Production and Immunogenicity of Chimaeric Human Papillomavirus-like Particle Vaccines

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ABSTRACT

Human papillomavirus (HPV) infection, specifically with oncogenic types, has been implicated in effectively all cervical cancer cases. Cervical cancer is a global health burden, especially in the developing world. Up to 18 types of HPV are considered oncogenic, of which HPV-16 and -18 cause 70% of cervical cancer cases worldwide. Two vaccines are available on the market: Gardasil®, targeted against HPV-16, -18; -6 and -11, and Cervarix™, against -16 and -18. Both vaccines are based on the L1 capsid proteins of the types they are targeted to and are efficient, prophylactic, type-specific vaccines. However, two problems remain: they do not protect against non-vaccine types, that may cause a significant proportion of cancers specifically in African and HIV-positive populations, and they cannot be used to treat existing infections. We therefore designed eight different chimaeric vaccines where regions of HPV-16 L1 were replaced with the same number of amino acids (aa) constituting different epitopes. Cross-neutralising epitopes derived from HPV-16 L2 protein between amino acids (aa) 108-120 (SAF), 56-81 (L2.56), 17-36 (L2.17) and BPV-1 L2 aa 1-88 (BPV) were inserted after aa 413 in HPV-16 L1. HPV-16 E7 aa 49-57 (E7M) and 86-93 (E7H), used in immunotherapy of HPV-related cancer in mice and humans respectively, were inserted after aa 417. Additionally, two variations of the L1/L2 (108-120) chimaera were created where the E7 epitopes replaced aa 433 to 441 (E+E7M and E+E7H). All eight constructs were expressed in insect cells and six of these (excluding E7H and E+E7H) were tested in animals. Results showed that L2.56, L2.17 and SAF elicited anti-L1 antibodies detected by ELISA, neutralised HPV-16 *in vitro* and expressed well in insect cells. L2.56 and L2.17 also elicited anti-L2 antibodies. L2.56 showed the most promise as a vaccine candidate, since it elicited the highest levels of anti-L1 and anti-L2 antibodies and could assemble into more immunogenic higher-order structures. BPV could be eliminated as a vaccine candidate based on its immunogenicity and expression levels. Both L1/E7 chimaeras elicited anti-L1 antibodies by ELISA, but E+E7M showed more promise as a therapeutic vaccine than E7M, because it primed the cellular arm of the immune system, whereas E7M primed the humoral arm. Chimaeric L1 vaccines incorporating cross-neutralising L2 and immunotherapeutic E7 peptides and replacing up to 27 aa of HPV-16 L1 of the helix 4 and the region to its C-terminal are promising second-generation vaccine candidates. More such chimaeras will be created and tested.

1. LITERATURE REVIEW

1.1 Introduction

Papillomaviruses (PVs) generally cause benign tumours, including warts. They are diverse and occur in many mammalian and bird species (de Villiers *et al.*, 2004). Because the L1 open reading frame (ORF), which codes for the "late" major capsid protein, is the most conserved gene within the genome, it has been used to classify new types over the past 15 years. Papillomaviruses constitute the family *Papillomaviridae,* which is divided into 16 genera, of which five, namely the Alpha-, Beta-, Gamma-, Mu- and Nu-papillomaviruses, affect humans and collectively they constitute the human papillomaviruses (HPVs), which are of interest in this study. The HPV genome encodes eight ORFs; namely L1, L2, E1, E2, E4, E5, E6 and E7. Figure 1.1 shows the genome organization of HPV and the genes encoded by its genome as well as a structure model for papillomavirus.

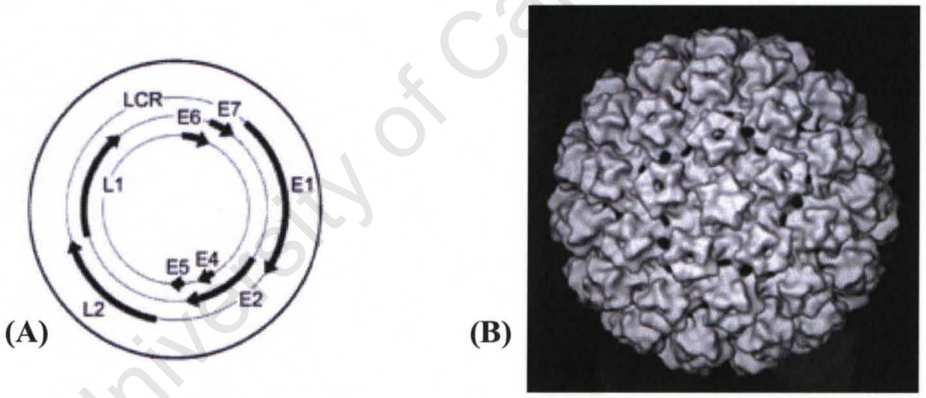


Figure 1.1 (A) *HPV genome organisation, indicating the different ORFs. LCR=Long control region. This region is a non-coding region involved in regulation of virus gene expression. (Modified from (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005)* ***(B)*** *Structure model of papillomavirus. (Taken from (Xu et al., 2006)*

HPVs can be divided into mucosotropic types, which affect the epithelial linings of the upper respiratory system and anogenital tract; and cutaneous types, which affect the skin (Castellsague, 2008; Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). Mucosal HPVs occur in benign and malignant lesions of the anogenital tract in both sexes. HPVs that only occur in benign lesions are designated as "low-risk", whereas HPVs found in malignant or

cancerous lesions are designated as "high-risk". Mucosotropic HPVs cause genital warts and cancers of the cervix, vulva, vagina, penis and anus. They also occur occasionally in the oral cavity, oropharynx, larynx and oesophagus. Cutaneous HPV cause cutaneous and plantar warts and are associated with *Epidermodysplasia verruciformis* (EV) and some skin cancers.

Of particular interest in our study are the cervical cancer-causing HPV types, which all fall into the alpha-genus of the family *Papillomaviridae* (de Villiers et al., 2004). Of all the human papillomavirus (HPV) types, about 15 types are considered high-risk for cervical cancer (Castellsague, 2008). These high-risk types, according to Castellsague (2008), are types 16, 18, 45, 31, 33, 52, 58, 35, 39, 59, 56, 51, 68, 73 and 82, but three types are considered as probable high-risk. These are HPV-26, -53 and -66 (Moodley *et al.*, 2009). HPV-16 is the causative agent in about 53% of cases and is thus the major type involved in carcinogenesis (Castellsague, 2008).

Infection with a high-risk HPV is the single causative factor for cervical cancer, with 95 to 99% of cases associated with the virus (Castellsague, 2008). In fact, there are four necessary steps in the development of cervical cancer, the first of which is infection with human papillomavirus (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). Secondly, the infection must persist; in fact, in 90% of cases, the infection will be cleared within 2 years (Cutts *et al.*, 2007). If the infection persists, it must then progress to precancerous lesions, at which stage the lesion could also still regress naturally, followed by invasion of the tissue by the transformed cells. (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005).

Cervical cancer is a global health burden, especially so in the developing world. About half a million women worldwide are affected annually and 260 000 die as a result (Cutts *et al.*, 2007). Of these women, 80% are in developing countries. In South Africa, HPV infection and cervical cancer prevalence is high: 20.4% of women with normal cytology are infected (Williamson *et al.*, 2002). It is the second most prevalent cancer in women (Williamson *et al.*, 2002; Mqoqi *et al.*, 2004) in South Africa with 6800 women facing new cases of this disease annually and 3700 deaths occurring

annually as a result (Sinanovic *et al.*, 2009). In the black population, it is the most common cancer amongst women (Mqoqi *et al.*, 2004).

There is a need for a vaccine, especially in developing countries where the mortality and morbidity rate is high (Cutts *et al.*, 2007). Prophylactic vaccination is supposed to be the most effective way of eradicating and preventing disease in general (World Health Organisation, 2005). In developed countries, screening programmes have reduced the incidence of cervical cancer, but this has not been the case in developing countries (Lowy *et al.*, 2008) and in South Africa (Sinanovic *et al.*, 2009). Limited resources prevent effective implementation of screening policies and so vaccination programmes could bridge the gap.

There are two prophylactic HPV vaccines on the market. Cervarix™ is manufactured by Glaxosmithkline, and is targeted against HPV-16 and -18, while Gardasil® is manufactured by Merck and is targeted against types -6 and -11 as well (Schiller *et al.*, 2008). Both vaccines are polyvalent vaccines based on L1 of the respective HPV types against which they are targeted (Schiller *et al.*, 2008). Although native capsids of HPV consist of both L1 and L2 proteins (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005), L1 by itself can self-assemble into virus-like particles (VLPs), which have been shown to be immunogenically similar to the native human papillomavirus, while being non-infectious (Kirnbauer *et al.*, 1992). Antibodies (Breitburd *et al.*, 1995), specifically those targeted against L1 (Kirnbauer *et al.*, 1992), have been shown to be the major factor in preventing PV infection in animals.

One problem is the high cost of current vaccines on the market. At current prices, vaccination against HPV is only cost-effective in countries that can afford to implement such a programme (Techakehakij & Feldman, 2008). In South Africa, the vaccine price needs to be reduced by at least 60% in order to make a vaccination strategy on top of a screening programme more cost-effective (Sinanovic *et al.*, 2009). These data imply that second-generation HPV vaccines should aim to be more affordable.

This review considers clinical trial data showing the high efficacy of current prophylactic HPV vaccines that are on the market. It also considers the two major problems that still need to be addressed in order to combat cervical cancer and that need to be considered in the development of second-generation HPV vaccines; namely, prevention against HPV types not included in current vaccines, and treatment of existing infections. It discusses the use of essential epitopes from HPV proteins other than L1, specifically L2 and E7, to combat these problems. The use of L1 as a carrier molecule for essential epitopes is also discussed.

1.2 The efficacy of currently licensed HPV vaccines

Gardasil® and Cervarix™ have been extensively tested and are still being tested in clinical trials. Both have shown exceptional efficacy in populations not previously exposed to HPV infection. Since the aim is to vaccinate girls before sexual debut, data on prevention of infection in such a population would be most important. It is also important to consider the different endpoints the vaccine should prevent. The vaccine may in some cases not prevent infection with an HPV type, but may help clear the infection before it progresses to cancerous lesions. Another consideration is whether the endpoint is due to a vaccine-specific type or not. Lastly, levels of antibodies secreted will also be important, since that would be an indication of the strength of an immune response mounted as well as an indication of the memory immune response.

Gardasil® showed a >90% efficacy rate in populations not previously exposed to HPV against all endpoints, such as infection with vaccine-specific types, cervical intraepithelial neoplasia (CIN) of all grades of severity, adenocarcinoma *in situ* (AIS), vulvar intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VaIN) and genital warts (Tay *et al.*, 2008; Villa *et al.*, 2006; Garland *et al.*, 2007; Ault, 2007; Joura *et al.*, 2007; Joura *et al.*, 2008). In most analyses, it was 100%.

In one study (FUTURE II STUDY GROUP, 2007), Gardasil® given to women who were positive for at least one, but fewer than 4 HPV types at the start of the study or became so in between vaccinations, showed a 91.1% efficacy against development of CIN and 93.8% efficacy against external vaginal or anogenital lesions. The efficacy was for lesions attributed to the vaccine type they were negative for throughout the

study. The vaccine was, however, 100% effective at preventing higher-grade lesions in this trial.

For Gardasil®, in the FUTURE I trial (Garland *et al.*, 2007), 99.5% of subjects seroconverted one month after the last dose. In combined results from FUTURE I and FUTURE II (Joura *et al.*, 2008), 89% or more of subjects had seroconverted to types 6, 11 and 16 44 months after the first vaccination. Seroconversion to type 18 was lower, with only 60% of subjects positive, but protection against lesions caused by type 18 remained as high as for the others. Five years after the first vaccination, participants in one of the clinical trials (Olsson *et al.*, 2007) had antibody levels at least as high as one would have from natural infection with HPV, in most cases higher. One study tested the memory immune response of Gardasil®. A challenge dose of vaccine was given 5 years after the first dose. Eighty-three percent of participants mounted a memory immune response with antibody levels higher than at month 60. Of those who had been seronegative at month 60, 75% seroconverted. More than 50% of them mounted an immune response higher than they did after the third vaccination. Antibody levels also rose more rapidly than for any of the other boosts. Gardasil® thus elicited high titres of antibodies.

For Cervarix™, in the main Phase III trial that was conducted (Paavonen *et al.*, 2009; Paavonen *et al.*, 2007), only women aged 15-25 years were included, thus representing a population of young women most likely to receive the vaccine. Endpoints included HPV infection, precancerous lesions and CIN2 and CIN3. In a population that was HPV negative at month 0 and month 6, the vaccine was determined to be efficient at preventing 92.9% of CIN2+ due to both vaccine types. Cervarix™ was also efficient at preventing 80.4 and 75.9% of 6 month and 12 month persistence of vaccine-type infection respectively (Paavonen *et al.*, 2007). Where existing lesions were assigned to specific types, 98.1% of CIN2+ due to type 16 or 18 could be prevented (Paavonen *et al.*, 2009).

For Cervarix™, it was shown that a very high number, more than 99.5% of women, seroconverted. Different age groups did not affect the strength of the immune response (Pedersen *et al.*, 2007; Schwarz *et al.*, 2009). No data is available to date on the memory response.

The data above would indicate that both Cervarix™ and Gardasil® are highly efficient type-specific prophylactic vaccines. They would be highly efficient at preventing HPV-16 and –18 related infection and lesions in young girls vaccinated before sexual debut as well as at eliciting high levels of anti-HPV-16 and –18 neutralising antibodies. L1 forms the basis of these highly efficient vaccines, therefore the use of this protein as a basis for the design of second-generation vaccines is a highly feasible approach.

1.3 The need for a more broadly neutralising prophylactic vaccine

1.3.1 L1 elicits type-specific antibodies

The question is whether current vaccines on the market would prevent against infection with types other than the types they are targeted to. It is known that neutralising antibodies elicited by vaccination with L1 VLPs are mostly type-specific. Rabbits vaccinated with bovine papillomavirus (BPV) particles, who are then challenged with cottontail rabbit papillomavirus (CRPV), are not protected against developing papillomas (Breitburd *et al.*, 1995). It is known that conformational epitopes are neutralizing sites and from monoclonal antibody (mAb) studies, it is known that most mAbs bind conformational epitopes and that they are type-specific. Many mAbs bind epitopes found on the surface loops of L1 (Bishop *et al.*, 2007b).

The reason for eliciting type-specific antibodies is related to the structure of VLPs. Even though the amino acid sequences of L1 proteins from different types are highly conserved, and their core β-sheet structures superimpose well, amino acid differences in their surface loops cause the loops to deviate significantly from one another in structure. This deviation was shown in a study by Bishop *et al.* (Bishop *et al.*, 2007b) where the structures of pentamers, which display surface loops in the same way that VLPs would, of types 16, 11, 35 and 18, were compared to one another. (Seventy-two L1 pentamers assemble to form VLPs) The deviation provides a structural reason for the type-specificity of anti-L1 antibodies. Figure 1.2 illustrates the distinct structural features of L1 pentamers from the four different types.

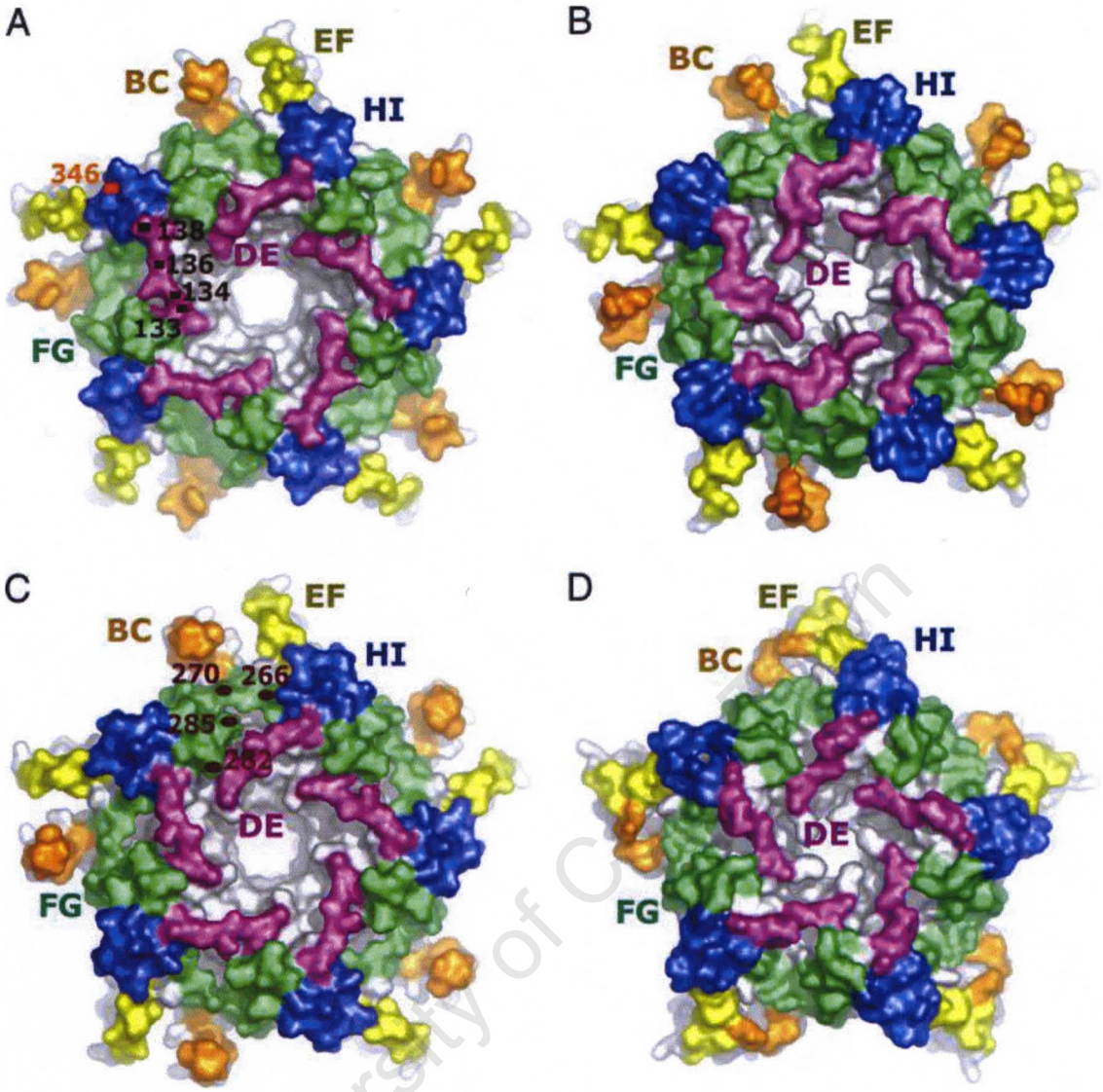


Figure 1.2 *The deviation in structure of surface loops on L1 pentamers from* ***(A)*** *HPV11,* ***(B)*** *HPV35,* ***(C)*** *HPV-16 and* ***(D)*** *HPV-18. Surface loops are shown in colour to distinguish them from the rest of the pentamer. BC loops are shown in orange, DE loops in violet, EF loops in yellow, FG loops on green and HI loops in blue. Indicated on HPV-11 and HPV-16 are amino acids essential to binding sites for neutralising mAbs. Black squares: H11.F1 and H11.G5 binding sites. Orange square: H11.H3. Red square: H16.V5 and H16.E70. Figure taken from Bishop et al. (Bishop et al., 2007b)*

In humans, L1-based vaccines elicit very low levels of cross-neutralising antibodies. As already mentioned above, Cervarix™ and Gardasil® are highly efficient type-specific vaccines, but are far less efficient against types not included in the vaccines. Data from clinical trials involving Gardasil® show a significant reduction in efficacy when lesions caused by types other than –16 or –18 are not excluded in intention-to-treat (ITT) populations (Garland *et al.*, 2007; Joura *et al.*, 2007; Ault, 2007; Barr *et al.*, 2008). These populations include all individuals without any exclusion criteria

applied; for example, they may be HPV-positive with abnormal cytology before vaccination. In HPV-naïve women, efficacy against types other than those included in the vaccine, was much lower than for the vaccine types (Brown *et al.*, 2009). Efficacy against infection with the most closely related types, types –45 and –31, was 40.3%, and against all members of the same species as types –16 and -18, 25%. Against CIN 1-3 or AIS, it was 43.6 and 23.4 % respectively and against CIN 2-3 or AIS, it was 58.7 and 32.5% respectively. The lower percentage for less related types suggests that efficacy can be positively correlated with relatedness, further supporting type-specific protection by Gardasil®. There was however, one study that showed cross-neutralisation of HPV-45 pseudovirions by human sera, although the neutralisation titres were substantially lower than those against the related type-18 (Smith *et al.*, 2007).

In the main clinical trial involving Cervarix™ (Paavonen *et al.*, 2009), the vaccine was efficient at preventing 70.2% of CIN2+ in HPV-naïve women when non-vaccine types were not excluded from the analyses. This figure is significantly lower than the 92.9% when these types are excluded. CIN2+ associated with 12 non-vaccine oncogenic types was prevented in 54% of cases. Cross-protection against closely related types –31 and –45, as well as –33, which belongs to the same species as type –16, was detected. In the interim analysis of this trial (Paavonen *et al.*, 2007), efficacy against preventing infection with oncogenic non-vaccine types 12 months after vaccination, was only about 27% as opposed to 75.9% mentioned previously.

1.3.2 Factors influencing HPV type distribution

An unexplored problem is that currently licensed vaccines that are targeted only against carcinogenic types 16 and 18 may not necessarily be as effective in all regions of the world, as there are regional differences in the proportions of cervical abnormalities due to different types of HPV (Adler *et al.*, 2008; Allan *et al.*, 2008; Bosch *et al.*, 2008). A global meta-analysis of all the data collected between 1999 and 2005 (Bosch *et al.*, 2008) provides data to this effect. Africa has a higher prevalence of any type of HPV at 22.1 % of all women with normal cytology infected, versus 10.4% of women worldwide. Figure 1.3 shows the percentage each HPV type contributes towards the total number of infections in women with normal cytology. In

Africa, HPV-16 only contributes towards 12.2 % of infections, whereas worldwide it contributes towards 25% of infections. Figure 1.3 also shows that type 35 is ranked sixth in Africa, whereas it is ranked ninth worldwide and its proportional prevalence is three times lower than in Africa. The data therefore indicates a greater burden of HPV types other than 16 and 18 in Africa, as compared to worldwide estimates, as well as a type distribution shift with respect to HPV-35. These data agree with another pooled analysis (Clifford *et al.*, 2005), where type 35 was observed to occur at significant levels in Africa. In fact, HPV-35 was shown to be as common as HPV-16 in women with normal cytology.

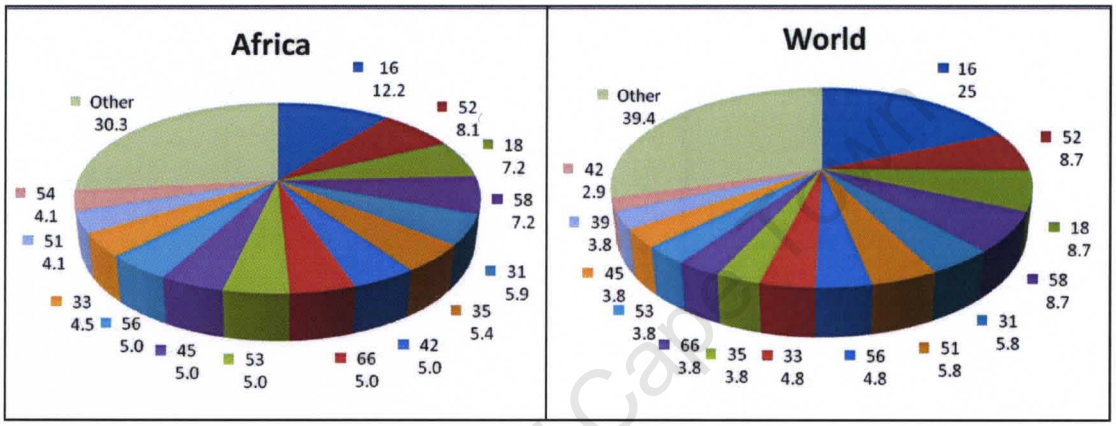


Figure 1.3.*Proportional prevalence of HPV types in women with normal cytology, in Africa and worldwide. Types are indicated next to the key, with the relevant percentage below each type. Data taken from Bosch et al. (Bosch et al., 2008)*

However, statistics for women with higher-grade lesions such as invasive cervical cancer (ICC) are more valuable, since it gives a better picture of the types associated with cancer. The percentages of HPV types detected in women with ICC is shown in Figure 1.4 for Africa and worldwide. Although types 16 and 18 contribute towards 70% of ICC both in Africa and worldwide, the third and fourth ranked types in both Africa and worldwide, types 33 and 45, show a higher incidence in Africa at 7.6 and 6.6 % respectively, compared to 4.3 and 3.7 % worldwide. In the case of type 35, it is ranked the fifth most common type in ICC in Africa, but only the eighth worldwide. A review on the epidemiology of HPV in sub-Saharan Africa (Louie *et al.*, 2009) provides similar statistics. It indicates that types observed in women with ICC and ranked third to fifth most prevalent, types 45, 33 and 35, each contribute towards 9.8, 7.1 and 4.5% of the types observed, which is significantly higher than the worldwide estimates for these types (Figure 1.4).

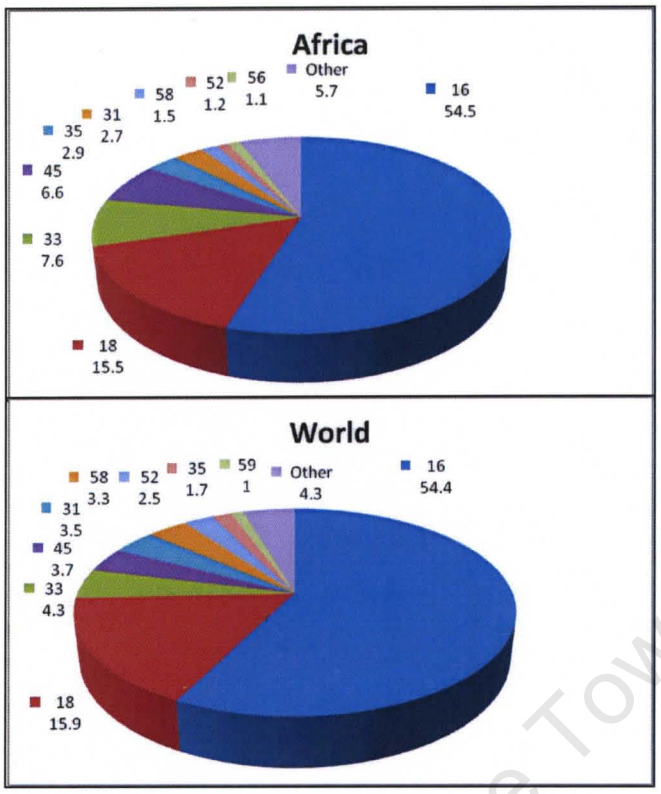


Figure 1.4. *HPV type prevalence in women with invasive cervical carcinoma, in Africa and worldwide. Types are indicated next to the key, with the relevant percentage below each type. Data taken from (Bosch et al., 2008)*

Other studies have looked at the type distribution in countries within Africa and have shown that HPV-16 does not necessarily predominate as it does worldwide. In Mozambique, HPV-35 was reported to be the most common type amongst women with no cervical abnormality and cervical dysplasia (a precursor to squamous cervical carcinoma), although in women with cervical cancer, types 16 and 18 still caused 69% of disease, but with co-infections with other types being common (Naucler *et al.*, 2004). In Uganda (Banura *et al.*, 2008), although the data does not give an indication of the cytology, of the carcinogenic types, HPV-18 and 52 were the most common, followed by type 16, 51 and 33 in order of prevalence. Another small-scale study in Uganda, which also didn't take lesion grade into consideration (Blossom *et al.*, 2007), showed that of the high-risk types, type 52 was by far the most common, followed by type 16, 58, 59 and 51. HPV-18 may also not necessarily be the second-highest ranked type in cervical cancer in African countries. In Senegal, one study found that HPV-16 and HPV-58 were most strongly associated with cervical cancer (Xi *et al.*, 2003) as opposed to HPV-16 and -18.

