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Pre-clinical immunogenicity of human papillomavirus alpha-7 and alpha-9 major capsid proteins

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ABSTRACT

Human papillomavirus (HPV) vaccines confer protection against the oncogenic genotypes HPV16 and HPV18 through the generation of type-specific neutralizing antibodies raised against the constituent virus-like particles (VLP) based upon the major capsid proteins (L1) of these genotypes. The vaccines also confer a degree of cross-protection against some genetically related types from the Alpha-9 (HPV16-like: HPV31, HPV33, HPV35, HPV52, HPV58) and Alpha-7 (HPV18-like: HPV39, HPV45, HPV59, HPV68) species groups. The mechanism of cross-protection is unclear but may involve antibodies capable of recognizing shared inter-genotype epitopes. The relationship(s) between the genetic and antigenic diversity of the L1 protein, particularly for non-vaccine genotypes, is poorly understood.

We carried out a comprehensive evaluation of the immunogenicity of L1 VLP derived from genotypes within the Alpha-7 and Alpha-9 species groups in New Zealand White rabbits and used L1L2 pseudoviruses as the target antigens in neutralization assays.

The majority antibody response against L1 VLP was type-specific, as expected, but several instances of robust cross-neutralization were nevertheless observed including between HPV33 and HPV58 within the Alpha-9 species and between HPV39, HPV59 and HPV68 in the Alpha-7 species. Immunization with an experimental tetravalent preparation comprising VLP based upon HPV16, HPV18, HPV39 and HPV58 was capable of generating neutralizing antibodies against all the Alpha-7 and Alpha-9 genotypes. Competition of HPV31 and HPV33 cross-neutralizing antibodies in the tetravalent sera confirmed that these antibodies originated from HPV16 and HPV58 VLP, respectively, and suggested that they represent minority specificities within the antibody repertoire generated by the immunizing antigen. These data improve our understanding of the antigenic diversity of the L1 protein *per se* and may inform the rational design of a next generation vaccine formulation based upon empirical data.

1. Introduction

Human papillomavirus (HPV) vaccines, Cervarix® and Gardasil®, comprise virus-like particles (VLP) based upon the major capsid protein (L1) of HPV16 and HPV18 and are highly efficacious at preventing persistent infection and more progressive disease associated with these two high risk genotypes in clinical trials [1]. Gardasil® also contains VLP representing HPV6 and HPV11, the principal genotypes associated with genital warts. HPV16 and HPV18 account for ca. 70% of cervical cancers worldwide [2,3] and recent epidemiological data for Australia [4], the USA [5] and the UK [6,7] demonstrate reductions in the prevalence of these two genotypes following the introduction of national HPV vaccination programs. Neutralizing antibodies against HPV16 and HPV18 can be detected in the serum and cervicovaginal secretions of vaccinees [8–10] and passive transfer of immune sera, purified immunoglobulin (IgG) and monoclonal antibodies (MAbs) can protect animals against papillomavirus challenge [11–13]. These observations have led to the reasonable assumption that vaccine-induced, type-specific protection is mediated by neutralizing antibodies [1,14].

Cross-protection has also been demonstrated against some genotypes within the Alpha-papillomavirus species groups, Alpha-9 (HPV16-like: HPV31, HPV33, HPV35, HPV52, HPV58) and Alpha-7 (HPV18-like: HPV39, HPV45, HPV59, HPV68) [1,15,16] and coincides with the detection of cross-neutralizing antibodies in the serum and cervicovaginal secretions of vaccinees [10,17–20]. Such

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antibodies may be effectors, or their detection may have utility as a correlate or surrogate of vaccine-induced cross-protection [21].

The development of potential next generation vaccines to improve the breadth of genotype coverage [1,22] is based upon two approaches: improving the immunogenicity of a conserved region of the minor capsid protein (L2) to generate broadly neutralizing antibodies [23], and using a multivalent L1 VLP-based vaccine that induces type-specific antibodies against a wider array of HPV genotypes (HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, HPV58; V503, Merck Research Laboratories). The latter approach is the most advanced and early clinical trial data show promising immunogenicity and efficacy profiles [24], whereas L2-based candidate vaccines are currently in pre-clinical development [23]. Reduced dosing schedules for the current HPV vaccines are also being investigated with data suggesting non-inferiority of type-specific antibody responses, although there is an impact on the development of cross-neutralizing antibodies [10,25–27].

Early pre-clinical immunogenicity [28–30] and MAb reactivity [17] data suggest a degree of inter-genotype antigenic similarity within the Alpha-7 and Alpha-9 species groups. The extent of this antibody cross-reactivity is unclear as only a limited number of immunogens and target antigens have been used. Some of these data have been generated using L1-based targets [28], rather than pseudovirus targets bearing both the L1 and L2 proteins, with both proteins being necessary for efficient infectivity and the appropriate presentation of L1 conformational epitopes [23,31,32].

We carried out a comprehensive pre-clinical evaluation of the immunogenicity of L1 VLP derived from multiple HPV genotypes within the Alpha-7 and Alpha-9 species groups and used L1L2 pseudoviruses, representing these same genotypes, as the target antigens in neutralization assays. Such data should improve our understanding of the antigenic diversity of the L1 protein *per se* and may inform the design of a next generation vaccine formulation that encompasses a limited number of antigens based upon empirical data.

