured.

Duck serum containing duck hepatitis B virus (DHBV) (1.4×10^9 vge/ml) was delipidated by mixing 2 ml of serum with 4 ml of organic solvent (40% analytical grade butanol and 60% peroxide-free DIPE) and gently rotated at approximately 28 rpm end-over-end for 1 h (Cham and Knowles, 1976a,b). The mixture was centrifuged at $400 \times g$ for 10 min and the aqueous phase (lower phase) was removed into a clean tube, and an equal volume of peroxide-free diethyl ether (DEE) added and mixed by end-over-end rotation at 28 rpm for 2 min. The mixture was then centrifuged for 10 min at $400 \times g$ to separate the serum and DEE phases. The aqueous serum phase was removed and was again mixed with an equal volume of DEE, centrifuged and the delipidated serum aqueous phase removed as before. Residual traces of DEE in the delipidated serum were removed by airing in a fume cabinet. All reactions took place at 20 °C.

Residual infectivity of the delipidated DHBV serum was tested by inoculating day-old ducklings with 4.6×10^7 vge or $>10^5$ times the 50% infectious dose (100,000 ID_{50}) of DHBV into the peritoneal cavity (Vickery and Cossart, 1996). Positive control ducks received an equal dose (100,000 ID_{50}) of undelipidated DHBV serum and negative control ducks received an equal volume of serum containing no DHBV. The ducklings were euthanased on day 12 and their livers removed for DHBV DNA analysis initially by dot blot hybridisation (Deva et al., 1996) and if negative by PCR of the PreC/core region of DHBV. Primers were 5′ CGGAATTCTCTTACATACACCCCTCTCTC and 5′ GGGGAAATTTGAGGTTTGGATCCCG and amplified a 1021 bp segment. The reaction mix consisted of 0.4 μM of each primer, 1.5 units of Taq polymerase, 2.5 mM of $MgCl_2$ and 0.2 mM dNTPs in 1× reaction mix. One of the positive control ducks inoculated with untreated DHBV serum died prior to 6 days of age and was excluded from further analyses. The Fisher's exact test was used to test for significant differences ($p < 0.05$) between groups of ducks subjected to different treatments.

Table 1
DHBV status of ducks injected with 10^5 times the 50% infectious dose of either treated (delipidated) or untreated (positive control) serum

	DHBV positive	DHBV negative
Untreated serum	6	0
Delipidated serum	0	7
Negative control	0	7

Delipidation of the positive serum pool completely inactivated DHBV. All seven ducks inoculated with delipidated serum remained DHBV negative while all six control ducks inoculated with untreated DHBV serum were infected ($p < 0.001$). All seven negative control ducks inoculated with serum containing no DHBV remained DHBV negative. A reduction in viral viability has also been shown with DIPE treatment of simian immunodeficiency virus (SIV) which leads to a $2 \log_{10}$ reduction in infectivity (Kitabwalla et al., 2005) (Table 1).

The second aim of the study was to determine if removing lipids from the envelope of DHBV would preserve the viral proteins in their antigenic form. The antigenicity of the delipidated DHBV positive serum was tested by comparing its ability to induce immunity and prevent infection in ducks when used as a vaccine with that of serum containing DHBV inactivated by glutaraldehyde treatment.

Ducks were inoculated with their respective vaccines on day 8 IP without adjuvant. On days 16 and 22 ducks were inoculated intramuscularly with their respective vaccines emulsified in Freunds Incomplete Adjuvant. The delipidated DHBV serum test vaccine was used to vaccinate six ducklings. The first and second doses were equal to 4.8×10^6 vge of delipidated DHBV and the third dose was equivalent to 2.8×10^7 vge of delipidated DHBV.

DHBV in the same serum pool was inactivated by incubation with 2% glutaraldehyde (Aidal Plus, Whiteley Industries, Sydney) for 10 min at room temperature and an equivalent dose of glutaraldehyde inactivated DHBV was used to vaccinate four ducklings using the same vaccination schedule as above. The six control ducklings were vaccinated in the same manner with an equivalent amount of untreated DHBV negative serum.

These ducks were then challenged with 1.8×10^{10} vge of DHBV (equivalent to $10^{4.7}$ time the ID_{50} dose for this age and weight duck) given intravascularly (IV) on day 29 posthatch. Ducks were bled prior to vaccination on days 1 and 10, prior to challenge on day 17 and 23 and post challenge on days 37, 43 and 52 and their sera tested for DHBV DNA by dot-blot hybridisation.

The presence of anti-DHBs antibody was tested for prior to challenge on day 29 and post challenge on day 43 by Dr. Jilbert as previously described (Jilbert et al., 1991). Briefly, 96 well flat bottom plates were coated with anti-DHBs (MAB I H ascites) diluted 1 in 5000 in bicarbonate buffer (pH 9.6) at 37 °C for 1 h, washed three times and unbound binding sites blocked with 200 μl of 5% skim milk in PBS for 1 h at 37 °C. prior to