Similarly to the situation in African countries, in a recent study in South Africa (Allan *et al.*, 2008), in black and mixed-race women with HSILs, the most prominent types emerged as types 16, 35, 31 and 52. Type 18 only emerged as the fourth most likely cause of high-grade squamous intraepithelial lesions (HSILs) or CIN2+. It is important to consider that many of the infections in this study were multiple, and that in many cases, type 35 only occurred as a co-infection with type 16. There were, however, some single infections with type 35. HPV-16 and –35 were associated with increased disease severity in this study, and more so compared to type 18. Another small-scale study involving 159 women from Pretoria in South Africa also showed the potential of the non-vaccine type HPV-35 to significantly contribute towards the cervical cancer burden, since it emerged as the predominant type in HSIL cases in this study (Said *et al.*, 2009). Marais *et al.* (Marais *et al.*, 2008) conducted a study on 1003 mixed-race and black women in South Africa and found that types 16, 53 and 52 were equally prevalent, regardless of the grade of the lesion.

In HIV-infected women there is also a shift towards types other than HPV-16 and HPV-18 (Clifford *et al.*, 2006; Allan *et al.*, 2008; Adler *et al.*, 2008). A 2006 meta-analysis of all published data on HPV types among HIV-positive women (Clifford *et al.*, 2006) revealed that the proportion of HPV infection caused by HPV type 16 is lower in HIV-positive women than in HIV-negative women, regardless of cervical cytology. As the severity of cervical lesions increased, the number of infections attributable to HPV-16 became more dominant, but less so than in the general population. HIV-positive women with HSIL were more likely to be infected with other high-risk types and also some types regarded as low-risk, but which may be responsible for HSIL in immunosuppressed individuals. The low-risk types, are, however, in the majority of cases, only a co-infection with a high-risk type, and may therefore be benign in itself. Once again, there were a few HIV-positive HSIL patients with single low-risk type infections. In Sub-Saharan Africa (Louie *et al.*, 2009) HPV-52 was shown to be more prevalent than HPV-18 in ICC cases. HPV-16 only contributed to 39.2% of ICC cases, compared to the about 50% estimated worldwide and other types, like HPV-35 and -58, contributed significantly to the number of ICC cases. In their review, co-infections involving HPV-16/18 and another type were often seen, which may confound the estimations, but it is not clear to what extent the co-infected type contributes to carcinogenesis.

Many studies in South Africa and other African countries have shown that HPV-16 does not predominate over other types as much as is seen in HIV-negative women. For example, in a South African study (Moodley *et al.*, 2009), among women with abnormal cervical smears, HPV-16, -58 and -51 were equally prevalent and in Zambia (Sahasrabuddhe *et al.*, 2007), the most prevalent types occurring in women with HSIL and SCC were HPV-52, -53 and -58, which were more prevalent than types 16 and 18. Other studies in African countries also indicate the lack of predomination by HPV-16 (Blossom *et al.*, 2007; Singh *et al.*, 2009; Banura *et al.*, 2008)

From the data above, it is therefore possible that Sub-Saharan Africa may have a bigger requirement for prophylactic vaccines targeted at non-vaccine types than other regions of the world. This requirement is due to a significantly higher burden of cervical cancer attributed to non-vaccine types compared to other regions of the world. Secondly, the higher number of people infected with HIV in the Sub-Saharan region means that HPV-16 predominates to a lesser extent in cervical cancer in this region. There is therefore an urgent need for vaccines effective against multiple types and against types other than HPV-16 and HPV-18.

1.4 The role of L2 in creating a more broadly neutralising HPV vaccine

The full-length minor capsid protein, L2, has been shown to elicit antibodies reactive against native HPV virions of types other than the type to which its sequence corresponds. When sheep were immunized with bacterially expressed HPV-6, -16 or-18 full-length L2, the obtained sera neutralised both homologous and heterologous types of pseudovirions, whereas immunisation with L1/L2 capsids did not have this effect (Roden *et al.*, 2000).

1.4.1 Cross-neutralising epitopes of L2

Much research has been done on mapping cross-neutralising epitopes of the ~500 amino acid L2. The N-terminal of L2 or the first 200 amino acids have been the focus of this work. Two mapping studies focused only on this region (Gambhira *et al.*,

2007a; Kondo *et al.*, 2007). However, the cross-neutralising region can be further narrowed down to the first 120 amino acids. Firstly, the first 120 amino acids of HPV-16 L2 are highly conserved across papillomavirus types (shown in Figure 1.5) and more so compared to the C-terminal of the protein (Lowe *et al.*, 2008; Yang *et al.*, 2003). Furthermore, in a study where HPV 16 L2 peptides were displayed on bacterial thioredoxin, amino acids 1-120 were used as a positive control, indicating that this region should contain all the possible epitopes of L2 to elicit neutralising and cross-neutralising antibodies to L1 (Rubio *et al.*, 2009).

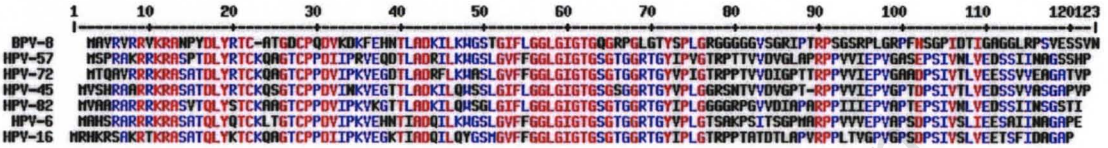


Figure 1.5. *Alignment of the first 120 amino acids of L2 from 7 divergent PV types, showing the high level of conservation. Red indicates a high level of conservation, whereas blue indicates regions that are semi-conserved. Sequences were aligned using MultAlin. (Corpet, 1988)*

In other studies, neutralising epitopes were consistently mapped to within the first 120 amino acids. In this region, there are three main regions of focus, of which the boundaries have not been clearly defined in all cases. The first such region stretches from amino acid 17 to 36. This peptide is highly conserved across HPV types, as can be seen in Figure 1.5, and it has so far been very promising as a region eliciting cross-neutralising antibodies. Linked to various haptens and immunostimulatory molecules, it can elicit antibodies which cross-neutralise up to 10 types of HPV tested so far, as well as the divergent BPV-1 (Gambhira *et al.*, 2007b; Alphs *et al.*, 2008). A monoclonal antibody, RG-1, was generated against this peptide, and neutralised both type 16 and 18 pseudovirions, as well as protecting mice against challenge with HPV-16 pseudovirions containing a luciferase gene (Gambhira *et al.*, 2007b). Depletion studies were also carried out. In these studies, polyclonal sera are depleted of antibodies targeted against a particular epitope, by removal of these antibodies with a column. Removal of antibodies targeted against amino acids 17-36, drastically reduced the ability of anti-L2 sera to cross-neutralise HPV-16 and –18, and in most cases, eliminated it (Gambhira *et al.*, 2007b). These data could indicate that 17-36 is by far the most important neutralising epitope within L2.

Other researchers have used similar regions to the RG-1 epitope in their studies. Schellenbacher et al (Schellenbacher *et al.*, 2009) inserted amino acids 18-31 into BPV L1, creating a chimaeric protein. Only when this protein was denatured, did it induce low titres of cross-neutralising antibodies. This result would indicate that residues 17 and 32 to 36 are probably essential to the epitope. The peptide 18-38, linked to keyhole limpet haemocyanin (KLH), did not induce significant cross-neutralising titres, supporting the neccessity of incuding amino acid 17 in this epitope, but when the same group inserted this peptide into HPV-16 L1, creating a chimaeric protein, cross-neutralisation was broader (Kondo *et al.*, 2007; Kondo *et al.*, 2008). Therefore the effect could have been due to poor immunogenicity of the peptide coupled to KLH. Another group used the peptide spanning from 20-38, displayed on bacterial thioredoxin (Rubio *et al.*, 2009) and obtained excellent neutralisation titres, while performing far better than other peptides used in this study. Therefore the necessity of residues 17-19 is still open to debate and may depend on the differences between studies, like linker haptens and display proteins.

Another region of significance lies between amino acids 56 and 81 of HPV-16 L2, overlapping with a highly conserved region of L2, as illustrated by Figure 1.5. Amino acids 69 to 81 were first tested by Kawana *et al.* (Kawana *et al.*, 1998) Monoclonal antibodies were raised against type 16 L1/L2 capsids. Of these, 11 bound linear epitopes of L2, and seven of those bound the peptide composed of amino acids 69 to 81. Sera against VLPs of types 16, 18, 58 and 6b bound to this peptide. Taken together, this would suggest that this peptide is a common immunodeterminant. The same peptide was also linked to KLH and inserted into BPV L1, used to raise antisera, and neutralisation of native type 11 virions as well as type 16 pseudovirions was tested (Slupetzky *et al.*, 2007). Antiserum to the peptide alone only neutralised type 16 pseudovirions, but antibodies to the displayed peptide also cross-neutralised native type 11 virions. Kondo *et al.* (Kondo *et al.*, 2007) tested the same peptide, and antisera to it cross-neutralised two of the three heterologous pseudovirions tested. A very similar region from amino acid 64 to 81 inserted into thioredoxin was also tested, (Rubio *et al.*, 2009) and antisera to it showed broad cross-neutralisation, although not as good as that elicited by peptide 20-38.

Kondo *et al.* (Kondo *et al.*, 2007) did a mapping study around this epitope, testing for neutralisation of type 18, 31 and 58 pseudovirions. Amino acids 64 to 81, 61 to 75 and 56 to 75 were tested. Only sera to the latter peptide cross-neutralised type 31, suggesting that amino acids 56 to 64 are essential to type 31 neutralisation and that amino acids 75 to 81 are not essential. This observation is supported by data where a peptide of amino acids 75 to 112 displayed on BPV L1 (Schellenbacher *et al.*, 2009), does not neutralise homologous or heterologous pesudovirions, indicating that the amino acids between 75 and 81 do not constitute an epitope by themselves. The peptide 56 to 75 was also displayed on HPV-16 L1 proteins, and neutralised all five pseudovirion types tested (Kondo *et al.*, 2008). However, according to Kawana *et al.* (Kawana *et al.*, 1998), the first group to test this epitope, amino acids 75 to 81 contain essential epitopes, because only one of the monoclonal antibodies raised against 69-81, also reacts with amino acids 63 to 75. Amino acids 56 to 68 may therefore also contain a cross-neutralisation epitope. In summary, not only does the region consisting of amino acids 56-81 contain multiple epitopes, but also incorporating all of these amino acids in a peptide used for immunisation should safely encompass all of them.

The third region of interest lies between amino acids 96 and 122. An epitope consisting of amino acids 108-120 was first discovered by Kawana *et al.* (Kawana *et al.*, 1999) These 13 amino acids also constitute a highly conserved region within L2, as seen in Figure 1.5. A panel of monoclonal antibodies raised against L1/L2 capsids, yielded 2 mAbs that bound to amino acids 108-120 and which also cross-neutralised type 6 pseudovirions. The same group has already vaccinated human volunteers with this peptide and obtained antibodies neutralising type 16 and 52 pseudovirions (Kawana *et al.*, 2003). This peptide, coupled to KLH, and injected into animals, raised sera which cross-neutralised native type 11 virions (Slupetzky *et al.*, 2007). However, these did not show cross-reaction with ROPV or CRPV (Embers *et al.*, 2002). Other peptides from the region of interest have also been tested. Pseudovirion neutralisation abilities of sera to peptides comprised of amino acids 90 to 111, 96 to 115 and 107 to 122 were compared (Kondo *et al.*, 2007). Of these three, sera to amino acids 96 to 115 were the only ones with broad-spectrum activity, taking into consideration that type 6 or type 52 pseudovirions were not tested for. These data suggest that amino acids 96 to 107 and 111 to 115 are essential for broad cross-neutralisation within this epitope.

This peptide displayed on type 16 L1, with residues 101 and 112 mutated, also showed broad-spectrum activity (Kondo *et al.*, 2008). The apparent redundancy of amino acids 108-120 is supported by Jagu *et al.* where amino acids 21 to 107 showed broad cross-reactivity, but 89 to 200 did not. The necessity of amino acids 111 to 115 is supported by Schellenbacher *et al.* (Schellenbacher *et al.*, 2009), where peptides omitting amino acids 113 to 115, did not elicit any neutralising activity, and by Kawana *et al.* (Kawana *et al.*, 1999), where no mAbs raised against L1/L2 capsids reacted with the peptide 95 to 107. Taken together, it seems as if there is a broader cross-reactive epitope to be found between amino acids 96 to 115, but there could be an epitope from amino acids 108-120 that induces cross-reactive antibodies against types other than those for amino acids 96 to 115. In summary, it may be safe to designate the complete region between amino acids 96 to 120 as a general cross-reactive epitope.

Regions of L2 from papillomavirus types other than type 16 have also been tested. The region between amino acids 1-88 of BPV L2 has been identified as a widely cross-reactive area (Gambhira *et al.*, 2007a; Pastrana *et al.*, 2005). Interestingly, the corresponding region from CRPV did not show the same cross-reactivity. The above-mentioned BPV L2 region showed better cross-neutralisation patterns than the corresponding region in HPV-16 L2, and sera raised against this peptide reacted to peptides found between amino acids 25 and 68 of HPV-16 L2.

1.4.2 Functions of cross-neutralising epitopes of L2

The localisation of neutralising and cross-neutralising epitopes of L2 can be attributed to these regions having a particular function, as conserved domains usually have an important function and are more likely to induce cross-neutralising antibodies. Recently published papers suggest a model for HPV infection (shown in Figure 1.6) that involves exposure of the N-terminal of L2 on the surface of the capsid and cleavage of the first 12 amino acids prior to cellular infection, thus exposing cross-neutralisation epitopes (Day *et al.*, 2008; Buck *et al.*, 2008; Kines *et al.*, 2009). This exposure of L2 is for a sufficient period of time to elicit antibodies (Day *et al.*, 2008; Kines *et al.*, 2009). It is not clear whether L2 is exposed on the surface of the viral capsid prior to infection, but recent work (Buck *et al.*, 2008) could not detect this, and

it is also known that L1/L2 capsids do not elicit more cross-neutralising antibodies than L1-only capsids (Roden *et al.*, 2000).

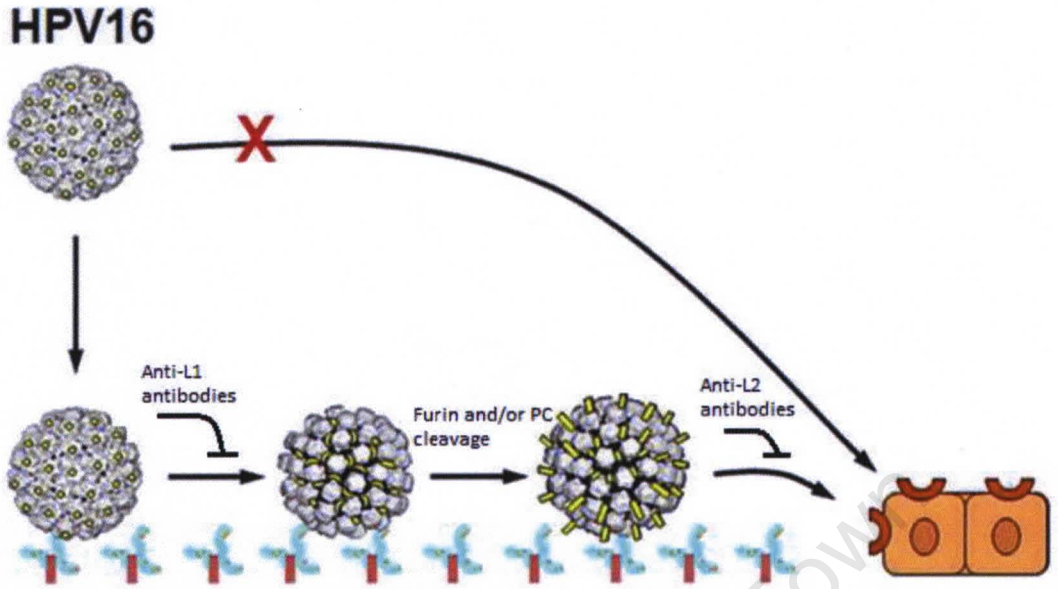


Figure 1.6. *A model for in vivo HPV infection. Mature virus capsids, on which anti-L2 antibody binding sites are hidden, bind to heparan sulphate proteoglycans on the cervicovaginal basement membrane. This interaction leads to a conformational change that exposes the N-terminal of L2 on the surface of the capsid, enabling cleavage of L2 amino acids 1-12 by furin or a proprotein convertase (PC). The capsid can then be transferred to a second receptor on the epithelial cells, which leads to endocytosis and infection. This process may be inhibited by anti-L1 antibodies, which inhibit the conformational change and exposure of L2, thus inhibiting infection. The process is also inhibited by anti-L2 antibodies, which bind to the exposed N-terminal of L2, thus preventing infection. Picture was taken from Kines et al. and modified. (Kines et al., 2009)*

The mAb, RG-1, and polyclonal sera against the L2 peptide 17-36, binds the exposed terminal epitope of L2 according to the proposed model (Day *et al.*, 2008; Kines *et al.*, 2009). RG-1 does not bind mature pseudovirions in solution prior to infection (Day *et al.*, 2008): therefore this region probably only becomes exposed after cell binding and furin cleavage. The RG-1 binding site is directly downstream from the furin cleavage site (Kines *et al.*, 2009). The position of the binding site makes this region the most likely part of L2 to be exposed on the surface of the capsid, but it is possible that a greater portion of the N-terminal of L2 is exposed on the surface, since vaccination with HPV-16 L1/L2 capsids have previously yielded monoclonal antibodies against amino acids 64-81 and 108-120 of HPV-16 L2 (Kawana *et al.*, 1998; Kawana *et al.*, 1999).

L2 has been shown to bind to the cell surface via a receptor different from L1, as well as greatly enhancing viral infectivity (Kawana *et al.*, 2001; Buck *et al.*, 2008; Day *et al.*, 2008; Kines *et al.*, 2009; Yang *et al.*, 2003). It contains several motifs that mediate these interactions. One such motif comprises residues 13 to 31, a region similar to the RG-1 epitope. When residues 18, 19, 21 and 22 were mutated, this interaction was abolished. The motif was also important for the infectivity of pseudovirions. Another motif is that found between amino acids 108 to 126, as tested by Kawana *et al.* (Kawana *et al.*, 2001). This motif was found to bind to COS-1 and HeLa cells, and to be essential in pseudovirion infection of these cells, particularly when residues 108 to 111 were substituted for. The data is supported by similar results from BPV L2 residues 91 to 129, confirming that this region is a conserved functional domain (Yang *et al.*, 2003). As mentioned before, amino acids 108-120 have been identified as a cross-neutralising epitope.

Another region of the L2 protein has been shown to be involved in HPV infection. L2 was shown to interact with β-actin, directing intracellular transport of papillomaviruses to the nucleus, where the virus replicates (Yang *et al.*, 2003). This interaction was common to both HPV-16 and BPV-1, which are distantly related PVs. The functional domain involved in this process comprised amino acids 25-45, but amino acids 13-31 did not show the same interaction. Amino acids 13-31 comprise a region that overlaps with the known cross-neutralising epitope of aa 17 to 36, which once again suggests the importance of residues 32 to 36. Antibodies against this β-actin-binding region of L2 may not prevent HPV infection, but could prevent subsequent intracellular transport and therefore neutralise viral infection.

In summary, from the above data, major conserved cross-neutralising regions of L2 emerge within the first N-terminal 120 amino acids. Those regions that have shown potential to cross-neutralise HPV, of types other than the type L2 is derived from, include HPV-16 L2 amino acids 17-38, 56-81 and 96-120; and BPV-1 L2 amino acids 1-88. These cross-neutralising regions of L2 should be considered in the design of more broadly neutralising HPV vaccines.

1.4.3 Chimaeric L1/L2 vaccines

One approach to the problem of creating a pan-papillomavirus vaccine, which avoids lack of surface exposure of L2 sequences, is to fuse L2, or cross-reactive L2 peptides, to L1. Because papillomavirus VLPs are made up of 360 molecules of L1, arranged as 72 pentamers, linking L2 to L1 results in a tandem array of L2 in close proximity to one another. This tandem array may increase its immunogenicity (Kirnbauer *et al.*, 1992; Bachmann & Zinkernagel, 1997). In native virions, the ratio of L1 to L2 can range from 5:1 to 30:1 (Buck *et al.*, 2008), making L2 the subdominant protein within the virion and L1 immunodominant. L1 immunodominance could be due to it being more closely spaced than L2. Another incentive for linking L2 peptides to L1 is that peptides by themselves are not very immunogenic and they need to be linked to haptens in order to increase their immunogenicity. Even when L2 peptides are linked to molecules such as KLH, they display low immunogenicity. L1 may be an ideal carrier molecule, since it can act as an adjuvant for various reasons, as discussed further below (Lenz *et al.*, 2001; Lenz *et al.*, 2003; Rudolf *et al.*, 2001a).

1.5 L1 as a carrier molecule

1.5.1 Advantages

The excellent immunogenicity of L1, as well as its capacity to accommodate modifications to its structure, are two reasons for using it as a vehicle to display heterologous epitopes. L1 is in itself an excellent adjuvant, as evinced by the fact that VLPs can in some cases be injected into animals without an adjuvant and still elicit an immune response (Ohlschläger *et al.*, 2003; Wakabayashi *et al.*, 2002; Reddy *et al.*, 2004; Greenstone *et al.*, 1998). L1 VLPs and L1/L2 VLPs also bind to multiple cells of the immune system, which accounts for their effectiveness in priming both the cellular and the humoral arms of the immune system (Lenz *et al.*, 2003; Da Silva *et al.*, 2001). Not only do they bind to dendritic cells, they also induce acute activation of dendritic cells and the repeptitive array of virion surface proteins were implied in this interaction (Lenz *et al.*, 2001; Rudolf *et al.*, 2001a). Dendritic cells are the most important antigen-presenting cells (APCs), and play a central role in adaptive immunity. Their activation and maturation are important steps towards activating

antigen-specific T- and B-lymphocytes and towards secretion of cytokines, which in turn leads to effective cell- and antibody-mediated responses (Janeway *et al.*, 1997a). It is interesting that assembly-deficient L1 protein was inferior in the induction of maturation of dendritic cells (Lenz *et al.*, 2001).

The close-packed, ordered structure of VLPs is a key factor in their immunogenicity and their induction of both T-cell and B-cell mediated responses (Kirnbauer *et al.*, 1992; Bachmann & Zinkernagel, 1997; Lenz *et al.*, 2001). In fact, this class of antigen is rarely encountered in an accessible form in vertebrate hosts and thus assists in identifying the antigen as foreign (Bachmann & Zinkernagel, 1997). Also, highly repetitive structures assist in cross-linking B-cell receptors, which is a necessary signal for T-cell independent B-cell activation and eventual secretion of antibody (Janeway *et al.*, 1997b). Only highly repetitive structures found on many viral capsids and bacteria, are able to bypass help from antigen-specific T-cells (Bachmann & Zinkernagel, 1997; Janeway *et al.*, 1997b).

1.5.2 Immunodominance

Caution may have to be exercised when L1 is employed as a carrier molecule for epitope vaccines, due to the phenomenon known as immunodominance (Sette & Fikes, 2003). Immunodominance occurs when the immune response to an antigen is focused on only a few or even only one epitope, to the exclusion of other subdominant epitopes. Immunodominance has been observed with polytope peptide vaccines (le Thuy *et al.*, 2001; Ju *et al.*, 1993). In fact, even when peptides from diverse organisms normally immunodominant within the context of their own antigenic system are combined, "superdominant" hierarchies emerge (Sandberg *et al.*, 1998). Therefore immunodominance may not necessarily be intrinsic to an epitope and may be dependent on the context in which it is presented (Sandberg *et al.*, 1998; Kim *et al.*, 2007a). The intrinsic high immunogenicity of L1 (Lenz *et al.*, 2003; Da Silva *et al.*, 2001) may indicate the presence of multiple immunodominant epitopes, which may dominate the immune response to the detriment of the inserted peptides. Stable tertiary or quarternary structures have been implicated in immunodominance (Ito *et al.*, 2003), which correlates with the situation in L1, where its higher-order structures, the surface loops, were responsible for eliciting the vast majority of neutralising

antibodies (Carter *et al.*, 2006; Sadeyen *et al.*, 2003; Christensen *et al.*, 2001). Immunodominant epitopes may even downregulate the immunogenicity of subdominant epitopes (Wieland *et al.*, 2010) and the deletion of immunodominant epitopes from antigens have been shown the enhance the immune response to other subdominant epitopes found within this antigen (Wieland *et al.*, 2010; Cleveland *et al.*, 2000b; Dai *et al.*, 2002). Although immunodominance has not specifically been studied in the context of L1, there may be indications of this phenomenon in some cases. For example, in one study (Bian *et al.*, 2008), when mice were immunised with chimaeric L1E7 capsomers, the dominant cellular immune response was directed towards L1 and not the inserted E7 peptide. The same effect was seen for antibody responses to E7 in the context of L1/E7 chimaeric proteins (Liu *et al.*, 2000) However, where chimaeric L1 proteins have been tested in mice for an immune response against the inserted peptide, in the vast majority of cases - provided the peptide was inserted into an optimum position - an immune response against the peptide could be detected. Evidence for this is presented in the discussion of optimum insertion sites within L1 below. There were also cases where the immunogenicity of an inserted epitope was improved in the context of L1 (Qian *et al.*, 2006; Slupetzky *et al.*, 2007).

1.5.3 Modifications of L1

The HPV capsid is made up of 72 pentameric capsomers, with complex loops protruding from the surface. L1, which self-assembles into VLPs nearly identical to the capsid, can be modified as a carrier for foreign epitopes, even to the extent of disrupting interpentameric bonds. When interpentameric bonds are disrupted, for instance by the insertion of epitopes into the L1 protein or other modifications, L1 will still form capsomers. Capsomers have also been shown to be highly immunogenic and to elicit neutralizing antibodies (Schädlich *et al.*, 2009; Thönes *et al.*, 2008; Varsani *et al.*, 2003; Murata *et al.*, 2009; Slupetzky *et al.*, 2007; Slupetzky *et al.*, 2001). It must be said, however, that they are less immunogenic than VLPs and therefore a higher dose may be required to elicit the same level of neutralisation (Thönes *et al.*, 2008). The immunogenicity of capsomers makes it possible to experiment with modifications of L1 towards more efficacious vaccines.