2. Materials and methods

2.1. L1 VLP immunogens

Cervarix® was obtained through the National Vaccine Evaluation Consortium, UK.

L1 VLP representing Alpha-7 and Alpha-9 HPV genotypes and control Bovine Papillomavirus (BPV) were expressed using the Bac-to-Bac® Baculovirus System (Life Technologies), as previously described [33,34], wherein the L1 genes shared 100% amino acid sequence identity with the L1 genes of the pseudovirus clones [20] used for the neutralization assay (see Section 2.3).

2.2. Immunization protocols

Five week old female BALB/c mice were immunized with saline (naïve) or 1/10th (2 μg each HPV16 and HPV18 VLP) the human dose equivalent of Cervarix® [35] by the intramuscular (IM) or subcutaneous (SC) routes. Two schedules were investigated whereby immunizations were carried out at week (W) 0, W3 and W7 or at W0, W4 and W12.

Eight to ten week old female New Zealand White (NZW) rabbits were immunized subcutaneously with saline (naïve) or 1/4th (5 μg each HPV16 and HPV18 VLP) the human dose equivalent of Cervarix® at W0, W4 and W12.

Eight to ten week old female NZW rabbits were immunized subcutaneously with 5 μg each of the indicated in house L1 VLP (or 5 μg each of HPV16, HPV18, HPV39 and HPV58 for the tetravalent preparation). VLP were absorbed onto 3% alhydrogel (250:1 (v/v), Superfos Biosector) for 1–2 h at room temperature under gentle rotation. For the final preparation of the rabbit inoculum, the VLP-alhydrogel mix was diluted in sodium phosphate buffer pH 6.5 (final concentration 2.7 mM NaH_2PO_4 and 3.3 mM Na_2HPO_4) with 150 mM NaCl, alhydrogel (250 μg/mL Al^{3+}), Sigma Adjuvant System (25 μg/mL monophosphoryl lipid), and incubated with gentle rotation at room temperature for a minimum of 15 min. Rabbits received additional immunizations at W4 and W12.

In all cases, serum samples were collected prior to the first immunization (pre-immunization) and two weeks following both the second and third doses.

All animal husbandry and regulated procedures were carried out in strict accordance with UK Home Office guidelines and governed by the Animals (Scientific Procedures) Act 1986 which complies with the EC Directive 2010/63/EU and performed under licences PPL 80/2537 and PPL 70/6562-3 granted only after review of all the procedures in the licence by the local Animal Welfare and Ethical Review Bodies.

2.3. Neutralization assay

L1L2 pseudoviruses representing Alpha-7 and Alpha-9 HPV genotypes and BPV, and carrying a luciferase reporter, were expressed from transiently transfected 293TT cells, purified and characterized as previously described [20,36]. The equivalent of a Tissue Culture Infectious Dose 50% ($TCID_{50}$) was estimated using the Spearman-Karber equation and a standardized input of 300 $TCID_{50}$ was used for all pseudoviruses. Serum samples were serially diluted and the 80% reciprocal neutralization titer estimated by interpolation. Heparin (H-4784; Sigma–Aldrich, UK) was included as a positive inhibitor control and as an indicator of inter-assay reproducibility. The median (Inter-quartile range, IQR) inhibitory concentrations (μg/mL) were as follows: HPV16 11.9 (9.5–22.3; $n=7$), HPV31 5.1 (3.3–8.1; 6), HPV33 13.1 (7.4–19.4; 6), HPV35 3.1 (2.9–4.9; 6), HPV52 25.2 (13.6–31.9; 6), HPV58 8.2 (3.6–19.4; 6), HPV18 3.9 (3.4–5.0; $n=6$) HPV39 5.8 (4.0–7.2; 5), HPV45 3.7 (3.5–3.9; 6), HPV59 13.6 (11.7–16.3; 6), HPV68 7.0 (6.5–12.1; 6) and BPV 73.5 (59.1–75.9; 5).

2.4. Competition of neutralizing antibodies using L1 VLP

Serial dilutions of selected final bleed rabbit sera were pre-incubated for 1hr at room temperature with 2 μg of L1 VLP (HPV16, HPV31, HPV33 or HPV58), followed by addition of 300 $TCID_{50}$ of L1L2 pseudoviruses representing the same HPV genotypes for 1 h at room temperature, before being transferred to 293TT cells for 72 h at 37 °C. The 80% reciprocal neutralization titers were estimated by interpolation.

2.5. Data analyses

For the comparison of inter-genotype neutralization data a heatmap representation of $\log_{10}$ titers (range 1.0–6.0 $\log_{10}$) was employed with titers below the assay threshold of 20 being censored with a value of 10 (1.0 $\log_{10}$). The phylogenetic relationship between L1 amino acid sequences (neighbor-joining [NJ] tree) and inter-genotype distance matrices ($n=500$ bootstrap replicates; heatmap range 0.0–1.0) were created using Mega v4.1 [37].