Modifications of L1 utilize its inherent immunogenicity and close-packed structure which primes the immune system against displayed epitopes. B-cells require binding of intact antigen to its receptors for the initiation of humoral immune responses (Janeway *et al.*, 1997b). Therefore, to generate an antibody response against an epitope, it needs to be displayed on the surface of a molecule. It follows, therefore, that insertion of peptides into the surface loops of L1 will ensure their display on the surface of the L1 molecule. Ideally, if anti-L1 antibody responses are desired, as in the case of prophylactic HPV vaccines, the insertion should not abolish an anti-L1 neutralising response, yet should generate a robust response against the inserted peptide. The formation of VLPs is also ideal, since VLPs are more immunogenic and would display more epitopes in closer arrays than other less ordered structures would.

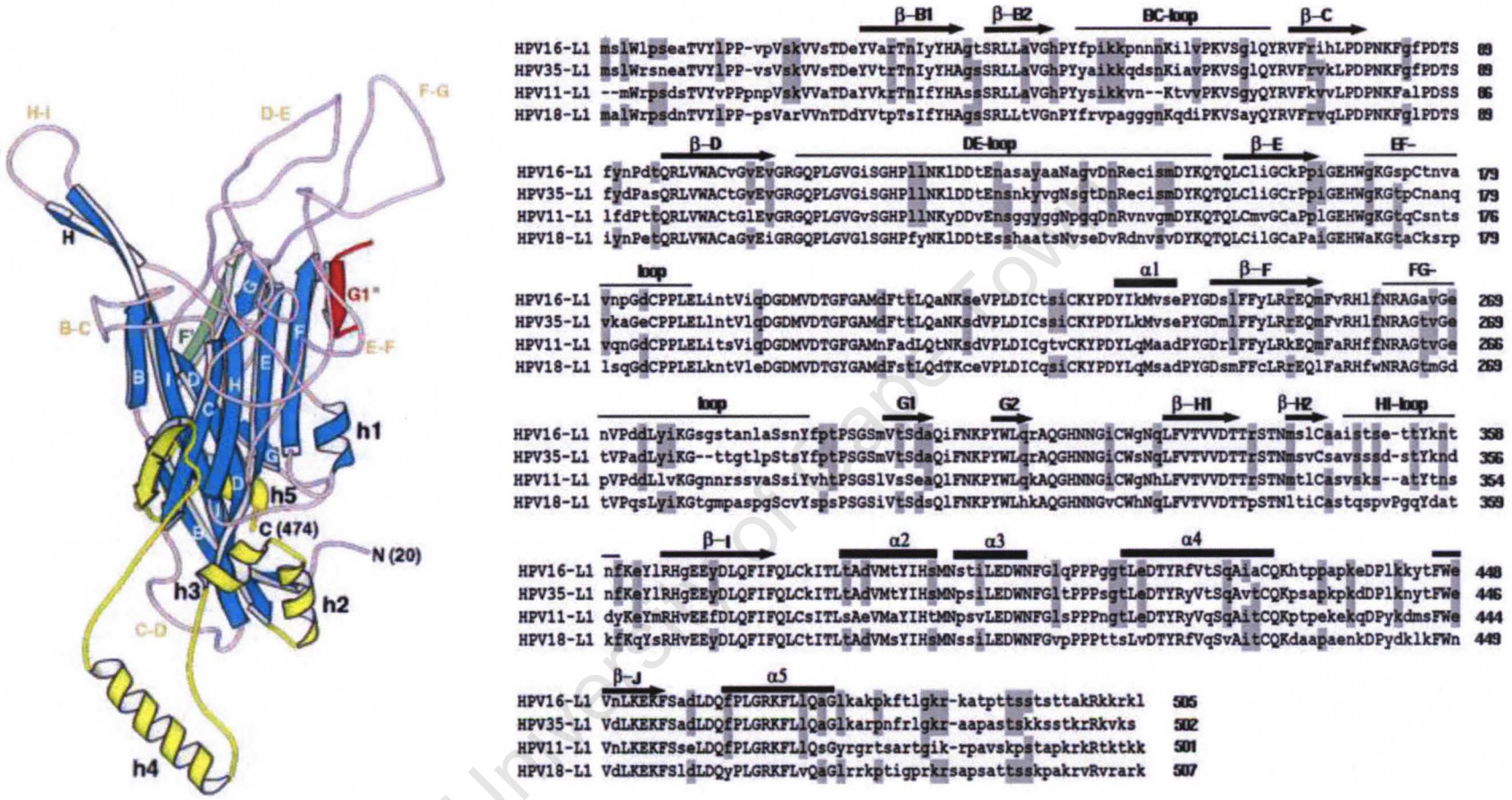
T-cell epitopes for presentation by MHC Class I molecules involved in cellular immunity can also be inserted into L1. Such a modification may seem counter-intuitive, since the classical pathway for MHC Class I presentation is via the endogenous pathway (Rudolf *et al.*, 2001a; Peng *et al.*, 1998; Liu *et al.*, 2000). This pathway requires that viral proteins be degraded in the cytosol into peptides that can be presented by MHC molecules. Exogenous antigens, like VLPs, have in many cases failed to elicit CTL responses (Peng *et al.*, 1998). However, papillomavirus VLPs do access the cellular immune system by interaction with immune cells (Lenz *et al.*, 2003; Da Silva *et al.*, 2001) and uptake by dendritic cells (Da Silva *et al.*, 2007).

Since display of T-cell epitopes for the induction of cellular immunity requires degradation of proteins in the cytosol, it may seem unnecessary to optimize the insertion sites of T-cell epitopes into L1. However, surface display or presentation of CTL epitopes as multiple copies in repetitive arrays, enhances induction of CTL responses (Kruger *et al.*, 1999). The importance of surface display was reiterated by Street *et al.* (Street *et al.*, 1999), who found that the insertion of a hydrophobic CTL epitope into a hydrophilic, surface-displayed region of the hepatitis B core antigen, caused the CTL epitopes to be invaginated. The invagination led to the abolishing of CTL responses and the reason given for this was that the CTL epitopes were not properly displayed to the afferent immune system or antigen recognition cells.

against the inserted L2 epitopes were therefore low (Varsani *et al.*, 2003; Kondo *et al.*, 2008).

L1 chimaeric proteins have also been created by fusing proteins or peptides to the C-terminal of L1. This has especially been done for L1/E7 constructs (Paz De la Rosa *et al.*, 2009; Ashrafi *et al.*, 2008; Kuck *et al.*, 2006; Peng *et al.*, 1998; Liu *et al.*, 2000; Wakabayashi *et al.*, 2002; Jochmus *et al.*, 1999), and in some cases L1/GFP (Windram *et al.*, 2008) and L1/HIV constructs (Liu *et al.*, 2000; Peng *et al.*, 1998; Liu *et al.*, 2002). In all cases, except when expressed in human cell lines, L1 retains its ability to form VLPs and to elicit antibodies, despite the deletion of up to 34 amino acids from the C-terminal of HPV-16 L1 or 25 amino acids from BPV L1. Antibody responses against epitopes fused to the C-terminal, which could indicate surface display of these epitopes, have not been clearly shown in the literature. Where they were tested, antibody responses were low, but the low response could be due to E7 epitopes being subdominant to L1 epitopes (Liu *et al.*, 2000). However, CTL responses against fused epitopes were better when VLPs were in their native form than when denatured (Liu *et al.*, 2002). It is likely, according to structural analysis of L1, that epitopes fused to the C-terminal are packed within the interior of the VLP shell (Chen *et al.*, 2000).

Full-length proteins have also been fused to L2, which were then co-expressed with L1 (Qian *et al.*, 2006; Rudolf *et al.*, 2001a; Xu *et al.*, 2007; Wakabayashi *et al.*, 2002; Greenstone *et al.*, 1998; Windram *et al.*, 2008). In all cases they form VLPs and retain their ability to elicit antibodies against L1, but the foreign proteins are unlikely to be displayed on the surface of the VLP.



A. B.

Figure 1.7. A. *L1 monomer. Surface loops are in pink and helices and the ß-J sheet are in yellow. Picture taken from Chen et al. (2000) (Chen et al., 2000) and modified.* ***B.*** *Sequence alignment of L1 from HPV-16, -35, -11 and -18, showing the position of structural features within L1. Conserved residues are shaded in grey, arrows indicate ß-sheets, thick lines indicate helices and the surface loops are also indicated. The picture was taken from Bishop et al. (Bishop et al., 2007b) and modified.*

1.6 The need for treatment of existing HPV-related cancers

Another problem not addressed by current HPV vaccines on the market is that of treating existing HPV infections and related precancerous and cancerous lesions. A clinical trial conducted in Costa Rica with women positive for HPV DNA at the start of the study and vaccinated with Cervarix™, showed no evidence of viral clearance even after 12 months (Hildesheim *et al.*, 2007). When subjects were stratified according to factors indicating disease extent, like viral load or cytological findings at entry, there was no correlation between disease severity and vaccine efficacy for viral clearance. HPV types other than 16 or 18 were also not cleared by the vaccine.

Currently, HPV-induced lesions are treated by removal of the abnormal tissues. Procedures include surgical removal of tissues, cryotherapy, laser evaporation therapy, loop electrosurgical excision procedure or cold-knife conization. These treatments are not only invasive, but also inefficient, since recurrence is a common outcome (Govan, 2005). Immunotherapy, where the immune system is primed to specifically target HPV-infected tumour cells, would be more specific and potentially more efficient at treatment. In addition, it would be far less invasive.

Therapeutic vaccination requires that a cell-mediated immune response be elicited. This is because tumour cells are targeted and killed by CTL (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). Since L1 and L2 are only expressed in terminally differentiated epithelial cells, and not in precancerous or cancerous tissue, they are not ideal targets for therapeutic vaccines (Govan, 2005). The "early" HPV proteins, especially E6 and E7 (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005), but also other early proteins such as E1, E2 and E5 (Govan & Williamson, 2007; Brandsma *et al.*, 2007; Liu *et al.*, 2007) are expressed in cancerous and precancerous lesions and are therefore better protein targets for immunotherapy. Therefore CTL epitopes derived from these proteins, should be presented in complex with MHC class I molecules on the surface of infected cells for recognition by CTL and their subsequent killing.

1.6.1 HPV-16 E7 T-cell epitopes

Preclinical studies have shown E7 to be an ideal target for therapeutic vaccines (Govan, 2005). Various E7-based vaccines are also being or have been tested in Phase I/II clinical trials (Roman *et al.*, 2007; Santin *et al.*, 2003; Fiander *et al.*, 2006; Einstein *et al.*, 2007). Like L2, E7 contains essential T-cell epitopes that can be used to prime cellular immune responses. Vaccinating with these epitopes instead of whole protein may be advantageous for many reasons, including that it circumvents the oncogenicity of whole protein, (Liu *et al.*, 2007; Ohlschläger *et al.*, 2006) increases memory immune responses, (Liu *et al.*, 2007) avoids viral regions with high inter-strain variability or regions to which tolerance might exist, (Kast *et al.*, 1994) and enhances immunity to subdominant epitopes displayed by MHC molecules on cell surfaces (Kast *et al.*, 1994).

Human T-cell epitopes derived from HPV-16 E7 have been mapped. Four CTL epitopes emerge as significant: these are amino acids 11-20, 12-20, 82-90 and 86-93. The binding of these peptides to the HLA-A2 allele was first demonstrated by Kast *et al.* (Kast *et al.*, 1994), and lysis of a human cervical cancer cell line by CTL stimulated by three of these peptides (excluding 12-20), was shown by Ressing *et al.* (Ressing *et al.*, 1995). Another research group (Jochmus *et al.*, 1997) stimulated peripheral blood lymphocytes from a healthy volunteer and cervical cancer patient with different peptides to obtain CTL lines. Only a small number of low-affinity CTL lines against the peptide 86-93 could be obtained and not against any of the other peptides used in stimulation, like 11-20, 7-15 and 82-90. Since then, 3 CTL epitopes have been tested in Phase I/II clinical trials. Amino acids 86-93 (TLGIVCPI) were linked to a universal T-helper epitope (PADRE) (Steller *et al.*, 1998) and elicited immune responses in all patients, but showed no clear clinical effect. In another trial, peptide aa 11-20 was mixed with aa 86-93 and a pan-DR T-helper epitope (van Driel *et al.*, 1999). There was no clear clinical response either. Lastly, patients were immunized with peptide consisting of amino acids 12-20, and some were immunized with the latter peptide as well as amino acids 86-93 fused to PADRE (Muderspach *et al.*, 2000). Once again, there was no clear clinical response, but only the peptide 12-20 had responders with respect to cytokine release from the patients. Another study specifically analysed the frequency of T-cells directed against HPV-16 E7 (86-93) and

(11-20) and found that these were upregulated in patients with HPV-16 positive carcinomas of the head and neck (Albers *et al.*, 2005).

The most significant E7 CTL epitope (amino acids 49-57, RAHYNIVTF) specific to mice carrying H2-D^b alleles, such as C57/BL6 mice, has also been identified and is used regularly in tumour regression and CTL response studies in mice (Bian *et al.*, 2008; Kuck *et al.*, 2006; Liu *et al.*, 2000; Peng *et al.*, 1998). This epitope was first identified by Feltkamp *et al.* (Feltkamp *et al.*, 1993) to bind strongly to H2-D^b MHC class I molecules and to protect mice against challenge with HPV-16 transformed tumour cells. In a recent article, this epitope was confirmed to be the only CTL epitope in mice (Khammanivong *et al.*, 2003). Immunotherapy with this peptide is highly successful in mice with the H2-$2D^b$ allele (Torrens *et al.*, 2005; Daftarian *et al.*, 2006; Paz De la Rosa *et al.*, 2009).

1.6.2 Problems related to vaccination with E7 T-cell epitopes

Several problems still need to be overcome before immunization with T-cell epitopes will be effective. The first problem is that immunisation with CTL epitopes has so far not been successful in cervical cancer patients, as can be seen from the clinical trials. In order to efficiently activate the MHC Class I pathway, appropriate adjuvants need to be co-administered (Peng *et al.*, 1998). The biggest reason why immunisation with CTL epitopes has not been successful, however, is immune evasion by HPV. When HPV infects epithelial cells, immunosuppressive cytokine expression is upregulated. Low viral protein expression, the lack of a danger signal and lack of cross-presentation are all factors that serve to aid HPV in evading the immune system (Daftarian *et al.*, 2006). It is also known that MHC Class I molecules may be downregulated in cervical cancer cells, therefore targeting CTL epitopes may not be such an efficient approach (Hohn *et al.*, 1999). Indeed, there are studies that suggest that CD4 T-cell epitopes play a significant role in mediating tumour regression: regression and loss of HPV infection was associated with cell-mediated immune responses to a CD4+ T-cell epitope (Kadish *et al.*, 2002; Peng *et al.*, 2007), CD4+ tumour-infiltrating lymphocytes have been suggested to directly mediate tumour regression by recognition of MHC Class II-presented antigens in the absence of sufficient MHC Class II-presented antigens (Hohn *et al.*, 1999) and CD4+ T-cell

responses have been far more frequent than CD8+ in cervical cancer patients (Piersma *et al.*, 2008). It is also becoming widely accepted that the inclusion of a T-helper or CD4+ epitope in a peptide-based vaccine, is important in stimulation of the immune system to eradicate tumours (Muderspach *et al.*, 2000; Steller *et al.*, 1998; van Driel *et al.*, 1999; Daftarian *et al.*, 2006; Doan *et al.*, 2005; Vambutas *et al.*, 2005; Tindle *et al.*, 1991; Xu *et al.*, 2009; Qin *et al.*, 2005).

The second problem still to be overcome is that of targeting HLA alleles other than HLA-A2 (HLA*A*0201), on which most, if not all, of the focus has been. The HLA*A*0201 allele is the most common allele in the Caucasian population (Madeleine *et al.*, 2008), but women of other races do not necessarily carry the same alleles to the same frequency. Furthermore, more analysis is needed to look at populations of tumour-infiltrating lymphocytes and their HLA specificity. The HLA-A2 allele has actually been shown not to confer as high a risk for cervical cancer as have others (Madeleine *et al.*, 2008). No region of E7 was shown to be preferred in anti-tumour responses, showing the variety of epitopes and their binding alleles potentially involved in cervical cancer. For MHC class II alleles, HLA-DQ and HLA-DP were shown to be preferred restriction elements, even though they are less abundantly expressed (Piersma *et al.*, 2008). There are a few studies that have focused on mapping E7 T-cell epitopes presented by a specific MHC molecule, like HLA-B8 (Oerke *et al.*, 2005) and HLA-B7 (Ellis *et al.*, 1995), which are more associated with cervical cancer, HLA-A24 (Morishima *et al.*, 2007), which is more abundantly expressed in Asian women, and HLA-B18 (Bourgault, I *et al.*, 2000). Some studies have focused on mapping epitopes within E6 and E7 binding to a variety of alleles simultaneously (Piersma *et al.*, 2008; Rudolf *et al.*, 2001b).

The third problem to be overcome is that of immunotherapy for patients infected with types other than type 16. Not many studies have focused on identifying CTL epitopes relevant to other types. Most studies on types other than type 16 have identified T-cell epitopes specific to HPV-18 (Smith *et al.*, 2005; Kather *et al.*, 2003; Castellanos *et al.*, 2001; Rudolf *et al.*, 2001b). CD4+ T-cell epitopes were identified for types 59, 68 and 33 (Sette *et al.*, 1993; Hammer *et al.*, 1993; Hohn *et al.*, 2000) and CTL epitopes have been mapped for HPV-11 (Xu *et al.*, 2008). However, the question remains to what extent immunotherapy with a T-cell epitope would cross-protect against other

types. An HPV-16E7 (11-20)-specific CTL line was tested for its ability to kill cells transfected with full-length type -16, -52, -45 and –31 (Youde *et al.*, 2005). Only cells transfected with type 52 could be killed. Tumour infiltrating lymphocytes were found to be specific to either HPV-16 or –18 respectively, and no cross-reaction was seen between the two types (Piersma *et al.*, 2008). A CD4+ T-cell epitope correlated with regression of CIN (Kadish *et al.*, 2002) showed non-type-specific effects, however, it couldn't be ruled out that patients could previously have been infected with type 16 and therefore these responses present memory responses. A cervical cancer cell line derived from an HPV-59 positive patient, TIL-CCA1, responded to B cells transfected with the E7 gene from HPV-59 and –68, but not –16 or –35 (Hohn *et al.*, 1999). Lastly, an HPV18/45 cross-reactive T-cell, HLA-A2-binding epitope has been identified, demonstrating that cross-reactive T cell responses against closely related HPV types can be induced *in vivo* (McCarthy *et al.*, 2006). All this evidence seems to suggest that cell mediated immunity may be as type-specific as humoral immunity for HPV.

1.6.3 Chimaeric L1/E7 vaccines

The leading candidate vaccines for the treatment or prevention of cervical cancer in humans are L1/E7 chimaeric virus-like particles (Rudolf *et al.*, 2001a). This approach is feasible for two reasons: These are creating vaccines that are simultaneously prophylactic and therapeutic, as well as seeking to enhance the immunity of E7 by utilizing the inherent immunogenicity of VLPs. So far, design of chimaeric VLPs containing targets for immunotherapy has exclusively focused on C-terminal fusion. Full-length E7 or the N-terminal part of E7 has been fused either to the C-terminal of L1 (Bian *et al.*, 2008; Kuck *et al.*, 2006; Ashrafi *et al.*, 2008; Kaufmann *et al.*, 2001; Kaufmann *et al.*, 2007; Jochmus *et al.*, 1999) or to the C-terminal of L2 (Qian *et al.*, 2006; Rudolf *et al.*, 2001a; Xu *et al.*, 2007; Greenstone *et al.*, 1998) creating L1/E7 or L1/L2/E7 cVLPs respectively. The efficiency of eliciting a cellular immune response in mice was compared between L1/L2 and L1 cVLPs and it was found that L1/E7 cVLPs were more efficient at doing so (Wakabayashi *et al.*, 2002). Only in two cases have CTL epitopes been used in chimaeric VLPs. One study inserted the mouse H2-D^b-restricted epitope into the C-terminal of BPV-L1 and was able to show efficacy in mice without co-administering adjuvant (Peng *et al.*, 1998). Another study fused 3

HPV-16 E7 epitopes, including the mouse-restricted epitope 49-57, the human-restricted epitope 86-93, and the human-restricted T-helper epitope 37-54, and one E6 epitope to the C-terminal of HPV-16 L1, of which the last 34 amino acids have been deleted (Paz De la Rosa *et al.*, 2009).

Phase I clinical trials in humans have been carried out only for L1/E7 chimaeric vaccines (Kaufmann *et al.*, 2007) and a non-significant trend was seen in efficacy. *In vitro* studies of cellular immune responses in human donors by L1/L2/E7 and L1/E7 vaccines have shown promise (Warrino *et al.*, 2005; Kaufmann *et al.*, 2001; Rudolf *et al.*, 2001a) and therefore L1/E7 chimaeric vaccines is a line of research worth pursuing.

1.7 Conclusion

L1 VLPs have proven to be highly immunogenic as prophylactic vaccines in humans. Their ability to prime and bind to cells of the immune system, as well as their repetitive nature, is the reason for their high immunogenicity. Therefore L1 is an adjuvant in itself. L1 can also easily be modified to carry foreign proteins and epitopes, while still forming immunogenic, higher-order structures.

Currently licensed vaccines are highly effective type-specific prophylactic vaccines, which also elicit high titres of neutralising antibodies. Even though the vaccines are directed against the most prevalent types worldwide implicated in cervical cancer, they may not be as effective in all populations, since type distribution is affected geographically as well as by concurrent HIV infection. A vaccine that will neutralise multiple types is therefore needed. A protein that elicits cross-neutralising antibodies is L2, the minor capsid protein. It contains multiple cross-neutralising epitopes that could be incorporated into L1 to produce a highly effective broad-spectrum vaccine.

Current vaccines also cannot be used to treat existing infections and physical treatment methods are not effective. Vaccination with proteins that are expressed in carcinogenic, HPV-infected cells, could boost cellular immunity that would eliminate tumour cells. E7 is such a protein and it contains CTL epitopes that have been shown

to reduce tumours in animals. Immunotherapy with CTL epitopes still has several problems to overcome. The first one is low immunogenicity in humans. Using CD4- T-cell epitopes in vaccination may be a key to overcoming this problem. Secondly, CTL epitopes are HLA-specific, and humans are highly polymorphic in this respect. Thirdly, CTL epitopes may be type-specific and little work has been done in mapping CTL epitopes against types other than –16 or –18.

Chimaeric L1/E7 vaccines are the leading candidate vaccines. Such vaccines can be both prophylactic and therapeutic and the adjuvanticity of L1 can be utilised. Although clinical trials have not shown high efficacy with respect to immunotherapy, chimaeric vaccines remain a good approach towards solving the problem of treating cervical cancer.

1.8 Objectives of this study

The aim of this project was first to address the need for a broad-spectrum prophylactic vaccine by incorporating the sequences of four different cross-neutralising L2 epitopes into the sequence of HPV-16 L1. The four epitopes that have been shown to elicit cross-neutralising antibodies neutralising divergent human papillomavirus types, HPV-16 L2 amino acids 17-36 (Gambhira *et al.*, 2007b), 56-81 (Kawana *et al.*, 1998) and 108-120 (Kawana *et al.*, 1999); and BPV-1 L2 amino acids 1-88 (Pastrana *et al.*, 2005), were employed in the design of these vaccines. The peptides were inserted into the helix 4 position, since such an insertion has been shown to be optimum both for anti-L1 and anti-L2 immunogenicity (Varsani *et al.*, 2003).

The second aim was to address the need for immunotherapeutic vaccines by incorporating the sequence of an HPV-16 E7 CTL epitope into the sequence of L1. HPV-16 E7 amino acids 49-57, a CTL epitope specific to the mouse H2-D^b allele and useful for testing in mice (Feltkamp *et al.*, 1993), as well as HPV-16 E7 amino acids 86-93, a human CTL epitope binding to HLA-A2 alleles (Kast *et al.*, 1994), were inserted into the helix 4 of HPV-16 L1, creating two different chimaeras respectively. Two chimaeras were also created where the cross-neutralising L2 epitope, consisting of HPV-16 L2 amino acids 108-120, was inserted into the helix 4 and one of the E7

epitopes mentioned above into the region between the helix 4 and the β-J sheet, also shown to be a region useful for insertion of foreign epitopes into HPV-16 L1 (Varsani *et al.*, 2003).

In this project, we aimed to express these eight chimaeric proteins in a baculovirus expression system and to test their ability to elicit neutralising and cross-neutralising antibodies (the latter only in the case of L1/L2 chimaeras) as well as the ability of L1/E7 chimaeras to treat tumours and to elicit cellular immune responses in an animal model.

2. EXPRESSION OF L1 CHIMAERIC PROTEINS IN INSECT CELLS

2.1 Introduction

Human papillomaviruses (HPVs) infect epithelial tissue and cause warts and other lesions, which are largely benign; however, high-risk genital lesions may become malignant (de Villiers *et al.*, 2004; Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). There are at least 120 HPV types (Bernard *et al.*, 2010) that are the cause of a large variety of symptoms or disease. The most significant disease, for which HPV is the causal agent, is cervical cancer. This disease is a significant global health burden, with 260 000 deaths occurring annually as a result, 80% of which occur in developing countries (Cutts *et al.*, 2007). Globally, HPV-16 and -18 are the types most frequently implicated in cervical cancer, with a 70% incidence rate (Bosch *et al.*, 2008). The other 30% is contributed by at least a further 13 types (Castellsague, 2008).

Vaccination has historically been the most effective way to eradicate infectious diseases, therefore vaccination against HPV is supposed to be the most effective way of combating cervical cancer (World Health Organisation, 2005). Vaccines against types 16, 18, 6 and 11 (the latter two types are most frequently associated with genital warts (de Villiers *et al.*, 2004)) are available. These are based on L1-only virus-like particles (VLPs), which differ from native HPVs where the capsid consists of L1 and L2 proteins, but are immunogenically similar. Merck manufactures Gardasil®, a vaccine consisting of a mixture of VLPs of the four types listed above. Each VLP type was independently expressed in *Saccharomyces cerevisiae*, followed by a two-step purification involving microfiltration and cation exchange chromatography. The final concentration of each VLP type range between 40 to 80 µg/ml (Shi *et al.*, 2007). The other presently available vaccine is manufactured by GlaxoSmithKline and consists of a mixture of HPV-16 and -18 VLPs at a concentration of 20 µg/ml each. These were expressed in insect cells or Hi-5 Rix4446 cells derived from *Trichoplusia ni* (Electronic Medicines Compendium, 2009). Both vaccines have shown high efficacy in preventing infection and disease by vaccine-specific types (Schiller *et al.*, 2008). However, problems have presented themselves, one of these is that screening for HPV

must still be continued, since current vaccines have limited efficacy against carcinogenic, non-vaccine types. It will also still be years until HPV is eradicated by vaccination, because of the high prevalence of infected women and the inability of current vaccines to clear existing infections. The pricing also makes them inaccessible to the developing world where they are needed most (Bosch, 2009).

Current research therefore focuses on finding broad-spectrum second-generation prophylactic vaccines (Huh & Roden, 2008). Some of this research is focused on L2 (Huh & Roden, 2008), the minor capsid protein, which can elicit cross-typic neutralising antibodies (Roden *et al.*, 2000). One approach is to vaccinate with cross-neutralising L2 peptides, (Jagu *et al.*, 2009; Kawana *et al.*, 2003; Rubio *et al.*, 2009; Alphs *et al.*, 2008) but much research has been done on replacing surface sections of L1 with L2 peptides or inserting L2 peptides into these sections without deleting any amino acids from L1. Possible modifications of L1 have been thoroughly researched (Varsani *et al.*, 2003; Murata *et al.*, 2009; Slupetzky *et al.*, 2001; Chackerian *et al.*, 1999). This strategy of creating a broad-spectrum vaccine is attractive, since, if L1 specific to a carcinogenic type is used, high titres of neutralising antibodies can be achieved, while also obtaining cross-typic antibodies. On top of that, VLPs - due to their ability to potently activate the immune system - serve as an adjuvant to the coupled peptides and could therefore be ideal carrier molecules (Lenz *et al.*, 2001; Rudolf *et al.*, 2001a).

The possibility of the L1 epitopes being immunodominant over the conjugated peptides must be considered (Sette & Fikes, 2003). It could be surmised that the immune response to L1 may downregulate the immune response to the peptides or even completely abolish it, especially since L1 is known to be an immunodominant protein capable of eliciting strong immune responses. However, there is no evidence for a humoral or cellular immune response to an inserted peptide being completely negated by the immune response to L1. There are examples, where E7 peptides have been inserted, showing that the dominant immune response might be targeted towards L1 and not E7 (Bian *et al.*, 2008; Liu *et al.*, 2000). There is, however, a study which showed the enhancement of humoral immunity to L2 peptides by incorporating them into L1, rather than injecting them in a form where they were conjugated to keyhole limpet haemocyanin (Slupetzky *et al.*, 2007). For these reasons, L1 chimaeric

vaccines remain a research direction to pursue in the search for a second-generation vaccine with the capability to elicit broad cross-protection.

Immunotherapy is another research focus, since mechanical ways of treating cervical cancer do not have a high success rate, and are invasive (Govan, 2005). Here the focus is on creating cellular antitumour immune responses by administration of E6- or E7-based vaccines (Govan, 2005). There are still many problems to be overcome in this area of research. One research focus is on improving the low immunogenicity by better adjuvanting of proteins or peptides (Daftarian *et al.*, 2006; Daftarian *et al.*, 2007; Steller *et al.*, 1998; Xu *et al.*, 2009; Govan *et al.*, 2008; Brandsma *et al.*, 2007; Yan *et al.*, 2009).

Low-cost vaccines for the developing world are under investigation. Vaccine cost can be reduced by tiered pricing or financing strategies for developing countries. It would be more effective if developing countries owned the technology for production of vaccines and if vaccines could be produced at a lower cost (Bosch, 2009). One way of producing vaccines at a lower cost would be to utilise expression systems that can be cultivated more cheaply on a large scale and can express high levels of protein.

Recombinant proteins have been expressed in cell cultures of bacteria, yeasts, fungi, mammals, plants, insects or via transgenic plants and animals (Demain & Vaishnav, 2009). HPV L1 has been expressed for the purpose of vaccine production in a variety of systems, including transgenic plants (Kohl *et al.*, 2007; Warzecha *et al.*, 2003; Regnard *et al.*, 2010), bacteria (Yuan *et al.*, 2001; Ohlschlager *et al.*, 2003a; Li *et al.*, 1997), yeasts (Sasagawa *et al.*, 1995; Carter *et al.*, 1991), the mold *Pichia Pastoris (Bazan et al., 2009)* and insect cells (Park *et al.*, 1993). Bacterial systems are beneficial in that they express high levels of protein rapidly and inexpensively, however, *E. coli* mostly produces capsomers and not more immunogenic VLPs (Yuan *et al.*, 2001; Li *et al.*, 1997). Another disadvantage with bacteria is the high level of endotoxins. Transgenic plants, yeasts and molds can produce correctly folded VLPs that are immunogenic. The advantages of these expression systems is that they express at much higher levels than even *E. coli,* making them highly cost-effective. They can all be cultivated cheaply, are more robust and can easily be scaled up (Demain & Vaishnav, 2009).

Insect cells are known for their superior ability to fold soluble mammalian proteins correctly. Other advantages include eukaryotic posttranslational modifications, proper disulphide bond formation (which is specifically important for VLP formation), safety due to the host specificity of baculovirus, expression of large proteins and efficient cleaving of signal peptides. Insect cells are also easy to scale up and express at relatively high levels, but they are inferior to some expression systems with respect to expression levels (Demain & Vaishnav, 2009). They are also more expensive to cultivate than yeast or bacteria and may be easily contaminated with other microorganisms.

L1 expressed from insect cells and administered to animals is accepted as the standard against which L1 produced in other systems are compared (Maclean *et al.*, 2007; Kohl *et al.*, 2007). For the latter reason, and because of their superior ability to produce correctly folded proteins that would be immunogenically active implies that in order to draw comparisons pertaining to immunogenicity between different VLP and/or capsomer-based vaccine candidates, it would be better to express them in insect cells before expressing them in other systems that may not produce correctly folded proteins.

Our study formed part of a project aimed at developing a local technology for affordable, broad-spectrum second-generation vaccines. In previous work done in our laboratory (Varsani *et al.*, 2003) regions of HPV-16 L1 were replaced with the cross-neutralising peptide, L2 amino acids 108-120 (Kawana *et al.*, 1999). From this work, the helix 4 and the region between the helix 4 and the β-J sheet in the C-terminal were identified as the best regions to display the L2 epitope, while at the same time retaining high anti-L1 immunogenicity. In our study, chimaeric L1 genes were designed to incorporate two other cross-neutralising regions of HPV-16 L2, amino acids 56-81 and 17-36 respectively (Kawana *et al.*, 1998; Gambhira *et al.*, 2007b), and amino acids 1-88 from bovine papillomavirus type 1 (BPV-1) (Pastrana *et al.*, 2005). They were inserted into the helix 4, replacing the same number of amino acids in HPV-16 L1, resulting in four different L1/L2 chimaeric genes. In the same way, MHC class I-restricted HPV-16 E7 epitopes, one with potential for immunotherapy in humans (Steller *et al.*, 1998) and another one designed specifically for pre-clinical studies in mice (Feltkamp *et al.*, 1993; Torrens *et al.*, 2005), were enigineered into

HPV-16 L1. These epitopes encompassed amino acids 86-93 and 49-57 respectively. The rationale of the latter approach was to create vaccines combining the prophylactic abilities of L1 with the therapeutic abilities of the E7 epitope, thus creating a dual vaccine. Further building on the sequence of the dual vaccine as a backbone, in order to create a dual vaccine also displaying cross-neutralising ability, two further chimaeras were designed. In this design, the sequence of ChiΔF-L2 previously constructed in our laboratory (Varsani *et al.*, 2003) was human codon-optimised and named SAF. SAF was used as the basis for chimaera construction, and the abovementioned E7 epitopes inserted into the region between the β-J sheet and the helix 4, replacing the same number of amino acids in SAF.

In work reported in this chapter, the eight constructed chimaeric genes (including SAF) were cloned into appropriate vectors and expressed in the standard insect cell expression system. Six of the chimaeras (excluding the two where the human MHC class I-restricted E7 epitope occurs) were then expressed at levels sufficient for the vaccination of mice. Expression levels were quantitated. Two possible methods for purification of chimaeric proteins were also tested.

2.2 Materials and methods

2.2.1 Synthesis of chimaeric L1 genes

The HPV-16 L1 sequence was derived from a South African patient (GenBank accession no. AY177679). This sequence is identical to the L1 domain encoded by the updated canonical HPV-16 L1 sequence (Swiss-Prot: P03101.2). It was then modified as follows: Amino acids 414-426 were replaced with amino acids 108-120 derived from HPV-16 L2. The nucleotide sequence was optimised for human codon usage, synthesised and cloned into PCR4Blunt-TOPO by GENEART AG, Germany. The resulting gene was named SAFMOD by GENEART, but hereafter will be referred to as SAF.

SAF was the basic genetic structure used for all further modifications, and since all modifications of the L1 (South African strain) gene were within the C-terminal, other

modifications were achieved by synthesising only the C-terminal amino acids 406-505, to replace a fragment of identical length in SAF.

The name of the chimaera, the amino acid sequences inserted into HPV-16 L1 (South African strain), their sites of insertion and the origin of the inserted amino acids are listed in Table 2.1.

Table 2.1. Modification of HPV-16 L1 (South African strain) gene to create chimaeric L1 genes.

Chimaera	**Amino acids inserted**	**Source of inserted amino acids**	**Amino acids replaced in L1**
L2.56	GGLGIGTGSGTGGRTGYIPLGTRPPT	HPV-16 L2 aa 56-81	414-439
L2.17	QLYKTCKQAGTCPPDIIPKV	HPV-16 L2 aa 17-36	414-433
BPV	MSARKRVKRASAYDLYRTCKQAGTCPPDVI PKVEGDTIADKILKFGGLAIYLGGLGIGTW STGRVAAGGSPRYTPLRTAGSTSSLASI	BPV-1 L2 aa 1-88	414-505
E7M	RAHYNIVTF	HPV-16 E7 aa 49-57	417-425
E7H	TLGIVCPI	HPV-16 E7 aa 86-93	417-424
E+E7M	LVEETSFIDAGAP RAHYNIVTF	HPV-16 L2 aa 108-120 HPV-16 E7 aa 49-57	414-426 433-441
E+E7H	LVEETSFIDAGAP TLGIVCPI	HPV-16 L2 aa 108-120 HPV-16 E7 86-93	414-426 434-441

Amino acids 406-505 derived from HPV-16 L1 (South African strain) containing the different modifications were synthesised by GENEART AG and cloned into PCR4Blunt-TOPO, except in the case of L2.17, where the fragment was cloned into pGA18.

2.2.2 Cloning

SAF was cloned from PCR4Blunt-TOPO into the vector pGA4, as shown in Figure 2.1(A). This vector contained another irrelevant gene, which had been cloned previously by another member of our research group. This irrelevant gene was replaced with SAF and clone into the *HindIII* and *XhoI* restriction enzyme sites. (The vector pGA4 can be obtained from GENEART AG). The cloning into pGA4 was done to avoid unwanted enzyme restriction sites in the PCR4Blunt-TOPO backbone.

All other chimaeric genes were created by cloning the synthesised modified fragments into pGA4 containing SAF, into the *PstI* and *XhoI* sites (Figure 2.1(B)). This destroys one of the *EcoRI* restriction enzyme sites.

All chimaeric L1 genes, excluding SAF, were then further subcloned into pFastBacDual (Invitrogen) under the polyhedron promoter (Figure 2.2). Chimaeric constructs were digested with *EcoRI and XhoI* and cloned into the vector's *EcoRI* and *SalI* sites. SAF was subcloned non-directionally using *EcoRI*, and the direction of the cloned gene was confirmed by *PstI* digestion.

Restriction enzyme digestion products were separated by gel electrophoresis using 0.8% agarose in Tris-borate buffer with ethidium bromide. The desired bands were excised under long wavelength UV light and DNA was eluted using the QIAquick® Gel Extraction Kit supplied by QIAGen. Vectors digested with restriction enzymes were dephosphorylated using shrimp alkaline phosphatase (Roche) and ligation reactions were performed using T4 DNA ligase (Fermentas).

Plasmid DNA was prepared and purified using the QIAPrep® Spin Miniprep Kit supplied by QIAGEN, according to the manual.

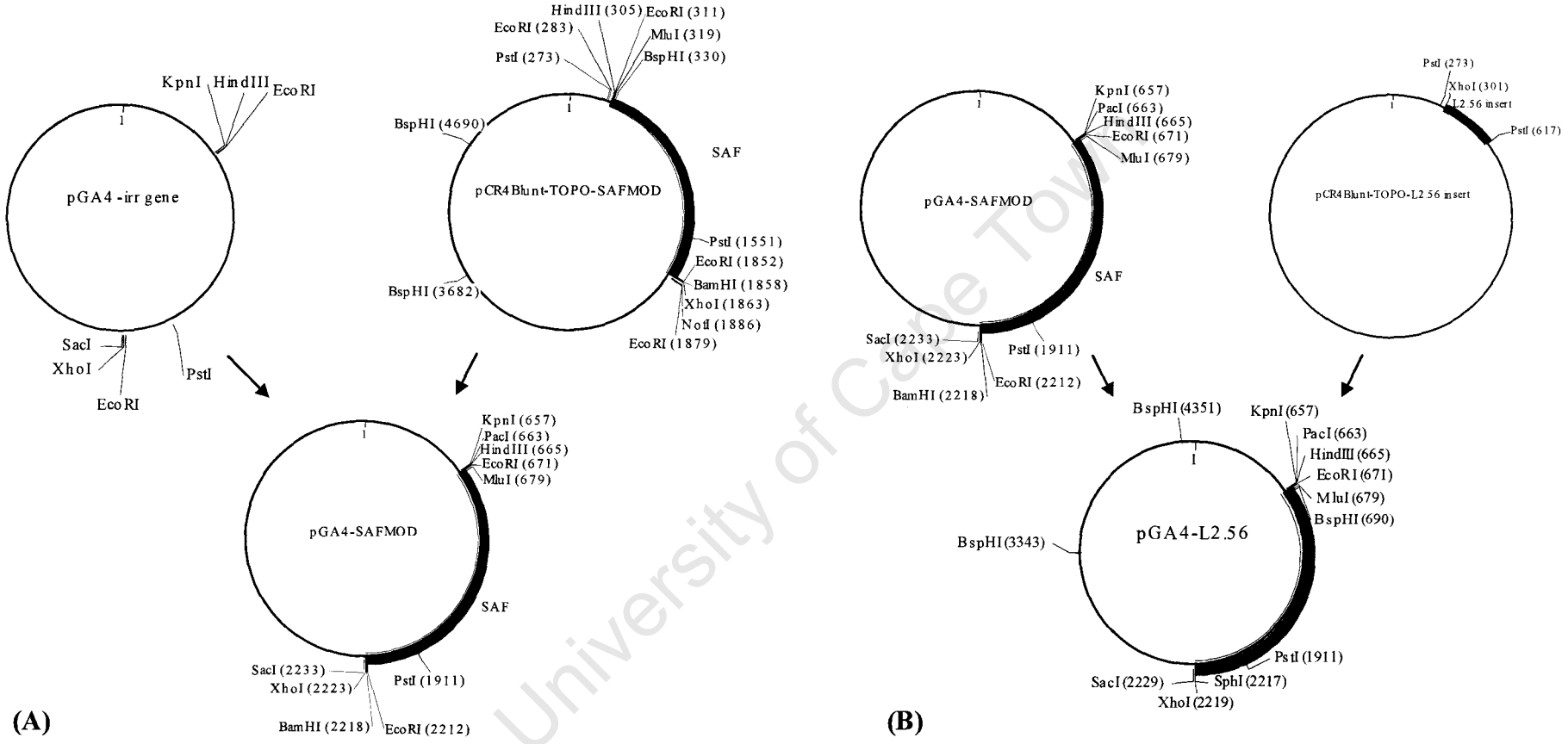


Figure 2.1. *(A) Cloning of SAF from PCR4Blunt-TOPO into the HindIII and XhoI restriction enzyme sites of pGA4 containing an irrelevant gene.* ***(B)*** *Creating chimaeric genes by replacing a fragment of SAF with the relevant modification for creating the new chimaeric gene. Shown as an example is the modification to create L2.56. The L2.56 insert was cloned into the PstI and XhoI restriction enzyme sites of pGA4-SAFMOD.*

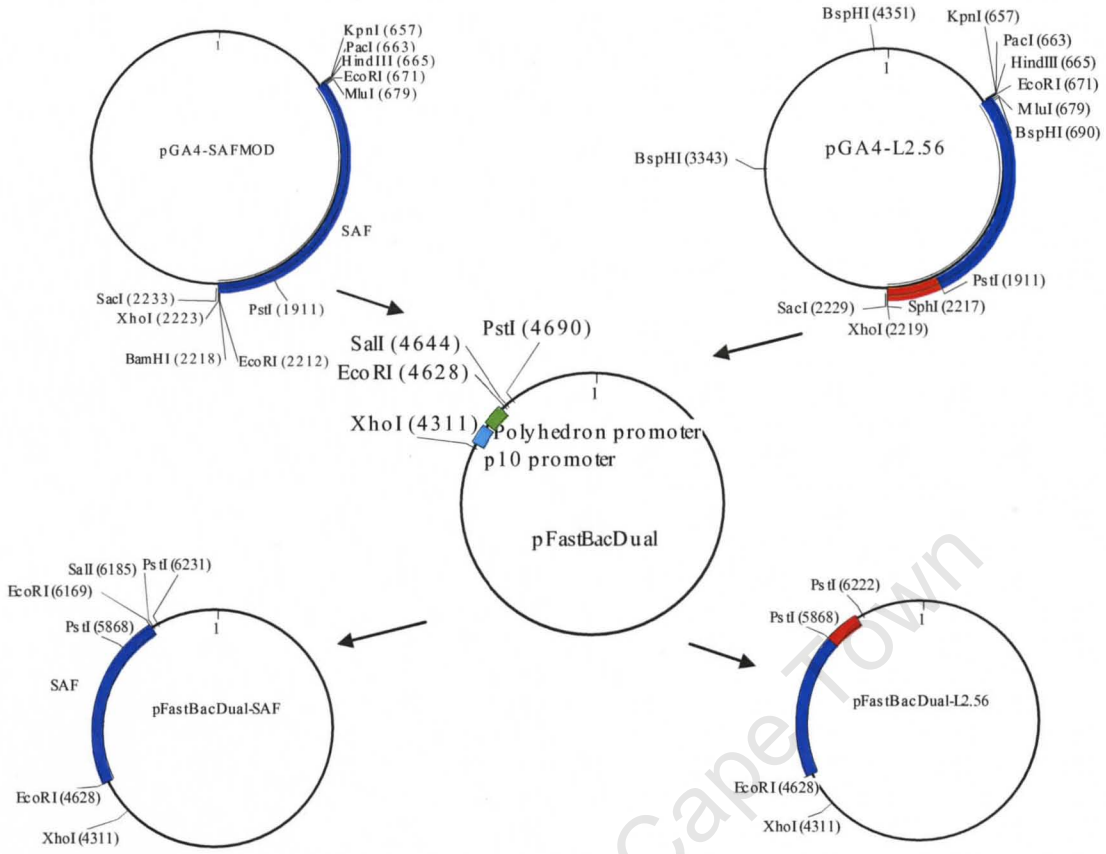


Figure 2.2. *Subcloning of chimaeric L1 genes into pFastBacDual. SAF was cloned non-directionally from pGA4 into the EcoRI sites of pFastBacDual. The direction of the cloned gene was confirmed by PstI digestion. The other chimaeric pGA4-constructs, of which pGA4-L2.56 is shown as an example, were digested with EcoRI and XhoI and the genes cloned directionally from pGA4 into the EcoRI and SalI restriction enzyme sites of pFastBacDual.*

2.2.3 PCR and primer design

Colony PCR was used to confirm the presence of a cloned insert after each cloning step. Details regarding the primers designed in our study are shown in Figure 2.3, while the details of the PCR reactions are shown in Table 2.2. The general PCR protocol, where only the annealing temperature would vary for each unique set of primers, is shown below. The final concentration of $MgCl_2$ in each reaction for all primers was 3mM. Reactions 1-5, 7, 9 and 11 were specifically used in colony PCR. The PCR products were visualised on a 0.8% agarose gel containing Tris-borate buffer (with ethidium bromide).

95 °C for 5min

95 °C for 30sec

Annealing temperature for 20 sec

72 °C for 30 sec

X25

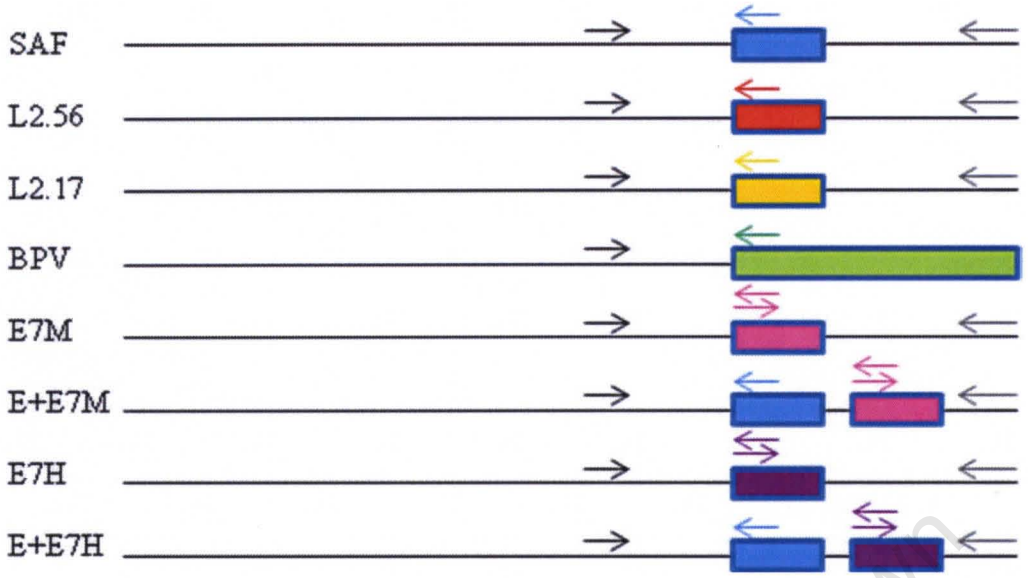
72 °C for 3 minutes

2.2.4 Sequencing

All constructs were sequenced to confirm sequence identity. Sequencing was carried out by the DNA Sequencing Service, Department of Molecular and Cell Biology, University of Cape Town.

2.2.5. Preparation of bacmid DNA

Purified pFastBacDual constructs were transformed into DH10Bac™ *E. coli* (Invitrogen), to create recombinant baculovirus DNA, according to the Bac-to-Bac® Baculovirus Expression System Manual. Colonies were selected using colony PCR as described above. Bacmid DNA was extracted from *E.coli* by the following method: 5 ml Luria broth was inoculated overnight and centrifuged for 3 minutes at 13000 rpm in a benchtop centrifuge. 100 µl of solution I (0.25 M Tris-Cl pH8, 0.5 M Glucose, 0.1M EDTA in water) was added and the resuspended pellet kept at room temperature for 5 minutes, then on ice. Then freshly made 200 µl Solution II (0.2M NaOH, 1% SDS in water) was added and the tube was kept on ice for 5 minutes. Cold Solution III (3M potassium acetate, made to pH 4.8 with acetic acid), 150 µl, was added, followed by 5 minutes on ice. The resultant mix was then pelleted at 14000 rpm in a benchtop centrifuge to obtain the supernatant. Nine hundred microliters of 96% ethanol was added and the DNA pelleted at 13000 rpm in a benchtop centrifuge. The air-dried pellet was resuspended in 300 µl Tris EDTA pH 8 (10 mM Tris, 1M EDTA), to which 15 µl 3M sodium acetate and 630 µl 96% ethanol was added. This was followed by a 20 minute centrifugation step at 13000 rpm at 4°C in a benchtop centrifuge. The DNA pelleting step was then repeated with 70% ethanol, the pellet air-dried and resuspended in 50 µl Tris-EDTA pH8 buffer.



Forward primers		
Name	**Sequence**	**Key**
FwdModNew	5'-CGACGACCTGTACATCAAGG-3'	→
TLGIFwd	5'-CTGGGCATCGTGTGCCCTATC-3'	→
RAHYFwd	5'-CCACTACAACATCGTGACCTTC-3'	→
Reverse primers		
Name	**Sequence**	
VEETRev	5'-GATGAAGCTGGTCTCCTCC-3'	←
RAHYRev	5'-GAAGGTCACGATGTTGTAGTGG-3'	←
TLGIRev	5'-TAGGGCACACGATGCCCAGG-3'	←
L2Rev	5'-GGATGTAGCCGGTCCTGC-3'	←
QLYKRev	5'-ACCTTGGGGATGATGTCAGG-3'	←
SAL1BPVRev	5'-TATCTAGGGCTTCCTCCAGC-3'	←
EndModRev	5'-CATCACAGCTTCCGTTTCTTCC-3'	←

Figure 2.3. *Primers designed for use in diagnostic and colony PCR reactions and their complementarity to L1 chimaeras. Each chimaeric L1 gene is indicated as a solid, black line, with its unique inserted sequence indicated as a coloured block. Primers are indicated as coloured arrows and are placed above their binding sites within the chimaeric genes. The key to the primers is shown in the table below the diagram, together with each primer's sequence. Diagram not drawn according to scale.*

Table 2.2. Annealing temperatures and the expected size of the PCR product of PCR reactions used to confirm the presence of an insert.

Reaction number	Chimaera	Annealing temperature (°C)	Primer combination	Size of band yielded (bp)
1	SAF	58	FwdModNew and VEETRev	410
2	L2.56	58	FwdModNew and L2Rev	442
3	L2.17	60	FwdModNew and QLYKRev	444
4	BPV	60	FwdModNew and SAL1BPVRev	559
5	E7M	55	FwdModNew and RAHYRev	419
6		64	RAHYFwd and EndModRev	224
7	E+E7M	55	ModFwdNew and RAHYRev	467
8		64	RAHYFwd and EndModRev	227
9	E7H	55	FwdModNew and TLGIRev	416
10		*61*	EndModRev and TLGIFwd	227
11	E+E7H	*55*	FwdModNew and TLGIRev	467
12		*61*	EndModRev and TLGIFWD	176
13	All Chimaeras except BPV	*60*	FwdModNew and EndModRev	700

2.2.6 Preparing viral infectant from *Spodoptera frugiperda* Sf-21 cells

Purified bacmid DNA was then transfected into Sf-21 insect cells. A 35 mm tissue culture dish was seeded overnight with $1x10^6$ cells per well in complete TC-100 (TC-100 supplied by Sigma, 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin). A volume of 5 µl of bacmid DNA was used regardless of concentration and the medium used was TC-100. Otherwise the protocol in the Bac-to-Bac® Baculovirus Expression System Manual was followed. Briefly, 5 µl of bacmid DNA was mixed with 100 µl of TC-100 and 6 µl of

Cellfectin® (Invitrogen) was mixed with 100 µl TC-100. The two solutions were mixed and left at room temperature for 15-45 minutes, after which it was made up to a total volume of 1 ml. The cell monolayer was washed twice with TC-100 to remove all traces of BSA and the DNA mixture added to the cells. The cells were then incubated for 5 hours at 27°C, after which another 1 ml of complete TC-100 was added. The primary supernatant was removed from the cells 48-72 hours post-transfection and clarified by centrifugation. Fresh medium was added to the cells and incubated for a further 48-72 hours and the secondary supernatant removed as for the primary.

Cells were then harvested in order to check protein expression by washing them off the bottom of the well with cold Dulbecco's PBS (DPBS), the cells were washed by centrifugation at 6000 rpm and the cell pellet resuspended in 100 µl DPBS. The cells were then subjected to three freeze-thaw cycles and protein expression verified by western blotting using J4 monoclonal antibody (a gift from Dr. Neil Christensen, Penn State Pathology and Laboratory Medicine).

If protein expression could be verified, the secondary supernatant was plaque-purified using Sf-21 cells. This was done to purify a single viral clone, which would enhance protein expression levels. The Bac-to-Bac® Baculovirus Expression System Manual was followed here. Briefly, $1.5x10^6$ Sf-21 cells in 2 ml of complete TC-100 were seeded in a 35 mm tissue culture dish and allowed to attach for 30-60 minutes. A tenfold dilution of the secondary supernatant was prepared in complete TC-100. Dilutions from 10^{-4} to 10^{-9} were used in this assay. The medium was removed from the cells and replaced with 1ml of the viral dilution. The virus was allowed to infect for 2 hours, after which the viral infectant was removed from the cells and the cells overlaid with 3ml per well of 1% SeaPlaque Agarose (Adcock Ingram Scientific) in Grace's Insect Plaquing Medium. The cells were then incubated for about 96 hours in an airtight container. The agarose-overlaid cells were then stained with 1ml of 100 µg/ml of neutral red in complete TC-100 for 3-5 hours, the dye removed and the cells incubated overnight at 27°C. Agarose from areas that stained white against the red background or one single plaque were then removed with a sterile pipette tip and resuspended in 1ml complete TC-100.

The medium containing the single plaque was then amplified twice serially. First, 500 µl infectant was used to infect $1x10^6$ Sf-21 cells per well in a 35 mm tissue culture plate for 1

hour, after which the infectant was removed and 2ml complete TC-100 added. The supernatant was removed after 96 hours and clarified by centrifugation, yielding Passage 1 infectant. One milliliter of Passage 1 infectant was then added to $5x10^6$ cells for 1 hour, after which it was removed and 10 ml complete TC-100 added. The supernatant was removed after 96 hours and clarified by centrifugation, yielding Passage 2 infectant. The Passage viral stock is then titreed in order to determine the plaque forming units per ml (pfu/ml) using a plaque assay according to the The Bac-to-Bac® Baculovirus Expression System Manual and as described above for single plaque purification, except that titration was generally done in duplicate at dilutions ranging from 10^{-6} to 10^{-9}.

2.2.7 Screening for purity of viral infectants

DNA extracted from plaque-purified, amplified viral supernatant was used to verify the identity of the construct and to eliminate the possibility of cross-contamination with other constructs. DNA was extracted using the RTP® DNA/RNA Virus Mini Kit (Invitek). The DNA was then used in a series of PCR reactions. This is described in Table 2.3. In each case positive controls, constructs that would yield a band with a specific primer combination, were included. BPV was the exception in that a set of primers (Reaction 13) binds to all the other chimaeras except BPV. Thus a negative result was used to confirm its identity. This was done because the reverse primer used in reaction 3 is semi-complementary to BPV. All other chimaeras were included as controls with the primers used in reaction 13.

2.2.8 Preparation of protein for vaccination purposes from *Spodoptera frugiperda* Sf-9 cells

Expression in *Sf-9* cells is not expected to differ in any way to expression in *Sf-21* cells. Therefore *Sf-9* cells were infected at a multiplicity of infection (MOI) of 1 and a cell density of $1x10^6$ cells/ml for 96 hours. Cells were counted, pelleted by centrifugation at 1000xg, washed with DPBS and pelleted again. Pellets were stored at -70°C and then resuspended in 20 times less volume of DPBS than the volume the cells were in. The cells were then lysed by three freeze-thaw cycles (-70 °C, 37 °C), the debris pelleted twice and the supernatant stored at -70°C, taking aliquots for analysis to avoid multiple freeze-thaw cycles of protein that could lead to degradation and structure destabilisation.

Table 2.3. Reactions used in a diagnostic PCR to confirm absence of cross-contamination

	Reaction number from Table 2								
Chimaera	1	2	3	4	6	8	10	12	13
SAF	+	-	-	-	-	-	-	-	NP
L2.56	-	+	-	-	-	-	-	-	NP
L2.17	-	-	+	-	-	-	-	-	NP
E7M	-	-	-	-	+	-	-	-	NP
E7H	-	-	-	-	-	-	+	-	NP
E+E7M	NP	-	-	-	-	+	-	-	NP
E+E7H	NP	-	-	-	-	-	-	+	NP
BPV	NP	NP	NP	+	NP	NP	NP	NP	-

NP=not performed. + and – denote positive and negative reactions respectively.

2.2.9 Quantification of protein

The quantity of L1 chimaeric protein was estimated by western blotting. Before electrophoresis, protein samples were diluted to the desired concentration in 5xloading buffer (2% SDS, 100mM Tris-HCl pH 7.5, 2mM EDTA, 91% glycerol, 7.6% beta-mercaptoethanol) and boiled at about 90°C for 5 minutes. Samples were then loaded onto a 12% acrylamide gel and electrophoresed for 2 hours at 20 mA. The gel was then blotted onto a nitrocellulose membrane for 1h30min at 15V. The membrane was blocked with blocking buffer (0.1% Tween-20 and 5% skim milk in 1xPBS pH 7.6), then incubated for at least 1 hour with a 1:10 000 dilution of primary antibody (Camvir-1, Abcam®) in blocking buffer. This antibody binds to the linear epitope, aa 230-236 (McLean *et al.*, 1990), which is not destroyed by any of the insertions. Secondary antibody was anti-mouse IgG (Sigma) used at 1:10 000 in blocking buffer for at least 1 hour. After each antibody step, the membrane was washed thrice for 15 minutes in wash buffer (0.1% Tween-20 in 1xPBS pH 7.6). Bands were visualised with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) substrate (Roche).

SAF previously quantified against wild-type L1 was used to construct a standard curve by loading a doubling dilution. Similarly, a dilution series of the sample to be quantified was also loaded. The intensities of the bands were measured by densitometry (GeneTools,

Syngene, Synoptics Ltd). A log curve was fitted to the readings of the standards using Microsoft Excel and the equation used to estimate the quantity of protein in each sample.

2.2.10 Purification of chimaeric L1 protein by ultracentrifugation

Ultracentrifugation using the density gradient medium iodixanol (the tradename is Optiprep™) was tested as a potential method to purify chimaeric L1 protein. Optiprep™ (Sigma), 60% w/v iodixanol in water, was diluted to 24% in DPBS. Cells were resuspended and lysed by freeze-thaw cycles in DPBS as described above. One millilitre of supernatant was then mixed with a Optiprep™-in-DPBS solution to a final concentration of 24% Optiprep™. The sample was centrifuged in a SW55Ti rotor at 35000 rpm for about 16 hours. The tube was then punctured with a needle at the bottom, either to collect ten 500 µl fractions, or on the side to extract the bands. Fractions were analysed by dot blot. Briefly, 2 µl of sample was dotted onto a nitrocellulose membrane. The membrane was then treated as for a western blot, using J4 as the primary antibody.

2.2.11 Protein purification by cation exchange chromatography

The method used was based on a method published by Cook *et al.* (Cook *et al.*, 1999). Diafiltration buffer (0.2 M (Na^+)MOPS, pH 7.0 1 0.4M NaCl), column Buffer A (0.05 M (Na^+)MOPS, pH 7.0 + 0.5M NaCl) and Column Buffer B (0.05 M (Na^+)MOPS, pH 7.0 + 1.5M NaCl) were made up according to this paper. Sf-9 cells were harvested by centrifugation at 1000xg. The pellet was washed with 1xDPBS and pelleted again. The pellet was resuspended at $12.6x10^6$ cells/ml (the count after infection) in diafiltration buffer containing protease inhibitor. Cells were sonicated, the cell debris pelleted and the supernatant filtered through a 0.45 um filter. Chromatography was first carried out using three cation exchange columns and the Akta Explorer 10 Chromatography System: Sulfopropyl (SP) Sepharose™ Fast Flow, carboxymethyl (CM) Sepharose™ Fast Flow and sulfopropyl (SP) Sepharose™ XL (supplied by GE Healthcare Life Sciences). The column was pre-equilibrated with 5 column volumes of diafiltration buffer. One millilitre of sample was loaded onto the column. The column was then washed with 10 volumes Column Buffer A. Protein was eluted using a 100% Column Buffer A-100% Column Buffer B linear gradient. The column was washed with 2 column volumes buffer B and the waste collected. The fractions were analysed by a dot blot as described above.

In another instance, the HS50 POROS® column (Applied Biosystems) was used. A flow rate of 15-75 ml/min is suggested for this column, however, the Akta Explorer 10 Chromatography System cannot perform at such a high rate, thus a flow rate of 10 ml/min was used. The sample prepared above was further diluted to $1x10^6$ cells/ml. Four milliliter of sample was injected. The protocol in Cook *et al.* (Cook *et al.*, 1999) was followed, except that the protein was eluted in 5 column volumes. Samples were analysed by indirect ELISA.

2.2.12 Indirect ELISA

A 96-well plate (Maxisorp, Nunc), was coated with 100 µl per well of 1:2000 Camvir-1 overnight at 4°C, followed by 2 hours blocking with 0.5% (w/v) polyvinyl alcohol (PVA) in PBS. The wells were then coated with 100 µl test samples of protein for 2 hours. The standard curve was wild type L1 with six doubling dilutions ranging from 9ng/ml to 300 ng/ml. Polyclonal rabbit anti-L1, 100 µl per well, diluted to 1:1000 in 0.5% PVA, was then added and incubated overnight at 4°C. Polyclonal swine anti-rabbit antibody, conjugated to horseradish peroxidise, was diluted to 1:2000 in 0.5% PVA, 0.8% polyvinylpyrrolidine in PBS and 100 µl added to each well for 30 minutes. The substrate, o-phenylenediamine dihydrochloride (OPD) tablets (supplied by Dako) was diluted as per instructions and 100 µl added per well. Development time was thirty minutes, the reaction was stopped with 0.5M H_2SO_4 and read at 490nm. All solutions were made up in PBS (pH 7.6) and between each step the wells were washed four times with 300 µl PBS per well.

2.3 Results

2.3.1 Verification of protein expression in Sf-21 cells

All eight chimaeric L1 genes, those listed in Table 2.1 as well as SAF, could be expressed as the full-length protein in Sf-21 insect cells after transfection with recombinant bacmid DNA and harvesting of the cells. Protein was harvested from the cells, because it is known from previous work that HPV L1 is expressed within the cell (Park *et al.*, 1993). The expression of the full-length constructs was confirmed using western blotting and probing with J4, a monoclonal antibody that detects a linear epitope (aa 261-280) (Christensen *et al.*, 1996) in HPV-16 L1. This epitope is not destroyed by any of the insertions. The western blots are shown in Figure 2.4. Figure 2.4E shows cell lysate from *Sf-9* cells infected with non-recombinant baculovirus. Expression in *Sf-9* cells is not expected to differ in any way to

expression in *Sf-21* cells. Camvir-1 reacted non-specifically with a protein about 40 kDa in size, but no 55 kDa band could be seen for protein samples derived from cells infected with non-recombinant baculovirus.

E7H and E+E7H would not be used in mouse experiments, because the inserted CTL epitopes are specific to human alleles. Therefore these two chimaeric proteins were not included in further analyses.

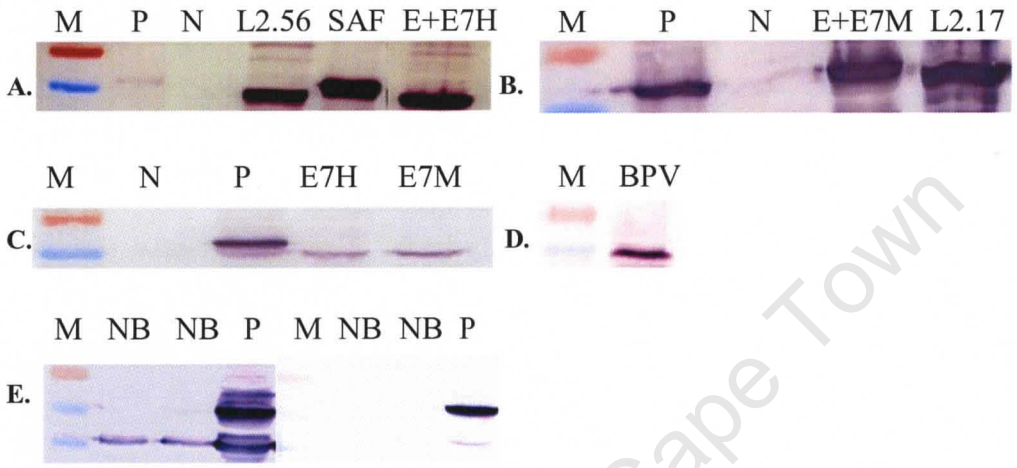


Figure 2.4. *Verification of expression of L1 chimaeric genes in Sf-21 insect cells after transfection with recombinant bacmid.* ***A.*** *L2.56, SAF and E+E7H.* ***B.*** *E+E7M and L2.17.* ***C.*** *E7H and E7M.* ***D.*** *BPV.* ***E.*** *Insect cell lysate after infection with non-recombinant baculovirus. The blot on the left was probed with Camvir-1, and on the right with J4 mAb. P=previously tested positive sample, N=mock-infected Sf-21 cells, NB=non-recombinant baculovirus, M=protein marker, showing the 55 and 70 kDa bands respectively and the 40 kDa band in the case of* ***E.*** *All the blots were probed with J4, an HPV-16-specific mAb, except as stated for* ***E.*** *All chimaeras were ~55kDa in size.*

2.3.2 Verifying chimaera identity by PCR

Since antibodies specific to the inserted epitopes were not available, the only way to distinguish between the different L1 chimaeric proteins and to ensure purity of the viral infectant, was to perform PCR on DNA extracted from viral infectant. This was done using primer sets that bind to the inserted epitopes as described in Section 2.2.7. Figure 2.5 shows examples of such PCR amplifications, as performed for SAF and BPV. The purity of all chimaeric proteins could be confirmed by PCR using this method.

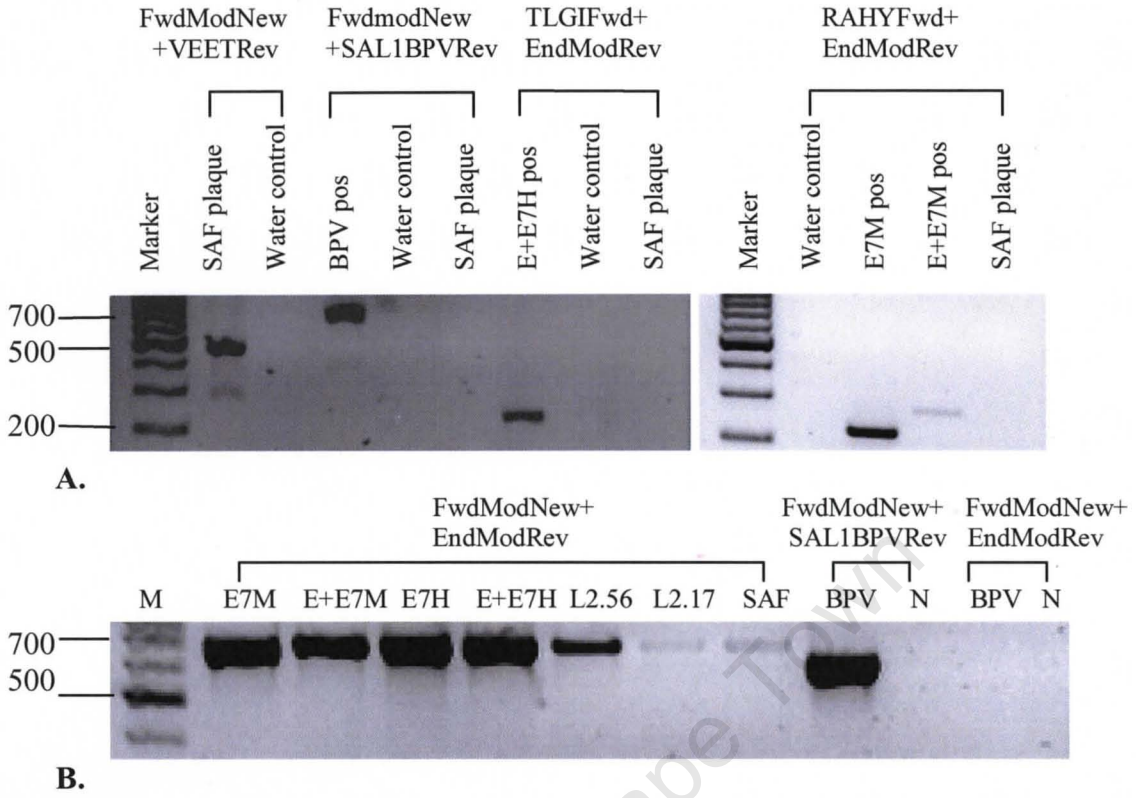


Figure 2.5. *Diagnostic PCR.* ***(A)*** *DNA extracted from infectant containing plaque-purified SAF-recombinant baculovirus (denoted as pl in the figure) was tested for purity with different primer sets in PCR. Pos=Positive control, either bacmid DNA or DNA extracted from viral infectant and previously tested from a construct that would yield a band with the relevant primer set. The primer set used in each reaction is denoted on top of the brackets.* ***(B)*** *DNA extracted from infectant containing plaque-purified BPV-recombinant baculovirus (denoted as BPV in the figure) was tested for purity, using a primer pair that would not yield a band with BPV, but would yield a band if used with all the other constructs. The primer set used in each reaction is denoted on top of the brackets. Bacmid DNA or DNA extracted from viral infectant confirmed to be positive for the relevant construct from all the other chimaeric constructs was tested as positive controls. N=no DNA control.*

2.3.3 Expression levels of chimaeric L1 proteins

When the chimaeric L1 proteins were expressed in insect cells under identical conditions, the expression levels were found to differ greatly when assayed by an indirect quantitative ELISA. The difference in expression levels indicates that expression levels of a protein could be influenced by the nature of the amino acids inserted, the length of the insertion or the exact position of insertion. This effect can be seen in Figure 2.6A, where the six chimaeric L1 proteins were expressed under identical conditions in insect cells originating from the same

flask of cells, and extracted under identical conditions. This trend roughly corresponds to the trend seen in a previous experiment where proteins were purified by Optiprep ultracentrifugation and visualised by western blotting (Figure 2.6B). It is possible that not all the proteins migrate to a similar position within the Optiprep gradient, however, a difference in migration among the different constructs was not observed to be the case (data not shown).

The range of concentrations obtained for the different L1 chimaeric proteins from various experiments are shown in Table 2.4. The highest levels seen were for L2.17 at 562 μg/ml per 10^6 cells, followed by L2.56 with 149 μg/ml per 10^6 cells, E7M with 53 μg/ml per 10^6 cells, SAF with 42 μg/ml per 10^6 cells, BPV with 10 μg/ml per 10^6 cells and E+E7M with 6 μg/ml per 10^6 cells. Expression levels were highly variable between experiments performed for the same construct; therefore the levels cannot be deemed significantly different between SAF, L2.56, L2.17 and E7M. However BPV and E+E7M consistently expressed lower levels of protein than the other chimaeras that were below or equal to 10 μg/ml per 10^6 cells.

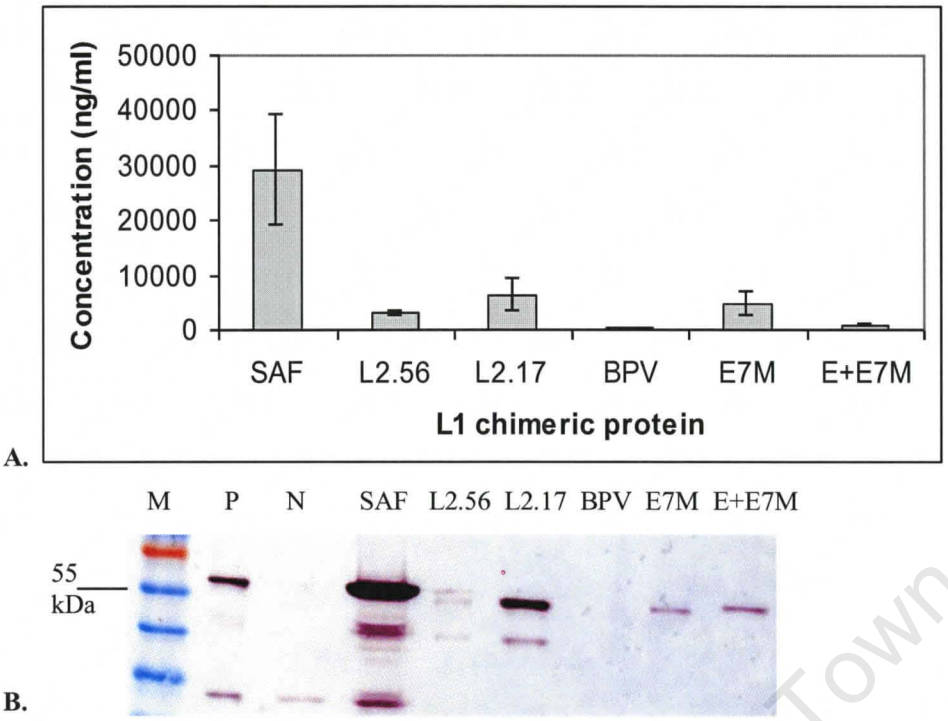


Figure 2.6. *L1 chimaeric genes yield different levels of protein when expressed in and extracted from insect cells under similar conditions.* ***A.*** *Indirect L1-ELISA was used to quantify protein levels. Sf-9 insect cells were infected at MOI=1 with cell density at 1x10^6/ml and harvested after 96 hours. Cell pellets were resuspended in equal volumes of DPBS containing protease inhibitor and sonicated. The supernatant containing total cellular protein was tested in the ELISA. The error bars indicate standard deviation between replicates.* ***B.*** *Western blot detection, using J4 antibody, of proteins expressed and extracted as above, and subjected to a 5%-25% step Optiprep ultracentrifugation. Equal volumes of protein (20ul each) were loaded onto the SDS-PAGE gel.*

Table 2.4. Concentration of protein obtained and the number of cells/ml post-infection for different batches of chimaeric L1

Chimaera	Concentration (µg/ml)	Cell count post-infection (million cells/ml)	Protein concentration per 10^6 cells (µg)
SAF	29.2	0.98	29.80
	18.5	1.6	11.56
	2.3	1.46	1.58
	43.7	1.05	41.62
L2.56	3.2	0.14	22.86
	68.5	0.46	148.91
L2.17	6.4	0.51	12.55
	22	1.07	20.56
	376.6	0.67	562.09
BPV	0.3	0.03	10
	3	0.82	3.66
E7M	4.8	0.09	53.33
	13.9	NA	NA
	53.3	NA	NA
E+E7M	0.9	0.24	3.75
	3	0.58	5.17
	7.6	1.85	4.11
	9.6	1.6	6

NA=information not available

2.3.4 Purification by ultracentrifugation

Optiprep™ is the tradename for 60% iodixanol (weight/volume in water) and is a desirable medium for ultracentrifugation for various reasons. It is a ready-made, sterile and endotoxin-free solution that is safe to be injected into animals. These properties make it desirable for purification of vaccines for mouse studies, because no dialysis is required, as long as high-salt buffer is not employed. Optiprep™ is also iso-osmotic and isotonic, which reduces destabilisation of proteins.

For these reasons, Optiprep™ was the first ultracentrifugation medium of choice for purification of L1 chimaeric proteins. A self-generated, continuous gradient consisting of 24% Optiprep™ in DPBS was used in 5ml tubes in a swinging bucket rotor, a SW55Ti rotor,

at 35000 rpm overnight. After centrifugation, the tubes yielded a constant banding pattern. A band could be seen within the bottom 1 ml of liquid, and another one towards the top. The bottom band was shown to contain L1 protein as determined by a dot blot, probing with J4 or Camvir-1. However, when cells infected with non-recombinant baculovirus were subjected to the same process, the same banding pattern was seen. Subsequently, after fractionation, it was observed that the bottom 1 ml of liquid contains all of the L1 chimaeric protein. When these fractions were subjected to electrophoresis on an SDS-PAGE gel, stained with Coomassie Brilliant Blue R, and compared to cell lysate, no difference was seen between non-purified and purified protein. The L1 chimaeric protein was not concentrated by this method, either. Therefore it was concluded that this particular gradient does not separate out insect cell produced proteins, but rather sediments all the proteins at the bottom of the tube. This is shown in Figure 2.7.

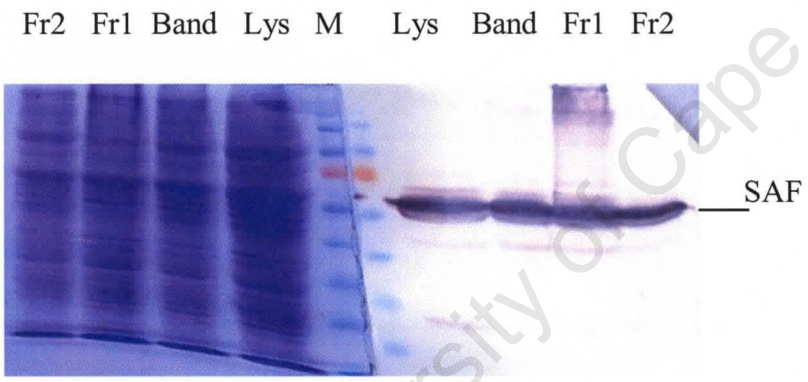


Figure 2.7. *A continuous gradient generated using 24% Optiprep does not concentrate or purify chimaeric L1 proteins. The figure shows a stained SDS-PAGE gel aligned with the other half of the gel as a mirror image used in a Western. Equal volumes of sample were loaded. Fr=500 ul fraction as counted from the bottom of the tube. Band=bottom band extracted from the ultracentrifuge tube, Lys=unpurified cell lysate.*

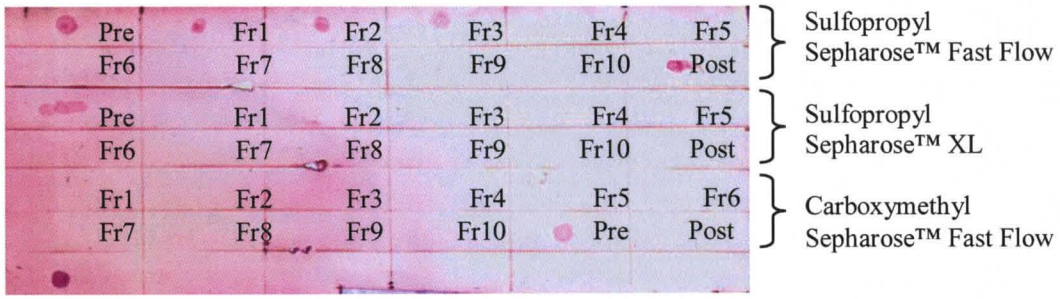
2.3.5 Cation exchange chromatography

Purification trials were performed with four different cation exchange columns. Cation exchange columns were used because the calculated pI for SAF indicated an overall positive charge, and previous work in our laboratory as well as literature (Cook *et al.*, 1999) indicates that a cation exchange column should be used. Previous work in our laboratory was based on the method by Cook *et al.* However, three other cation exchange columns were tested,

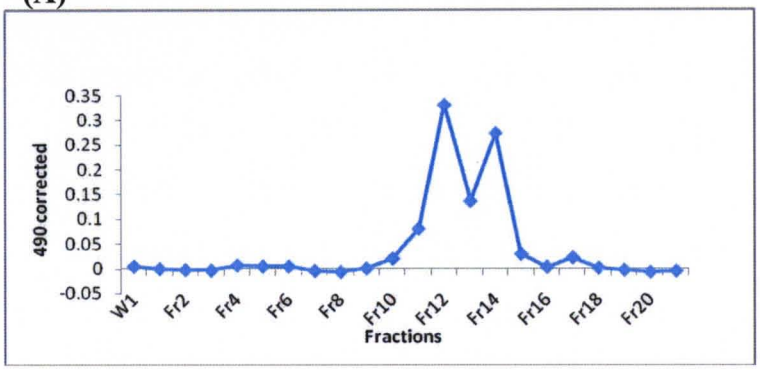
because these columns are better suited to the chromatography system and are routinely used in our laboratory. Two columns were strong cation exchangers with sulfopropyl functional groups. One column was a weak cation exchanger with carboxymethyl functional groups. SAF was tested as a proof of concept for the other chimaeras. Figure 2.8A indicates that the first three columns tested were not feasible for use. SAF eluted mostly in the pre-elution wash, except in the case of column 2, where SAF also eluted in fractions 1-3 and in the post-elution wash. The elution of SAF both in the pre-elution and post-elution wash is an aberrant result that could not be explained. It remains, however, that neither of the three columns tested first are feasible for purification of SAF. Figure 2.8B shows results for the HS50 POROS® column. SAF elutes from fraction 11 to 15. These fractions were also tested on a western blot, and the correct size of protein could be detected (data not shown).

2.3.6 Western blot quantification

The concentration of protein in the vaccine preparations was estimated by western blot. It is known from literature that the relationship between absorbance and protein mass on a western blot is not linear (Heidebrecht *et al.*, 2009). Saturation occurs, causing the curve to plateau. For this reason, a log curve was fitted to the points of the standard curve and the equation of this curve was used to calculate values for test samples. An example of how this quantitation was done is shown in Figure 2.9 for E+E7M.



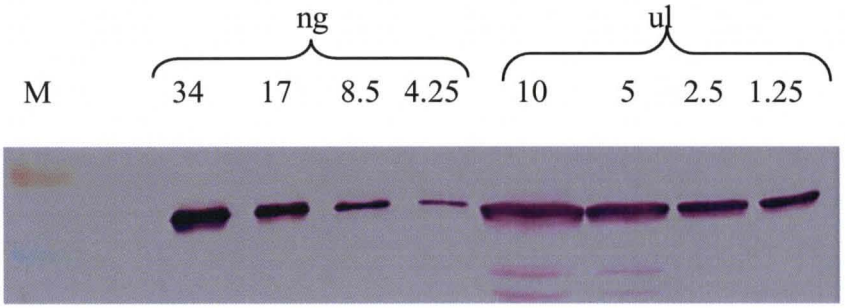
(A)



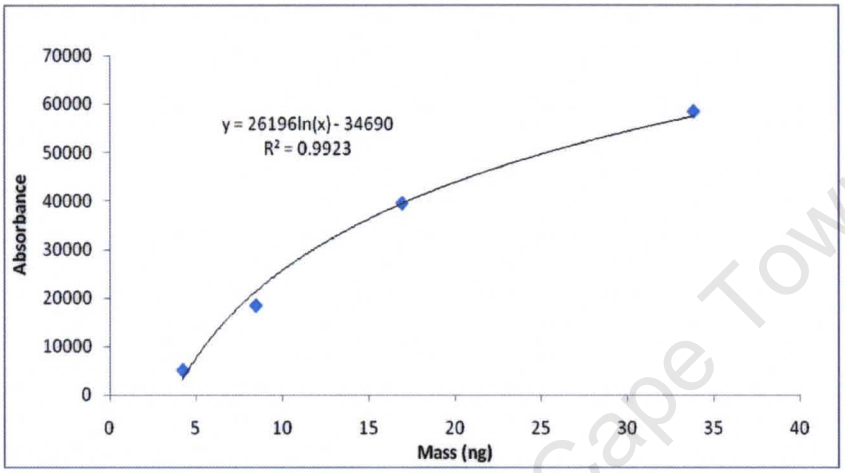
(B)

Figure 2.8. A. *Testing purification of SAF by cation exchange. Three different columns with either sulfopropyl (strong cation exchange) or carboxymethyl (weak cation exchange) functional groups were tested. Fractions were eluted over an increasing 0.5M to 1.5M NaCl gradient, dotted onto a membrane and probed with J4 antibody to detect the presence of SAF. The columns to which the fractions correspond are indicated to the right of the dot blot. Fraction descriptions are in the righ-hand bottom corner of each block containing a dotted fraction. Fr=fraction, Pre=Pre-elution wash, Post=Post-elution wash.* ***B.*** *Testing purification of SAF by cation exchange employing the HS50 POROS column, containing sulfopropyl functional groups. (strong cation exchange) Fractions were eluted over an increasing 0.5M to 1.5M NaCl gradient and tested for the presence of SAF by indirect ELISA. Fractions are numbered according to elution time. W1=pre-elution wash.*

A.



B.



Volume (µl)	Absorbance	ln(mass)	Calculated mass (ng)	Calculated concentration (ng/µl)	Average concentration (ng/µl)
10	74547.3	Out of range			
5	58039.4	3.540	34.461	6.892	9.612
2.5	46585.7	3.103	22.256	8.902	
1.25	38433	2.791	16.304	13.043	

Figure 2.9. *Estimating the concentration of protein by western blotting quantification.* ***A.*** *Western blot of E+E7M, probed with Camvir-1.The standard curve samples were loaded into lanes 3-6, with the total mass loaded per well indicated on top. Lanes 7-10 contain the test samples and the total volume of sample loaded is indicated above.* ***B.*** *The absorbance values of each band were plotted on a graph and a log curve was fitted to the data points. The R^2 value is displayed as well as the equation used in calculations. Below it is a table showing how the concentration of protein in the test sample was estimated. The equation indicated in B was used, where y=absorbance and x=mass in ng. The calculated mass was divided by the volume loaded to obtain the concentration.*

2.4 Discussion

2.4.1 Expression of chimaeric L1 constructs

All the chimaeric constructs could be expressed in insect cells as shown by western blot in Figure 2.4. The absence of a 55kDa band in any of the mock-infected or non-recombinant baculovirus samples confirmed that the bands seen for the recombinant constructs were indeed specific. However, it became clear that not all the chimaeric proteins expressed at similar levels in insect cells, as can be seen in Table 2.4 and Figure 2.6. The order of maximum expression from highest to lowest levels was L2.17, L2.56, E7M, SAF, BPV and E+E7M.

Expression levels were highly variable between experiments performed for the same construct and therefore expression levels between SAF, L2.56, L2.17 and E7M were not deemed to be significantly different. However, E+E7M and BPV were consistently expressed at lower levels than the other chimaeric proteins. These two chimaeras differ from the others in that they have amino acid replacements to the C-terminal end of aa 439. They also differ in that a longer region of L1 is modified; for E+E7M a region of effectively 27 aa is modified, whereas BPV has the whole C-terminal replaced from aa 414 to 505. In contrast, L2.56 also has a region of 25 amino acids modified, almost as long as for E+E7M, and it is expressed to high levels. For this reason, the lower expression levels obtained for E+E7M and BPV may be due to the L1 protein having amino acids replacements to the C-terminal end of aa 439, but whether it is due to the length of region modified, is not clear.

The inferior quantity of BPV may perhaps be ascribed to the deletion of nuclear localisation sequences. This effect was observed in other studies. Maclean *et al. (Maclean et al., 2007)* showed that expression in plants of a chloroplast-targeted construct where 22 amino acids were deleted from the C-terminal, thus truncating the nuclear localisation signal (NLS), was much lower than for the other constructs, where the NLS was not deleted. Senger *et al.* (Senger *et al.*, 2009) also reported inefficient production of HPV-16 L1ΔE7$_{(1\text{-}60)}$, which has the 34 aa deleted from the C-terminal of HPV-16 L1, in insect cells. In contrast to these two studies, Kohl *et al. (Kohl et al., 2007)* found that HPV-11 L1 could only be expressed in plants if the NLS was removed; other chimaeric L1 vaccines have also been expressed in insect cells with up to 34 aa deleted, yet expression levels comparable to that of wild type L1

could be obtained (Wakabayashi *et al.*, 2002; Liu *et al.*, 2002; Kuck *et al.*, 2006). It is therefore not entirely clear whether the deletion of nuclear localisation signals affect expression of HPV L1 protein.

The stability of BPV may be another reason for its low accumulation in insect cells. On a western blot, many degradation products were observed, but the number of degradation products was not markedly different from those obtained from any of the other constructs (data not shown).

The modification of a sequence by only a few amino acids can affect its expression and this may explain the differences in expression levels observed for different constructs in our study. Other studies in the literature have shown that different gene variants can often express at different levels. This difference in expression was seen when the expression of the South African HPV-16 L1 isolate was compared to its human codon-optimised and plant codon-optimised versions' in plants, (Maclean *et al.*, 2007) yielding vastly different expression levels between the different genetic variants. The human codon-optimised variant expressed at the highest levels, whereas the plant codon-optimised variant did not express at all. The authors suggested that the disruption of inhibitory sequences in the N-terminal of the human codon-optimised L1 gene could be one of many factors responsible for the higher expression of this variant. In our study, all nucleotide changes to HPV-16 L1 were in the C-terminal, but it is still possible that some of the sequences inserted contained inhibitory sequences, whereas others did not. It should be mentioned here that the entire sequence, both that of L1 and the inserted L2 sequences, of each construct in our study were human codon-optimised to optimise expression.

Another study (Touze *et al.*, 1998) also looked at expression levels for different L1 gene isolates. It was observed in one case that even one amino acid difference could majorly influence the expression levels of L1, and it was speculated that the difference in expression levels could be the result of truncated L1 RNA transcripts (Neeper *et al.*, 1996). However, all the mutations in Touze *et al.*'s study occurred in the region between residues 83 to 97 of L1, which does not correspond to the modifications made to L1 in our study. It is still conceivable, though, that the replacement of amino acids within L1 could affect the protein's expression levels, either at the transcriptional level or the translational level. Senger *et al.* (Senger *et al.*, 2009) found, though, that the length of mRNA transcripts did not correlate to

expression levels and concluded that translation rate and protein stability have a stronger impact on yield. It is likely that the different amino acids inserted may prevent the protein from folding properly, leaving the protein more prone to degradation.

The exact insertion site could be another factor influencing expression levels. In our study, all the chimaeric constructs, except those bearing E7 epitopes, had epitopes inserted after aa 413. However, due to the differences in length of the peptides replacing amino acids in L1, the exact insertion region differs between the constructs. According to Murata *et al.* (Murata *et al.*, 2009), when the proline residues flanking the helix 4 are not deleted, either when the helix 4 is deleted or replaced with a similarly-sized peptide, this impairs quantity and quality of capsomers produced. Whether monomers are still produced in their case, is not clear. The authors suggest that the proline residues impose structural constraints on the structure of the protein and that it affects quantity and quantity of capsomers produced. In our study, the proline residues are removed up to the C-terminal end of helix 4 in the case of L2.17, L2.56 and E+E7M, but not to the N-terminal end. In the case of SAF and E7M, proline residues flank the insertion on both sides, but in contrast to Murata *et al.*'s results, these two constructs were not inferior to the other constructs in our study with respect to expression levels. Because total protein content, regardless of whether higher-order structures assembled or not, were assessed in our study, and not capsomer quantity, as in the case of Murata *et al.*, their results may not be directly comparable with ours. However, as stated before, the inability to form higher-order structures may affect the stability of the protein. Their data does support that the notion that precise amino acid position where an epitope is inserted into L1 may affect quantity and quality of chimaeric protein produced due to the loss or retaining of key residues that have an impact on the structure of the protein.

Infectious constructs in this study were infected at the same MOI, with cells at the same density and harvested after the same period of time. The optimal conditions had been previously determined for SAF by our research group. However, optimal conditions may need to be separately determined for each construct. Cell density and infection time are the factors most likely to affect expression levels (Pillay *et al.*, 2009). Protein production can also be optimised by expression in a different cell line. It was determined for chimaeric HIV-1 VLPs that *Trichoplusia ni Pro*^{TM} expressed protein at slightly higher levels than *Sf-9* cells (Pillay *et al.*, 2009). Protein production levels can also be enhanced by the use of the MultiBac baculovirus expression system (Senger *et al.*, 2009), which has several

improvements over the conventional system. It was shown that by using this system, constructs that could not be produced efficiently by the conventional system, could now be expressed at much higher levels. Ways of improving the yield of low expressers like BPV or E+E7M therefore include using a different MOI, cell density, infection time, cell line or the MultiBac baculovirus expression system.

The stability of the baculoviruses after several passages may also differ from construct to construct. After plaque purification, passaging of the viral infectant was kept to a minimum for all the constructs in this study and each infectant was titreed with respect to plaque forming units per millilitre. However, the stability of each construct is not known.

2.4.2 Verifying the identity of the chimaeric protein

It was essential to develop a method that would distinguish between any two of the chimaeras, which would ensure that only one species of chimaeric L1 protein was present in a sample and that no cross-contamination had taken place. In order to make meaningful comparisons between the different chimaeric L1 proteins with respect to their immunogenicity, which was the ultimate aim of this project, each sample had to contain only one species of chimaeric L1 protein. Even if only low levels of contaminating protein is present, it may still have an effect, since L1 is highly immunogenic.

The different constructs could not be distinguished from one another by immunological techniques, unless mAbs binding to the specific L2 epitopes are used Such antibodies were not available in our laboratory and may not generally be available for all the epitopes that we had inserted into HPV-16 L1. Therefore recombinant baculovirus DNA was extracted from the viral infectant to be used in protein production, using the RTP® DNA/RNA Virus Mini Kit. A diagnostic PCR was then performed with this DNA. The PCR used unique combinations of primers that would either yield or not yield a PCR product with the construct to be tested, or would yield products of different sizes. The PCR method is set out in Tables 2.2 and 2.3 and in Figure 2.3.

As can be seen in Figure 2.5, the purity of each protein sample could be confirmed. No doubt can therefore exist that cross-contamination with other chimaeric L1 proteins could have influenced the immunogenicity results shown in Chapter 2.3.

The RTP® DNA/RNA Virus Mini Kit proved a very successful tool in isolating viral DNA from viral infectant, but also from crude insect cell lysate supernatant. Low levels of baculoviruses are likely to be present in the cells, despite the majority of them being secreted into the extracellular medium. We were therefore also able to distinguish between the different constructs, not only prior to infection, but also after extraction of total protein, before any purification steps have been attempted.(data not shown)

2.4.3 Purification of chimaeric L1 protein

It is desirable to purify and concentrate a protein to a high degree of purity before vaccination. This is to avoid immune responses against proteins that are not of interest. Such immune responses may interfere with the immune response against the protein of interest by downregulating it. Also, high levels of antibodies against many different proteins increase the chances of obtaining cross-reactive antibodies. Cross-reactive antibodies may cause high levels of background in an ELISA or western blot, which may mask the true immune response to the protein of interest. We therefore attempted purification of the six different chimaeric L1 proteins by two methods, ultracentrifugation and cation exchange.

2.4.3.1 Ultracentrifugation

We attempted to purify the different chimaeric L1 proteins by ultracentrifugation, using Optiprep™ and a self-generated gradient. This has not been attempted to our knowledge for empty L1 VLPs or capsomers.

L1 VLPs, especially chimaeric VLPs, when prepared for animal vaccination studies, are most commonly purified by a two-step purification involving sucrose and caesium chloride as gradient media (Varsani *et al.*, 2003; Kondo *et al.*, 2008; Kuck *et al.*, 2006; Schellenbacher *et al.*, 2009). The disadvantages to these media are that dialysis is required, a step that would involve loss of protein. Both media can also destabilise proteins due to being hyperosmotic, whereas Optiprep™ is an isoosmotic gradient medium (Axis-Shield, 2007). Optiprep™ does not need to be removed from protein samples prior to animal vaccination. The disadvantage is its expense, but optimisation of a method to purify chimaeric VLPs by ultracentrifugation is worth undertaking. Self-generated gradients, such as the 24% gradient we employed, are

convenient in that the results are highly reproducible and multiple samples can be processed rapidly.

As can be seen in Figure 2.7, SAF did not separate out sufficiently according to buoyant density, which indicates that the gradient was not steep enough. For a steeper gradient a higher percentage Optiprep should be used and centrifugation should be at a higher speed. Because swinging bucket rotors are not suitable for self-generating gradients, vertical or near-vertical rotors should be used (Axis-Shield, 2009).

Alternatively, a pre-formed continuous gradient could be employed. Buck *et al.* (Buck *et al.*, 2004) optimised a method for harvesting of HPV pseudovirions by using three steps of Optiprep at 27, 33 and 39% respectively. This method may have to be optimised to be applied to our work, since the chimaeric L1 particles in our studies are most likely to be capsomers not containing DNA and have a different buoyant density range, high-salt PBS unsuitable to vaccination studies is employed in their method, which is likely to change the density gradient, and the pseudovirions are purified from mammalian cells, not insect cells. However, purification of protein by Optiprep for animal vaccination studies should be investigated further, as it could be a simple, rapid method for preparing protein on a small scale without losses incurred by dialysis.We did not investigate this method further, due to time constraints.

2.4.3.2 Cation exchange chromatography

Another method employed in the purification of L1 protein is by cation exchange (Cook *et al.*, 1999). In fact, it is by this method that the VLPs contained in the Gardasil® vaccine have been purified (Shi *et al.*, 2007). For this reason, cation exchange was attempted in the purification of SAF as a prototype for all the other chimaeric L1 proteins. This was done using Sepharose columns routinely used in our laboratory that are well adapted to the Akta Explorer 10 Chromatography System. Unfortunately these columns could not be used in the purification of SAF. Figure 2.8A indicates that most of the protein is eluted in the pre-elution wash when the sepharose columns were tested. The elution of protein in the pre-elution wash would mean that the protein does not bind to the column in the first place.

Because the sepharose columns did not bind SAF, we tested another cation exchange column, the HS50 POROS column, previously tested in our laboratory and shown to purify SAF. From Figure 2.8B, it was clear that the HS50 POROS column does bind and elute SAF.

The most likely reason why the Sepharose columns did not bind SAF, whereas the HS50 POROS column did may be the difference in the make-up of the matrix. The sepharose columns consist of a 6% highly cross-linked beaded agarose matrix, and in the case of the SP XL column, it has long dextran chains coupled to the matrix to increase surface exposure of the functional groups. The POROS column has a matrix consisting of cross-linked polystyrene divinylbenzene. This matrix may be more permissible to binding of large particles like capsomers than the agarose matrix. It is likely that the functional groups are not responsible for the differences in binding of SAF, since the POROS column has the same sulfopropyl functional group as the SP Sepharose columns. Because the same buffers were used in both cases, it could also not be due to the ionic strength or pH of the buffer.

The HS50 POROS column is therefore a feasible method to be used in purification of SAF, and it also shows potential for the other chimaeric proteins, since all of them had an overall positive charge as indicated by their calculated pI, which were all very similar (data not shown). This method as applied to the other chimaeric proteins still needs to be tested, because the calculated pI is for the linear protein and does not take into account the surface charge of the protein. Isoelectric focusing could be employed in the future to determine the overall charge of the protein and whether this method may be used for all the chimaeric proteins.

2.4.4 Quantification of protein by western blot

In this study, the concentration of protein in samples to be used in vaccination studies was estimated by immunoblotting, as shown in Figure 9. There were several reasons for choosing this method over the conventionally used ELISA. Firstly, both Camvir-1 and J4, the antibody bound to the wells of the ELISA plates, showed cross-reaction with other proteins. Therefore this would give artificially high readings. On a western blot, because the proteins are separated according to size, the protein corresponding to the size of L1 can be quantitated, excluding cross-reactive proteins. Another reason for quantifying protein in a denatured state, as on a western blot, rather than in its native conformation, as would be the case in an ELISA,

is that it is not known how the binding of antibodies are affected by the epitope inserted into the protein. The protein could fold in such a way that the linear epitopes to which J4 and Camvir-1 antibodies bind are hidden.

This method was also chosen over using bovine serum albumin to construct a standard curve on a stained SDS-PAGE gel. Using SDS-PAGE was not a feasible method, because of the impurity of the protein extracts and the possible co-migration of other proteins of similar size to L1.

This method is a semi-quantitative method. A more accurate indication of the concentration may have been obtained if replicates of experiments were performed and if the standard and samples were loaded in replicate. However, different dilutions of samples were loaded, which allowed for replicate samples by which the average concentration of protein could be better determined.

Even though the standard and the sample were subjected to the same conditions within the experiment, there may still be differences within the blot. Not all bands necessarily transfer similarly from the protein gel to the nitrocellulose membrane, due to non-constant distribution of electrical resistance, which depends on protein amount and other factors, such as blot or gel irregularities (Heidebrecht *et al.*, 2009).

The relationship between the absorbance measured for each protein band on the western blot and the protein content is actually more complex than the one assumed in our study. Heidebrecht *et al.* (Heidebrecht *et al.*, 2009) showed that the relationship is more complex and is based on a hyperbolic function derived from the Michaelis-Menten equation. Applying their method of regression could have resulted in more accurate results. However, even though the absolute amount of protein in each sample may be inaccurate, the amounts of protein relative to one another could still be obtained in this manner, since all protein amounts were estimated against the same standard and in the same manner. It is also not uncommon for protein quantities to be estimated, rather than precisely determined, and used in vaccination studies. Estimation was previously done for insect cell lysate orally administered to mice where different formulations containing the same amount of protein were compared to one another (Thönes & Muller, 2007).

2.5 Conclusion

In conclusion, eight HPV-16 L1 chimaeric proteins were constructed with cross-neutralising and immunotherapeutic epitopes from L2 and E7 respectively. All could be expressed in insect cells. Crude extracts of six of the eight chimaeras were prepared from insect cells and quantified for purposes of animal studies. Of these, BPV and E+E7M expressed at lower levels than the others. Ultracentrifugation, using iodixanol as a centrifugation separation medium, may be a rapid, effective way of preparing protein on a small scale, however, a steeper gradient needs to be generated to obtain separation of proteins by this method. For large-scale preparation of proteins, column purification may be used in combination with other methods. The HS50 POROS cation exchange column bound and eluted SAF and this method should be tested further for the other chimaeric proteins.

3. IMMUNOGENICITY OF CHIMAERIC L1 VACCINES

3.1 Introduction

The selection of vaccine candidates is based mainly on its immunogenicity. Vaccines are first tested in animal models in order to obtain insight into their immunogenicity. Often a challenge model exists. These animals can be infected with the same infectious agent and the prophylactic or therapeutic ability of the vaccine can be tested directly. There are papillomaviruses that infect animals and cause similar disease to that caused by HPV, for example cottontail rabbit papillomavirus (CRPV). Vaccination strategies can be tested in these animal models utilising the species-specific virus, but the conclusions drawn from these studies may not necessarily be directly applied to HPV. Mice are a preferred animal model, because they are genetically well-characterised, easy to breed, easy to house and care for and biological reagents are readily available. Mouse cells can also be transformed with high-risk oncoproteins and implanted into laboratory mice to model tumours caused by HPV (Fausch *et al.*, 2003). Indicators of immune responses, such as antibody levels, cytokine release or T-cell counts, can also easily be assessed in mice.

There are two branches of the immune system, the humoral and the cell-mediated. The humoral immune system involves the binding of antibodies to extracellular pathogens and toxins for uptake and destruction by cells of the immune system. The cell-mediated immune system combats pathogens that replicate intracellularly and involves the killing of infected cells, thus preventing replication and release of new viruses (Janeway *et al.*, 1997a). Of the two branches of immunity, humoral immunity, or levels of neutralising antibodies, have been found to be the most important factor in preventing infection with papillomavirus (Breitburd *et al.*, 1995). In fact, the majority of antibodies are targeted against L1, the major capsid protein, and not L2, the minor capsid protein (Roden *et al.*, 2000). However, antibodies against L2 have been shown to elicit antibodies that neutralise pseudovirions *in vitro* (Roden *et al.*, 2000; Kawana *et al.*, 2003). Therefore mice or rabbits can be vaccinated with an HPV vaccine and levels of antibodies against both L1 and L2 in the blood can be assayed for as an indicator of how immunogenic the vaccine is.

A challenge model does not exist for HPV, however, antibody-mediated neutralisation of HPV *in vitro* can be tested. Pseudovirions, consisting of the capsid proteins L1 and L2,

encapsidating a reporter plasmid, can be produced in mammalian cells and employed in a pseudovirion neutralisation assay (Pastrana *et al.*, 2004). Human embryonic kidney cells over-expressing the SV40 large T-antigen (HEK293TT) are transfected with plasmids that contain an SV40 replication origin. These plasmids express the capsid proteins L1 and L2 of a particular HPV type as well as a reporter gene. The plasmid expressing the reporter gene is encapsidated by the assembled pseudovirions and is therefore of optimal size for encapsidation (Buck *et al.*, 2004). The pseudovirions are then harvested and used in a neutralisation assay. The pseudovirions infect HEK293TT cells in a way similar to that of native virus infection, resulting in the expression of the reporter plasmid. The reporter gene, as in this case, expresses alkaline phosphatase (AP) and the enzyme is secreted into the extracellular medium. Levels of this enzyme can then be assayed for in the extracellular supernatant by adding a substrate. When sera containing antibodies against HPV are added to the pseudovirion - infected cells neutralization of the pseudovirions occurs, and is quantified as a reduction in the level of AP in the extracellular supernatant. The percentage reduction in AP activity is therefore an indication of the levels of neutralising antibodies in the sera (Pastrana *et al.*, 2004).

The cell-mediated immune response has been found to be important in the clearance and regression of existing tumours caused by HPV (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). For therapeutic HPV vaccines, therefore, indicators of cell-mediated immunity must be used. The spleens of vaccinated mice can be removed and the splenocytes, amongst which are vaccine-specific T-cells, can be co-cultured with cells presenting the specific vaccine antigen in order to asses the function of vaccine specific T cells. Cytokines secreted by vaccine specific T cells in response to this stimulation indicates responses to the test vaccine. These cytokines can be assayed by a flow cytometric bead array (CBA) assay, a modification of ELISA (Janeway *et al.*, 1997d), and it has been one of the assays employed in this study to evaluate vaccine efficacy. The type of cytokine secreted also indicates whether the response is skewed towards a Th1 (cell-mediated) or Th2 (antibody) response (Janeway *et al.*, 1997c). The frequency of vaccine specific cells secreting a specific cytokine can be determined using an ELIspot assay. In this study the IFN-γ.ELIspot assay was used to determine the frequency of vaccine specific T cells secreting IFN-γ. Specific cytotoxic T-cell activity can also be assessed by ^{51}Cr-release or ^{3}H-thymidine release/retention assays (Janeway *et al.*, 1997d) but such assays have not been performed in this study.

Therapeutic HPV vaccines can also be tested directly for regression of tumours in mice by implanting tumour cells subcutaneously. Two cell lines are available for these purposes, C3 and TC-1. C3 cells are mouse cells that have been transformed with the complete HPV genome and the *ras* oncogene (Feltkamp *et al.*, 1993), while TC-1 cells have been transformed with the transforming proteins, E6 and E7, as well as *ras* (Torrens *et al.*, 2005). The tumour regression capacity of a vaccine can be assessed as the ability to cause regression of tumour size induced by either of these 2 cell lines. Vaccines can also be assessed for their ability to prevent tumour formation in a tumour challenge experiment (Torrens *et al.*, 2005). For this, animals are first vaccinated with the vaccine, then challenged with tumour cells and subsequently the tumour size is compared to that in the absence of vaccination. Tumour regression experiments are accepted as superior to tumour challenge experiments for evaluating vaccines because C3 or TC-1 cells do not have a 100% rate in forming tumours (Personal communication, Peter Öhlschläger). It is important to note here that when C3 cells are employed in tumour formation, vaccination with L1, which otherwise cannot be used to treat infections in humans (Hildesheim *et al.*, 2007), does mediate protection against tumours in mice (De Bruijn *et al.*, 1998; Ohlschlager *et al.*, 2003b).

If a peptide-based vaccine is tested for its therapeutic ability in mice, the peptide should be one restricted by the immune system of mice, unless transgenic mice expressing human MHC molecules are employed (Schreurs *et al.*, 2005). If mice that are not transgenic are employed, the results are not necessarily a true reflection of reality. In cases where the E7 peptide restricted by H2-D^b MHC class I molecules in mice was administered to mice, very good tumour regression was achieved (Torrens *et al.*, 2005; Paz De la Rosa *et al.*, 2009; Feltkamp *et al.*, 1993) However, when a similar approach is followed in humans, using a peptide restricted by human MHC class I molecules, no significant trend in the regression of tumours were seen in human volunteers (Muderspach *et al.*, 2000; Steller *et al.*, 1998; van Driel *et al.*, 1999). Tumour regression experiments in mice have therefore not been a good indicator of peptide-based vaccines' therapeutic efficacy in humans, but they remain the best way to ascertain therapeutic efficacy of HPV vaccines in preclinical studies.

In order to elicit a measurable immune response, whether humoral or cellular, in mice, an adequate dose needs to be administered. Previously, in other studies, a wide range of doses have been administered subcutaneously. Thönes *et al.* (Thönes *et al.*, 2008) observed measurable IgG titres, pseudovirion neutralisation titres and IFN-γ levels with as little as

three doses at 3ng each of VLPs injected subcutaneously without adjuvant. Biemelt *et al.* administered three injections of 40ng of plant- and insect cell-produced VLPs subcutaneously and detected an antibody response, however, the vaccine was mixed with adjuvant. On the other hand, Maclean *et al.* (Maclean *et al.*, 2007) detected a poor neutralising antibody response after vaccination of mice with one dose of 1 μg of insect-cell derived VLPs containing no adjuvant. The poor response may be attributed to the lack of booster vaccinations given. The number of boosts given is important as boosts are important for magnifying the immune response (Janeway *et al.*, 1997a).

For tumour challenge experiments, it seems at least 30 μg of L1/E7 chimaeric VLPs (cVLPs) were necessary to achieve an 80% survival rate amongst mice (Wakabayashi *et al.*, 2002) This necessity for a high dose may also be a result of applying only a single dose and no boosters. However, immunisation with a single dose of 10 μg of L1/L2/E7 without adjuvant afforded complete protection against tumour challenge in another study. (Greenstone *et al.*, 1998).

From the literature there emerges no clear optimum dose of VLPs to be administered to mice. Extracting data from a variety of sources, however, without distinguishing between humoral and cellular immune responses, indicates that any dose of L1, L1/E7, L1/L2 or L1/L2 E7 VLPs ranging from 5 to 100 μg, with or without adjuvant, with any number of injections ranging from 1 to 4, administered subcutaneously, may elicit an immune response (Greenstone *et al.*, 1998; Kawana *et al.*, 1998; Slupetzky *et al.*, 2007; Varsani *et al.*, 2003; Sadeyen *et al.*, 2003; Maclean *et al.*, 2007; Wakabayashi *et al.*, 2002; Greenstone *et al.*, 1998; Paz De la Rosa *et al.*, 2009; Qian *et al.*, 2006; Reddy *et al.*, 2004; Kim *et al.*, 2007b; Christensen *et al.*, 1996; Fleury *et al.*, 2006; Ryding *et al.*, 2007).

However, when L1 is in pentamer, as opposed to VLP formation, a higher dose is necessary to elicit the same immune response. According to Thönes *et al,* 2008, three injections of 48 ng of L1 pentamers each, elicited a detectable immune response that is very low, yielding only 34% neutralisation in an HPV-16 pseudovirion neutralisation assay. Adjuvants may rescue the immunogenicity, but this may depend on the adjuvant applied, because in their study, two injections of 10 μg each mixed with one adjuvant but not another, elicited an immune response. *E. coli*-produced pentamers (Ohlschläger *et al.*, 2003) in another study had to be at a total dose of at least 1 μg without adjuvant to elicit a significant CTL and

humoral immune response. In another study, 5 μg of pentamers without adjuvant were injected twice and a humoral immune response obtained (Schädlich *et al.*, 2009).

The immunogenicity of L1 depends in part on its assembly into higher structures (Lenz *et al.*, 2001; Thones *et al.*, 2008; Kuck *et al.*, 2006; Schädlich *et al.*, 2009). In fact, it seems to depend strongly on its ability to form at least capsomers (Kuck *et al.*, 2006) and it is also known that denatured L1 does not elicit the desired immune response (Schellenbacher *et al.*, 2009). The ability to assemble into "small VLPs" or T=1 particles of about 30-40 nm in size also enhances humoral immunity and the levels elicited may be comparable to those elicited by VLPs or T=7 particles (Schädlich *et al.*, 2009). Assembly into T=1 particles did not show to be of benefit to the cellular immune response and therefore the formation of T=7 particles remains preferable in such a case. Despite the lower immunogenicity of capsomers, they may still be employed in vaccine formulations, since adjuvants can compensate for their lowered immunogenicity.

The assembly of L1 into VLPs or capsomers may be influenced by modifications of L1. In our study, most modifications, except for BPV, disrupted the helix 4. This modification has repeatedly been observed to abolish formation of any higher-order structure other than capsomers (Schädlich *et al.*, 2009; Murata *et al.*, 2009; Bishop *et al.*, 2007a; Varsani *et al.*, 2003). All the chimaeras created and used in our study have the helix 4 deleted and are therefore expected to assemble into capsomers.

In our study, we immunised mice with insect cell lysates containing six different chimaeric vaccines as constructed in Chapter 2. Vaccination with crude preparations is not uncommon in the literature and may be a rapid way of assessing immunogenicity of a particular construct without having to purify it (Maeda *et al.*, 1996; Fan *et al.*, 2007; Cusi *et al.*, 1995; Sullender & Britt, 1996; Thönes & Muller, 2007). Vaccination with crude preparations can be employed as long as the negative control is also cell lysate negative for the protein of interest and the assays used to evaluate immunogenicity specifically selects for the response to the protein of interest.

In this chapter, the 6 chimaeras constructed, expressed in and extracted from insect cells and quantified as described by Chapter 2, are compared with respect to immunogenicity for purposes of selecting the best vaccine candidates. All the chimaeras were assessed with

respect to their ability to elicit protective homologous and heterologous antibodies, whereas the L1/E7 chimaeras were assessed for their ability to elicit cell-mediated immune responses essential for treatment of existing HPV-induced lesions and to effect tumour regression in mice. All the chimaeras were also analysed with respect to their ability to assemble into higher-order structures by electron microscopy.

3.2 Materials and Methods

3.2.1 Mice and cell lines

Female C57/BL6 mice were purchased from the South African Vaccine Producers Animal Unit (Johannesburg, South Africa) and maintained under BSL2 conditions according to European Guidelines in force in the Animal Unit of the Health Science Faculty, University of Cape Town. All mice were 7 weeks of age at the time of vaccination and prebleeds were taken 5-7 days before vaccination. Permission for this work was obtained from the Research Ethics Committee, University of Cape Town (REC REF 008/037).

C3 cells and EL-4 cells were kindly provided by Peter Ohlschläger. C3 cells were cultured in RPMI with glutamax, supplemented with 10% FCS, 0.1 mg/ml kanamycin, 0.8 mg/ml of G418 and 1% penicillin-streptomycin. The G418 was only added to the medium 3 days after cell culture was started. EL-4 cells were cultured under the same conditions, but without G418 or kanamycin.

3.2.2 Immunisation

For assessment of humoral immune responses, mice were vaccinated with SAF, L2.56, L2.17, BPV and a negative control. The details of this experiment are listed in Table 3.1. The negative control was cell lysate previously infected with wild-type baculovirus and prepared as for the other vaccines, as described in Chapter 2. All cell lysates were adjusted such as to contain the dose listed in Table 3.1 in the total volume of DPBS listed in Table 3.1. The vaccine was then homogenised by the syringe-extrusion technique (Koh *et al.*, 2006) in Incomplete Freund's Adjuvant (Sigma) at a 1:1 ratio of vaccine to adjuvant. The volume of vaccine plus adjuvant injected per subcutaneous site was 200 µl.

Table 3.1 Details of immunisation for the assessment of humoral immune responses

Vaccine	Number of mice (groupsxnumber in group)	Dose	Volume of DPBS	Injection site	Vaccination schedule	Final bleed
SAF	3x3	5 µg	200 µl	Subcutaneous	Day 0, 14, 42, 70	Day 77-80
L2.17	2x3 and 1x4	5 µg	200 µl	Subcutaneous	Day 0, 14, 42, 70	Day 77-80
L2.56	3x3	5 µg	200 µl	Subcutaneous	Day 0, 14, 42, 70	Day 77-80
BPV	3x3	1.8 µg	600 µl	Subcutaneous	Day 0, 14, 42, 70	Day 77-80
Negative control	2x3 and 1x4	N/A	200 µl	Subcutaneous	Day 0, 14, 42, 70	Day 77-80

N/A=not applicable

For assessment of cell-mediated immune responses, mice were vaccinated with SAF as the negative control, E7M, E+E7M and a DNA vaccine (HPV-16E7SH), expressing a shuffled E7 gene and thus having no transforming potential (Ohlschläger *et al.*, 2006), as a positive control. The details of this experiment is shown in Table 3.2. The DNA vaccine was kindly provided by Peter Ohlschläger and was prepared by using the EndoFree® Plasmid Giga Kit from Qiagen. SAF, E+E7M and E7M were adjusted to their final concentration, homogenised with adjuvant and administered as described above. The DNA vaccine was not mixed with adjuvant.

Table 3.2 Details of immunisation for assessment of cell-mediated immune responses

Vaccine	Number of mice (groupsxnumber in group)	Dose	Volume of DPBS	Injection site	Vaccination schedule	Final bleed and spleen removal
SAF	2x3 and 1x4	5 µg	200 µl	Subcutaneous	Day 0 and 14	Day 21
E7M	2x3 and 1x4	5 µg	200 µl	Subcutaneous	Day 0 and 14	Day 21
E+E7M	2x3 and 1x4	3 µg	600 µl	Subcutaneous	Day 0 and 14	Day 21
HPV-16E7SH	2x3 and 1x4	100 µg	50 µl	Intramuscular	Day 0 and 14	Day 21

For tumour regression experiments, mice were subcutaneously injected with $0.5x10^6$ C3 cells resuspended in 200 µl Dulbecco's Phosphate Buffered Saline (DPBS) on the right flank. When the tumours reached a size of 2-3mm in diameter, mice were vaccinated with SAF, E7M, E+E7M, HPV-16E7SH as a positive control and cell lysate previously infected with wild-type baculovirus as a negative control. SAF, E+E7M, E7M and the negative control were adjusted to their final concentration, homogenised with adjuvant and administered as described previously. The DNA vaccine was not mixed with adjuvant. Tumour size was then measured every 2-3days. Mice were killed and bled by cardiac puncture as soon as the tumour volume reached 2500 mm^3 or at the end of the experiment.

Table 3.3 Details of immunisation in tumour regression experiments

Vaccine	Number of mice (groupsxnumber in group)	Dose	Volume of DPBS	Injection site	Vaccination schedule	End of experiment
SAF	2x3 and 1x4	5 µg	200 µl	Subcutaneous	Day 0 and Day 14	Day 78
E7M	2x3 and 1x4	5 µg	200 µl	Subcutaneous	Day 0, 14, 39	Day 60
E+E7M	2x3 and 1x4	3 µg	600 µl	Subcutaneous	Day 0, 14, 39	Day 60
HPV-16E7SH	2x3 and 1x4	100 µg	50 µl	Intramuscular	Day 0 and Day 14	Day 78
Negative control	2x3 and 1x4	N/A	200 µl	Subcutaneous	Day 0, 14, 39	Day 60

N/A=not applicable

3.2.3 ELISA for detection of anti-L1 antibodies

3.2.3.1 Extraction of plant-produced SAF for use as antigen in ELISA

Because SAF had been previously tested in our laboratory, it served as a positive control for our experiments. Therefore SAF was used in an ELISA to detect antibodies in mouse sera. The protein was expressed in and purified from plants, because SAF produced in insect cells, even if semi-purified, would also detect antibodies against other insect cell proteins, since

mice were immunized with insect cell lysate. *Agrobacterium tumefaciens* were transformed with pTRAkc-rbcs1-cTP, into which the SAF gene had been cloned by Cathy Pineo, as well as pBIN-NSs, according to the method outlined by Maclean *et al.* (Maclean *et al.*, 2007) *Nicotiana tabacum* plants were infiltrated by injection as outlined by the same publication, but as many leaves as possible were infiltrated per plant. Five days post-infection, the leaves were harvested and the big stems excised. Leaves were ground up in liquid nitrogen, using a mortar and pestle. The leaf material was then suspended at 1g of leaf material per 12.5 ml of high-salt phosphate-buffered saline (HSPBS) with 0.5 M NaCl and EDTA-free protease inhibitor (Roche). The material was allowed to defrost and ground again, after which it was homogenised for about 10 minutes. The material was then sonicated for 9x30 sec with 20 sec rests after which it was filtered through Miracloth (supplied by CALBIOCHEM), a filtration material for gelatinous grindates, to get rid of the particulate matter. It was then centrifuged for 5 minutes at 25 000xg, the supernatant retained and centrifuged again under the same conditions. The final supernatant was stored at -80°C.

3.2.3.2 Purification of SAF from plant extracts

SAF was purified from crude plant extract by CsCl centrifugation. Crude supernatant was made up to 40% CsCl in HSPBS containing protease inhibitor. Samples were then subjected to centrifugation for about 23 hours at 36000 rpm in a SW55Ti rotor. Fractions (~500 µl each) were taken after centrifugation and 2 µl per fraction was spotted onto a nitrocellulose membrane. The membrane was blocked with blocking buffer (0.1% Tween-20 and 5% skim milk in 1xPBS pH 7.6), then incubated for at least 1 hour with a 1:10 000 dilution of primary antibody (Camvir-1, Abcam®) in blocking buffer. This antibody binds to the linear epitope, aa 230-236 (McLean *et al.*, 1990), which is not destroyed by any of the insertions. Secondary antibody was anti-mouse IgG conjugated to alkaline phosphatise (Sigma) and used at 1:10 000 in blocking buffer for at least 1 hour. After each antibody step, the membrane was washed thrice for 15 minutes in wash buffer (0.1% Tween-20 in 1xPBS pH 7.6). Spots were visualised with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) substrate (Roche).

Densitometry (GeneTools, Syngene, Synoptics Ltd) was used to measure the intensity of absorbance in each spot by placing a circle of the same size on each spot. A similar spot was placed on a blank section of the membrane and served as background. The mean number of

pixels per spot were then divided by those of the background. Only fractions with values at least twice as much as that of the background were pooled and served as the purified sample to be used in ELISA.

3.2.3.3 Quantification of SAF

SAF was then quantified by Western blotting as follows: Before electrophoresis, protein samples were diluted to the desired concentration in 5xloading buffer (2% SDS, 100mM Tris-HCl pH 7.5, 2mM EDTA, 91% glycerol, 7.6% beta-mercaptoethanol) and boiled at about 90°C for 5 minutes. Samples were then loaded onto a 12% acrylamide gel and electrophoresed for 2 hours at 20 mA. The gel was then blotted onto a nitrocellulose membrane for 1h30min at 15V. The membrane was blocked with blocking buffer (0.1% Tween-20 and 5% skim milk in 1xPBS pH 7.6), then incubated for at least 1 hour with a 1:10 000 dilution of primary antibody (Camvir-1, Abcam®) in blocking buffer. This antibody binds to the linear epitope, aa 230-236 (McLean *et al.*, 1990), which is not destroyed by any of the insertions. Secondary antibody was anti-mouse IgG (Sigma) used at 1:10 000 in blocking buffer for at least 1 hour. After each antibody step, the membrane was washed thrice for 15 minutes in wash buffer (0.1% Tween-20 in 1xPBS pH 7.6). Bands were visualised with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP) substrate (Roche).

SAFMOD previously quantified against wild-type L1 was used to construct a standard curve by loading a doubling dilution. Similarly, a dilution series of the sample to be quantified was also loaded. The intensities of the bands were measured by densitometry (GeneTools, Syngene, Synoptics Ltd). A log curve was fitted to the readings of the standards using Microsoft Excel and the equation used to estimate the quantity of protein in each sample.

3.2.3.4 ELISA

ELISA was performed as follows: 96-well plates (Maxisorp, Nunc) were coated with 30 ng of purified SAF per well diluted in 1xPBS pH 7.6 overnight at 4°C. Plates were then blocked with blocking buffer, (1% low-fat milk in 1xPBS) 300 µl per well, for 2 hours at room temperature. Sera from mice vaccinated for assessment of antibody production, as well as those vaccinated with E7M and E+E7M for assessing cell-mediated immune responses, was

pooled for each group, resulting in three groups per vaccine. Sera were diluted in a fourfold series, in triplicate, ranging from 1:50 to 1:51200, in blocking buffer and 100 µl was added to each well. Positive control wells, containing V5 monoclonal antibody binding to conformational epitopes at 1:2000 (a gift from Dr Neil Christensen) and blank wells containing no antibody were included. Sera were incubated in the wells for 2 hours at room temperature. Anti-mouse antibody conjugated to horseradish peroxidise at a 1:2000 dilution in blocking buffer was added at 100 µl per well and incubated for 1 hour at 37°C. Here, and in between each previous step, plates were washed 4 times with 300 µl per well of 1xPBS. O-phenylenediamine dihydrochloride (OPD) tablets (supplied by Dako) were then diluted as per instructions and 100 µl added per well. Plates were developed in the dark for 30 minutes at room temperature. The reaction was stopped with 0.5M H_2SO_4 and the absorbance read at 490 nm.

A two-tailed, non-paired t-test was used to calculate statistical significance as for the tumour volumes.

3.2.4 Western blot detection of anti-L2 antibodies

3.2.4.1 Preparation of L2 protein for use as antigen in Western blotting

His-tagged L2 produced in *E. coli* was used in a Western blot to detect anti-L2 antibodies in the sera of mice vaccinated with L1/L2 chimaeras. L2 was produced and purified by extracting inclusion bodies as follows: L2 in the pProEX htb vector. 100ml *E.coli* was grown to an optical density of 0.6 and induced by addition of iso-propyl-β-D-thiogalactoside (IPTG) to 0.6 mM. The culture was grown for another 3 hours under shaking. The cells were then harvested by centrifuging at 3800xg for 15 minutes at 4°C. The pellet was retained and weighed. Cells were resuspended in 4 volumes lysis buffer (50mM Tris pH 8.5, 5 mM β-mercaptoethanol) and phenylmethanesulfonyl fluoride (PMSF) added to 0.4mM, as well as lysozyme (Roche) to 0.08 µg/µl. The cells were then left on ice for 20 minutes. Triton-X was then added to 1% final concentration and the cells left for a further 20 minutes at 37°C until the solution was viscous. DNAse and RNAse were then added to 4 µg/ml and 40 µg/ml final concentration respectively. Cells were then left at 30 minutes at room temperature until it wasn't viscous any more and centrifuged at 13000 rpm in a benchtop centrifuge for 15 minutes at 4°C. The pellet in each microcentrifuge tube was resuspended in 1 ml lysis buffer

(2.5 mM Tris pH 8.0, 3.125 mM β-mercaptoethanol, 0.2mM EDTA, 0.0025% Triton-X diluted in water) and left to lyse for ten minutes at room temperature. Another 13000 rpm centrifugation step was carried out for 15 minutes at 4°C, followed by four washes of the pellets with 1xPBS. The final pellet was resuspended in 1 volume of the weight of the pellet of 1xPBS.

3.2.4.2 Western blot

His-tagged L2 protein was then subjected to electrophoresis on a 10% acrylamide gel. However, instead of using a gel with 10 wells, a comb was used which has one well on the left to load the marker and the other nine wells fused together, to form a large well on the right, into which a total of 1.8 µg of purified His-tagged L2 protein was loaded and equally spread across the width. The rest of the protocol was identical to the one described above for Western blotting quantification. However, after blotting of the gel onto a membrane, only the area on the blot containing proteins between 55 and 100 kDa (since L2 is ~70 kDa in size) was excised. This strip was then divided into enough equally-sized strips to probe with different mouse sera. The resulting blocks were transferred to 25-well tissue culture plates and blocked with blocking buffer (0.1% Tween-20 and 5% skim milk in 1xPBS pH 7.6) Sera from all nine or ten mice vaccinated per construct in the antibody assessment experiments as well as mice vaccinated with E+E7M in the cellular immune response experiments were pooled, diluted in blocking buffer and added to different wells containing a section of membrane. One section was probed with mouse anti-His antibody (Serotec), at a 1:2000 dilution for a positive control. The same was done for the prebleeds on a different gel and blot. Sera were incubated with the membrane sections for 1 hour. The rest of the protocol as described for western blotting quantification was then followed.

3.2.4.3 Cross-absorption of non-specific antibodies

Because of high levels of non-specific interactions with the L2 protein by prebleed and negative control sera, cross-absorption of the sera with *E.coli,* insect cell and baculovirus proteins was also attempted. One set of strips of nitrocellulose membrane, 20x7.5 mm each, was coated overnight at 4°C with *E. coli* lysate, prepared as described above, but containing no L2 protein. In some cases, *E.coli,* expressing another unrelated his-tagged protein and prepared as described above, was used, or insect cell lysate expressing E7M. Another set was

coated, also overnight at 4°C, with Sf-9 insect cell lysate infected with wild-type baculovirus or, in some cases, with baculovirus containing an unrelated gene. The insect cells were at $1x10^6$ cells per ml, resuspended in 1xDPBS. The strips were washed thrice with blocking buffer. Two strips were then placed together into a well in a 25-well tissue culture plate. Sera pooled as described above were diluted in blocking buffer and added to different wells. Cross-absorption was for 2 hours at room temperature by shaking. The diluted sera were than removed from the cross-absorption membranes and transferred to the membrane sections onto which L2 protein had been transferred.

3.2.5 Pseudovirion neutralisation assays

3.2.5.1 Pseudovirion production

Protocols for this procedure can be obtained from http://home.ccr.cancer.gov/lco/protocols.asp. All work with pseudovirions performed in our study was based on these protocols, but there were modifications. HEK293TT cells were maintained in complete Dulbecco's Modified Eagle Medium (cDMEM) which contains GlutaMAX™ and high glucose content (supplied by Gibco), containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml Fungin™.

For pseudovirion production, cells were preplated in complete DMEM in a 175 cm^2 flask so they would reach 70-80% confluence the next day. The medium was replaced with fresh medium the next day. FuGene6 (Roche), 175 µl, was added to 5.7 ml of DMEM with glutamax and incubate for 5 minutes at room temperature. A total of 80 µg of DNA was added and the mixture incubated for a further 30 minutes. The mixture was then added dropwise to the cells. After 4-6 hours, the medium was replaced with fresh complete DMEM. The flask was incubated for 40-48 hours in a 37°C, 5% CO_2 humidified incubator.

Pseudovirions were harvested as follows. Cells were trypsinised and the reaction inactivated with complete DMEM. The cells were then transferred to a conical polystyrene tube, -which is very important, since pseudovirions adhere to polypropylene tubes- centrifuged and the pellet washed with DPBS. The pellet was then resuspended in the same volume of DPBS supplemented with extra 9.5 mM $MgCl_2$ as the volume of the pellet itself. Brij-58 was added to a final concentration of 0.5% (w/v). Benzonase (Sigma) and Plasmid-Safe™ ATP-

dependent DNase were added to 0.2% (v/v) each. The mixture was incubated at 37°C for 45 minutes to effect lysis and then transferred to the preferred temperature for maturation of pseudovirions overnight. (25° for HPV-16 and 37° C for the others) After maturation, the cell lysate was clarified by adjusting the NaCl concentration to 850 mM and incubation at 4°C for 10 minutes. The lysate was then centrifuged for 10 minutes at 10 000xg and 4°C. The supernatants were aliquoted into polystyrene conical tubes and stored at -80°C until further use.

3.2.5.2 Neutralisation assays

Pseudovirion neutralisation assays were performed as described in the protocols on the website listed. Doubling dilutions ranging from 1:200 to 1:25600 were used to titrate type 16. Because the titre was expected to be lower for types other than type 16, dilutions from 1:100 to 1:12800 were used to titrate types 45 and 52. The dilution of pseudovirions to be used in the neutralisation assay was chosen as one being within the linear range of the resulting curve of luminescence versus concentration of pseudovirions.

Sera from mice vaccinated with SAF, L2.56 and L2.17 in the antibody assessment experiment, as well as from mice vaccinated with the L1/E7 chimaeras for ELIspot were tested for neutralisation of HPV-16. Only sera from SAF, L2.56 and L2.17 were further tested with types 45 and 52. Final bleed sera were pooled per group and each group was diluted fourfold in triplicate with dilutions ranging from 1:40 to 1:10240. Prebleeds were also pooled as for the final bleeds and tested at 1:40 in triplicate and in some cases 1: 160. Sera from all ten mice vaccinated with negative control cell lysate were pooled and the group was tested at 1:40 in triplicate.

Each 96-well plate included controls: four wells with pseudovirions, and two wells with no pseudovirions or antibodies for a background reading of cell culture supernatant. Positive control antibodies were also titrated against the pseudovirions. Monoclonal antibodies were kindly donated by Dr Neil Christensen and these included H16.V5 and H45.N5 ascites as well as H52.D11 and H52.C1 supernatants. H16.V5 neutralises HPV-16 and was used at a tenfold dilution ranging from 10^{-4} to 10^{-7}. H45.N5 neutralises HPV-45 pseudovirions and was used at a four-fold dilution range from 1:400 to 1:25600; and on another occasion at a tenfold dilution ranging from 1:100 to 1:100 000. H52.D11 was used at a tenfold dilution of 1:40 to

1:40 000 and H52.C1 at a tenfold dilution from 1:100 to 1:100 000. H52.D11 and H52.C1 neutralise HPV-52.

SEAP activity was assayed for by using the Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech Laboratories, Inc.) according to the instructions in the manual. However, due to the expense of the substrate, the volumes used per well were adjusted to be 0.6 of that given in the protocol.

The neutralisation titres of the test sera were calculated as the highest dilution of serum which reduced the maximum SEAP activity, as measured in the wells where only pseudovirions were added to the cells.

3.2.6 Assays to determine cell mediated immune responses to vaccination

3.2.6.1 Preparation of splenocytes

Splenocytes were used in all cell mediated immune response assays. The spleens of each group of mice within the given experiments were pooled. Splenocytes were separated by meshing the pooled spleens of each group through a metal sieve (pore size 70μm) in RPMI medium (Gibco). Suspensions were transferred to 50 ml Sterilin™ tubes and centrifuged at 400 g for 5 min. The resulting cell pellet was resuspended in 50 ml of RPMI and the centrifugation step repeated. Resulting pellets underwent two further washes. Fibrin clots were removed using a Pasteur pipette before the final centrifugation step. Red blood cells (RBCs) were lysed in aliquots of the splenocytes by suspending $50x10^6$ cells in 1ml of RBC lysis buffer (5 mM Tris-HCl, 140 mM NH_4Cl, pH 7.3, Sigma) for 2 minutes. The cells were then pelleted by centrifugation at 400 g for 7 minutes. The supernatant containing the lysed red cells was discarded and splenocytes were suspended at a final concentration of 5×10^6 cells/ml in R10 medium (RPMI supplemented with 1% penicillin G/streptomycin, 10% FCS and 0.1% 2-mercaptoethanol).

3.2.6.2 IFN-γ ELIspot assay

The IFN-γ ELIspot assay was one of the assays used to measure the cell-mediated immune response induced by the vaccines expressing the E7 epitope. This assay directly quantifies the

frequency of ex vivo vaccine-specific effector CD8 T cells in the spleen that release IFN-γ when splenocytes are stimulated with the E7 peptide. An IFN-γ ELIspot assay kit supplied by BD Biosciences was used.

The membranes of the ELIspot plates were pre-wet in 35% ethanol (15 µl/well) for 60 seconds and then washed three times with 200 µl PBS (supplied by Gibco, pH 7.2). Plates were then coated with 100 µl purified anti-mouse IFN-γ antibody, diluted to 5µg/ml in PBS and stored overnight at 4°C. The next day, the antibody was discarded, the wells washed once with R10 and then blocked with 200 µl R10/well for two hours at room temperature. Blocking buffer was discarded and the wells and the membranes were washed once with R10. The stimulants, in 100 µl R10, were then added. These were either medium only (to determine background responses); IL-2 (to determine background responses to IL-2); E7 peptide; E7 peptide and IL-2; or Concanavalin A (assay control). Recombinant mouse IL-2 expressed in *E. coli* was supplied by R&D and used at a concentration of 10ng/ml, of which 1.6 µl was added to every 100 µl splenocytes. The peptide was the H2-D^b restricted HPV-16 E7 peptide, amino acids 49-57 (RAHYNIVTF) and used at 1 µmol per well. It was obtained from Dr Öhlschlager. Concanavalin A (final concentration 0.5 µg/ml) was supplied by Sigma. Each pooled group of splenocytes was assayed in triplicate.

Plates were incubated for 24 hours at 37°C in a 5% CO_2-humidified incubator. After the incubation, the cell suspensions were discarded from the plates and the membranes were washed three times with deionised water. The water was removed from the wells, and the membranes were washed three times with PBS+ 0.05% Tween 20 (Sigma-Aldrich, USA). The PBS + 0.05% Tween 20 was then discarded and a volume of 100 µl of biotinylated anti-mouse IFN-γ detection antibody (2µg/ml in PBS with 10% FCS) was added to each well. The plates were incubated at room temperature in the dark for two hours after which the antibodies were discarded and the membranes washed three times with PBS+ 0.05% Tween 20. A volume of 100µl of avidin-horseradish peroxidase (Avidin-HRP) (5 µg/ml in PBS with 10% FCS) was added to each well and the plates were incubated at room temperature in the dark for one hour. The membranes were washed three times with PBS+ 0.05% Tween 20 then three times again with PBS (Sigma-Aldrich, USA). The substrate solution Nova Red (Vector) was prepared in distilled water and a volume of 100 µl was added to each well. The plates were incubated at room temperature in the dark for 5 minutes for spot development.

The reaction was stopped by washing five times with distilled water. The plates were dried in the dark before analysis.

The ELIspot plates were scanned using an ImmunoSpot CTL Reader and the spots in each well were counted using ImmunoSpot Software version 3.2. For each reaction, the mean number of spots from triplicate wells and the standard deviation (SD) of this mean were calculated and adjusted to 1 x 10^6 splenocytes and expressed as spots forming units per 1 x 10^6 splenocytes (SFU/10^6 splenocytes). Positive responses of the splenocytes to the stimuli for each group were considered to be those greater than twice the background response. For all positive responses, the background responses of the group were subtracted from the E7-specific responses and the response then expressed as net SFU/10^6 splenocytes.

3.2.6.3 Flow cytometric bead array assay

Splenocytes were also used in a flow cytometric bead array (CBA) assay. This assay detects the range of cytokines secreted into the supernatant by effector CD8 T cells in the spleen in response to stimulation with the E7 peptide. This assay was done to determine the Th1 or Th2 type of immune response induced by the vaccine. It was also done because IFN-γ may not necessarily be the only cytokine secreted, as measured by the IFN-γ ELIspot assay. Other cytokines may also be secreted.

3.2.6.3.1 Stimulation of splenocytes for 48 hours

To a 96-well plate, 100 µl of each stimulant, as listed in Section 3.2.6.2, was added, as well as 100 µl of splenocytes at a cell density of 15x10^6/ml. The splenocytes were cultured for 48 hours, the predetermined time required for maximum secretion of cytokines . After this 150 µl of supernatant was removed and stored at -20°C until analysis by the BD™ flow cytometric bead array (CBA) assay, using the mouse inflammation kit from BD Biosciences, according to the instructions of the manufacturer.

3.2.6.3.2 Stimulation of splenocytes for 6 days

In vitro stimulation of splenocytes for 6 days with the E7 peptide was performed to expand the E7-specific CD8 T cell population. During the peptide stimulation period E7-specific

CD8 T cells expand through proliferation to effector cells. Prolonged peptide stimulation allows detection of vaccine induced memory cells which would not be detected in the IFN-γ ELIspot assay and the 48 hour stimulation protocol (Ohlschläger *et al.*, 2003; Ohlschläger *et al.*, 2006).

For this, pooled splenocytes were cultured for 6 days at a starting cell density of $10x10^6$ cells/ml in R10, together with 4 µg/ml of the E7 peptide. After 6 days, live lymphocytes were harvested from the cultures as follows: Cells were pelleted, resuspended in RPMI and counted. Cell concentration was adjusted to $15\text{-}30x10^6$ cells/ml (including dead cells) and 5 ml of cells transferred to polystyrene conical-bottomed tubes. The cells were then underlaid with 5ml of lympholyte M (Cedarlane, Canada) and the tubes centrifuged at 930 g for 20 minutes at room temperature, with no brake applied. After centrifugation, the supernatant was discarded and the interface containing live lymphocytes carefully removed. The lymphocytes were pelleted, washed three times with RPMI and then resuspended in complete RPMI. The lymphoyctes were further cultured as follows: $3x10^6$ EL-4 cells (antigen presenting cells) $+3x10^6$ lymphocytes+4 µg/ml E7 peptide in a final volume of 600 µl R10. The cultures were stopped at 4 hours, 8 hours and 24 hours respectively and harvested supernatant used in the BD™ flow cytometric bead array (CBA) assay, using the mouse inflammation kit from BD Biosciences, according to the instructions of the manufacturer. This assay was done to determine the array of cytokines release by E7 specific CD8 T cells in the lymphocyte population.

3.2.7 Calculation of tumour volume

Tumour volume was calculated as $0.5x\text{width}^2x\text{length}$. The tumours were measured with calipers every 3-4 days. When tumours reached a size of 2500 mm^3, the mice were killed. GraphPad Prism® 5.01 was used for statistical calculations and construction of a survival curve. A two-tailed, non-paired t-test was used to calculate whether the mean tumour volumes were significantly different. The Kaplan-Meier method was used to construct a survival curve, detailing the time points where tumours reached a cut-off size of 250 mm^3. A log-rank (Mantel-Cox) Test and a Gehan-Breslow-Wilcoxon Test were used to determine significant differences between the survival curves. P values smaller than 0.05 were deemed significant.

3.2.8 Electron microscopy analysis

Protein samples were treated as they would before vaccination. Protein samples were defrosted overnight at 4 °C, resuspended in DPBS to the concentration at which they would be injected and incubated at 37 °C for 30 minutes. The aliquots were then diluted a further 20 times in DPBS. Human codon-optimised L1 was expressed in insect cells (see Chapter 2) in the same way as for the other constructs. Neat cell lysate containing L1 protein at 9 μg/ml was treated as for the other constructs and diluted a further 20 times in DPBS. Protein was captured onto carbon-coated copper grids (200 mesh, obtained from Electron Microscopy Sciences, Pennsylvania) using the HPV16-conformation-specific antibody V5 (a gift from Dr Neil Christensen), which should capture L1 pentamers and VLPs onto the grids (Christensen *et al.*, 1996; Varsani *et al.*, 2003). Grids were incubated on 20 μl of a 1:1000 dilution of V5 in 1XPBS, washed twice with water, then incubated on 20 μl of protein sample for 20 min and washed with water three times. Grids were stained with 2% uranyl acetate and visualised under a transmission electron microscope (Zeiss 912 OMEGA CRYO EFTEM) at 50,000 times magnification.

3.3 Results

3.3.1 Humoral immune responses against the major capsid protein, L1

Antibodies, specifically neutralising antibodies against the immunodominant major capsid protein, L1, have been shown to confer excellent protection against infection with HPV (Schiller *et al.*, 2008). Therefore the ability of the L1/L2 chimaeric vaccines to elicit antibody production against L1 was assessed by ELISA. The L1/E7 chimaeras were also tested, but, since mice were only vaccinated twice, instead of four times, the results were not comparable to the other chimaeras. The results of the ELISA is shown in Figure 3.1.

L2.56 elicited production of significantly higher levels of antibodies than the negative control (P=0.0002), L2.17 (P=0.0114) and SAF (0.0184) at a 1:50 dilution. As expected, E7M and E+E7M did not elicit as high levels of antibodies. At a 1:50 dilution, the absorbance of E7M was significantly different from that of the negative control, but this was not the case for E+E7M. The mean absorbance for E+E7M, however, was higher than that for the negative control. BPV did not elicit a response higher than the negative control. (P=0.3553) SAF and L2.17 were not significantly different from one another. (P=0.3938) The prebleeds did not elicit a response (data not shown) The titres of the chimaeric vaccines are shown in Table 3.4. According to the titres, the three L1/L2 chimaeras, excluding BPV, elicited similar immune responses, E7M and E+E7M elicited positive but low responses and BPV no response.

In summary, all chimaeric vaccines except BPV elicited some anti-L1 immune response, as measured by ELISA. Of these responses, that to L2.56 was the highest.

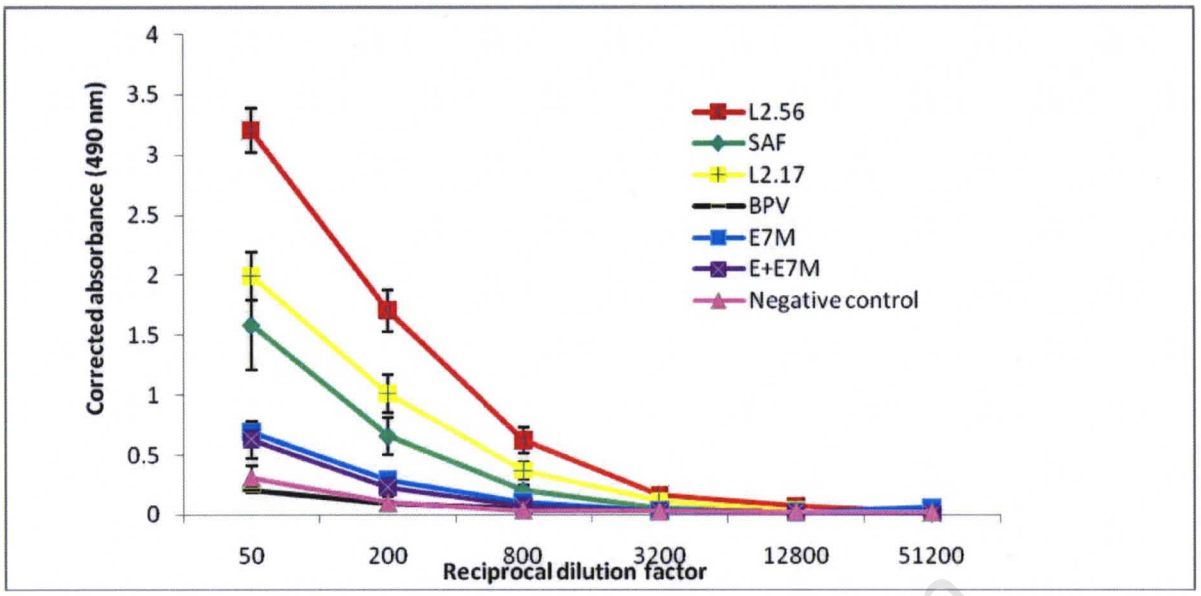


Figure 3.1. *Anti–SAF responses elicited by immunisation with different chimaeric L1 vaccines and detected in the final bleed serum. Three groups of mice were vaccinated on day 0, day 14, day 42 and day 70 and bled 7-10 days after the last vaccination for the L1/L2 chimaeras. Mice were vaccinated twice at 2 week intervals and sacrificed 7 days after the last vaccination for all the L1/E7 chimaeras. The negative control is wild-type baculovirus-infected insect cell lysate. Sera from the immunised mice were bound to semi-purified plant-produced SAF in a direct ELISA. Sera from each group of mice vaccinated were pooled and assayed in triplicate. The error bar indicated here is standard error of the mean of the three groups per vaccine.*

Table 3.4. Antibody titres elicited by L1 chimaeric vaccines.

Chimaera	**Titre**[a]
SAF	800
L2.56	800
L2.17	800
BPV	50
E7M	200
E+E7M	200
Negative Control	50

[a]Titres were calculated as the dilution at which the mean absorbance was twice that of the mean absorbance of the prebleed at a 1:50 dilution.

3.3.2 Humoral immune responses against the inserted L2 peptides

We tested for the presence of antibodies in the sera of mice vaccinated with SAF, L2.56, L2.17, BPV and E+E7M or all those chimaeras including L2 peptides. This was done by Western blotting. If antibodies against L2 are present in the sera, they should bind to the

linearised form of full-length L2. Antibodies against L2 would indicate whether the peptides inserted into L1 are displayed on the surface of the particle, so that they are detected by the immune system. It is also antibodies against L2 that would be responsible for cross-neutralisation of HPV types other than type 16, since antibodies against L1 are type-specific. Various attempts were made at detecting the response on a western blot, without cross-absorption and with cross-absorption as described in Sections 3.2.4.2 and 3.2.4.3, because the prebleeds and the negative control showed non-specific reactions. Cross-absorption was attempted with both E7M-infected insect cell lysate, which might get rid of cross-reacting antibodies against L1, insect cells and baculovirus, as well as an irrelevant His-tagged protein prepared from *E. coli* in a similar manner to the His-tagged L2 protein, which might get rid of all the cross-reacting anti-*E.coli* and anti-His tag antibodies. However, despite this extensive cross-absorption, non-specific reactions remained for the prebleeds and the negative control. Despite the non-specific reactions, the consistent result was that anti-L2 antibodies could only be detected for sera from L2.56- and L2.17-vaccinated mice with an intensity above that of the background. A representative result is shown in Figure 3.2, showing darker and more pronounced bands for sera from L2.56- and L2.17-vaccinated mice than for sera from any of the prebleeds or negative control-vaccinated mice.

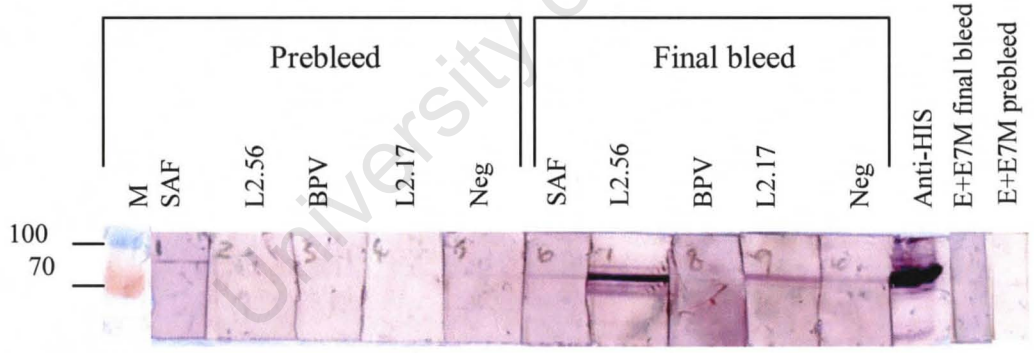


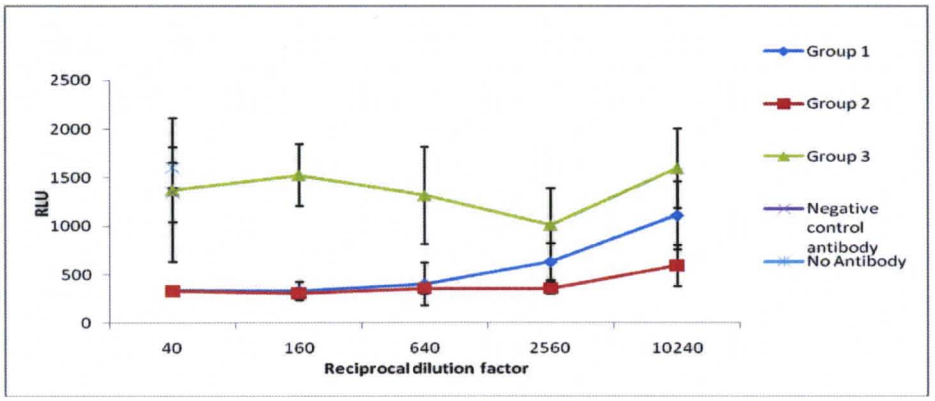
Figure 3.2. *Detection of antibodies against L2 elicited by vaccination with chimaeric vaccines. Three groups of mice were vaccinated on Day 0, Day 14, Day 42 and Day 70 and bled 7-10 days after the last vaccination for the L1/L2 chimaeras. Mice were vaccinated twice at 2 week intervals and sacrificed 7 days after the last vaccination for E+E7M. The negative control is wild-type baculovirus-infected insect cell lysate. His-tagged L2 expressed in E. coli was subjected to electrophoresis and transferred unto a nitrocellulose membrane. Sera from different groups of vaccinated mice were bound to L2 and visualised. M=marker, indicating the size in kDa; prebleed=sera from blood taken before mice were vaccinated; Final bleed=sera from blood taken after vaccination; Neg=negative control; Anti-HIS=antibody against histidine tag attached to L2 protein.*

3.3.3 Homologous protection against HPV-16

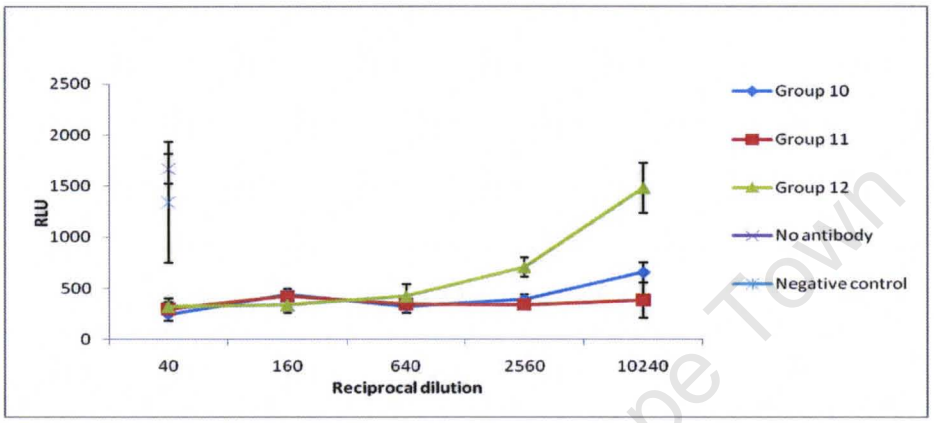
HPV-16 is the most common cancer-causing type overall and in Africa (Bosch *et al.*, 2008). For this reason, and because the chimaeric vaccines were based on HPV-16 L1, sera from mice vaccinated with the chimaeric vaccines were tested for protection against HPV-16 *in vitro,* by pseudovirion neutralisation assays. Since no antibodies were detected for BPV by ELISA, its sera were not tested. Pseudovirion neutralisation assays were also carried out for sera from E7M and E+E7M, as for the ELISA, but no response was detected (data not shown). The lack of response could be attributed to fewer vaccinations than for the other chimaeras. The prebleeds also showed no response (data not shown). Figure 3.3 shows the results of the neutralisation assay and Table 3.5 shows the titres. For SAF, as seen in Figure 3.3(A), only two of the three vaccinated groups, groups 1 and 2, could neutralise HPV-16 pseudovirions at titres of 2560 and more than 10240 respectively. Group 3 showed no neutralisation. Sera from mice vaccinated with L2.17, shown in Figure 3.3(B), from all three groups showed neutralisation with titres of 2560 for one group and >10240 for the other two groups. The same was seen for sera from mice vaccinated with L2.56 (Figure 3.3(B).

SAF, L2.56 and L2.17 therefore show potential as vaccines capable of eliciting neutralising antibodies against HPV-16, as can be seen from the significantly high titres, and there was no significant difference between the three vaccines' ability to elicit neutralising antibodies.

A.



B.



C.

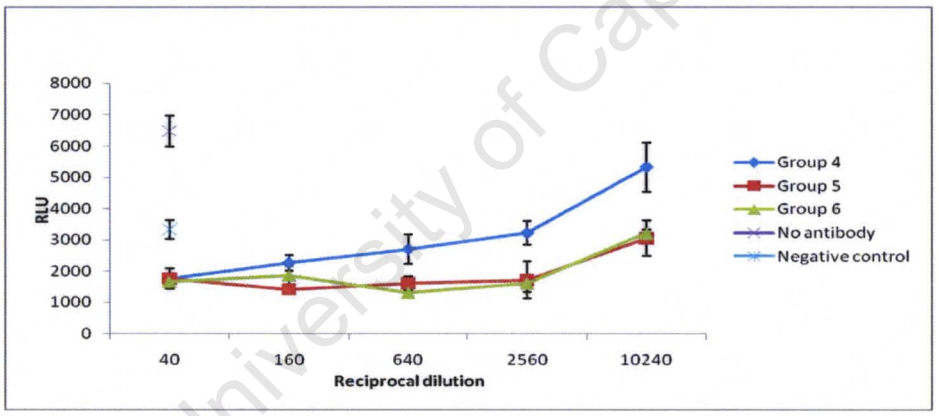


Figure 3.3. *Neutralisation of HPV-16 pseudovirions by sera from mice vaccinated with (A) SAF (B) L2.17 and (C) L2.56. Three groups of mice were vaccinated on Day 0, Day 14, Day 42 and Day 70 and bled 7-10 days after the last vaccination. The negative control is wild-type baculovirus-infected insect cell lysate. Sera from each group of mice vaccinated were pooled and assayed in triplicate. The "no antibody" control wells contained only pseudovirions, but no antibodies. The error bars indicate standard deviation between triplicates.*

Table 3.5. Titres of neutralising antibodies against HPV-16 pseudovirions

Chimaera	**Titre**[a]
SAF	0 to >10240
L2.17	2560 to >10240
L2.56	2560 to 10240
E7M	40
E+E7M	0 to 40
Negative control	0 to 40

[a]The titre was calculated as the highest dilution of serum where the SEAP activity is reduced by at least 50% as compared to the maximum SEAP activity.

3.3.4 Heterologous protection by chimaeric L1 vaccines

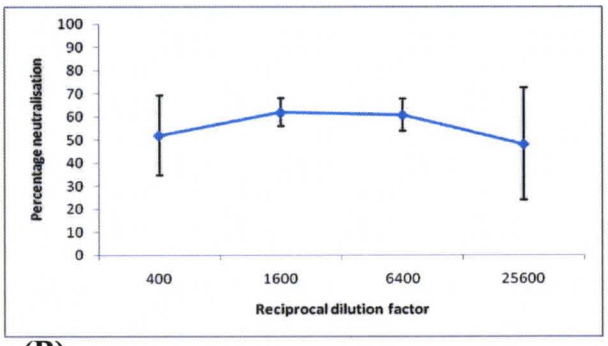
As many as 12 other types are implicated in cervical cancer (Castellsague, 2008). An aim of our study was to create a monovalent vaccine with cross-neutralising ability that would also protect against types other than type 16. The HPV-16 L2 peptides included in the L1/L2 chimaeras, SAF, L2.56, L2.17 and E+E7M, have previously been shown to mediate neutralisation against various other types of HPV (Schellenbacher *et al.*, 2009; Rubio *et al.*, 2009; Kondo *et al.*, 2007; Kondo *et al.*, 2008; Alphs *et al.*, 2008; Gambhira *et al.*, 2007b; Embers *et al.*, 2004; Kawana *et al.*, 2003; Kawana *et al.*, 1998; Kawana *et al.*, 1999). All three peptides have shown to mediate neutralisation against HPV-18. This type of HPV is ranked as the second-highest cause for cervical cancer (Bosch *et al.*, 2008) and therefore is an important type to consider when testing vaccines for their ability to mediate cross-neutralisation. Due to the unavailability of monoclonal antisera to HPV-18, we tested sera from mice vaccinated with SAF, L2.56 and L2.17 for cross-neutralisation of the closely related type (de Villiers *et al.*, 2004), HPV-45, instead, as an indication of HPV-18 neutralisation ability. We also chose to test neutralisation of HPV-52 by these sera, because this type has emerged in a recent study as major cause of cervical cancer in South Africa (Personal communication, Anna Salimo). Sera from mice vaccinated with E+E7M was not tested against HPV-45 or -52 pseudovirions, since these sera did not contain high levels of neutralising antibodies against HPV-16 L1. Since L1 is the immunodominant protein, it was therefore highly unlikely that an immune response to L2 could be detected in sera from mice vaccinated with E+E7M.

Figure 3.4 shows the results we obtained for type 45 pseudovirions. None of the sera seemed to neutralise type 45 pseudovirions, however, the results are not conclusive. A monoclonal antibody neutralising HPV-45 pseudovirions, H45.N5, did not behave as expected with respect to neutralisation of pseudovirions, which would be neutralising almost 100% of pseudovirions at high concentrations of the antibody, with this percentage decreasing with decreasing antibody concentration. Instead, as seen in Figure 3.4A, H45.N5 only neutralised up to 50%-60% strength, even at high concentrations of antibody and the level of neutralisation stayed constant, even when the antibody was diluted 1:25600. Even though the antibody neutralised pseudovirion infectious activity by 50%, none of the sera tested could do so, as seen in Figure 3.4B. Therefore it seems like sera from mice vaccinated with SAF, L2.56 and L2.17 were not capable of neutralising HPV-45.

Figure 3.5 shows results for type 52 pseudovirions. Similar to the results for type 45, none of the test sera could neutralise HPV-52, but once again this is not conclusive. H52.D11, the positive control monoclonal antibody known to neutralise HPV-52 pseudovirions, did not neutralise HPV-52 pseudovirions in our experiments at all, as can be seen in Figure 5A. Another monoclonal antibody, H52.C1, delivered the same result (Figure 3.5A). This result was not due to the pseudovirion concentration being too high and not in the linear part of the curve, since, even at half the concentration of pseudovirions, the same effect was observed. Once again, none of the test sera neutralised HPV-52 pseudovirions, as seen in Figure 3.5B, but whether this is due to the conditions of the experiment or the inability of the sera to neutralise, is not clear.

In summary, none of the sera from mice vaccinated with SAF, L2.56 or L2.17 could neutralise the HPV types other than type 16 that we tested for *in vitro,* but the result is not conclusive, due to the lack of neutralisation by positive control monoclonal antibodies.

(A)



(B)

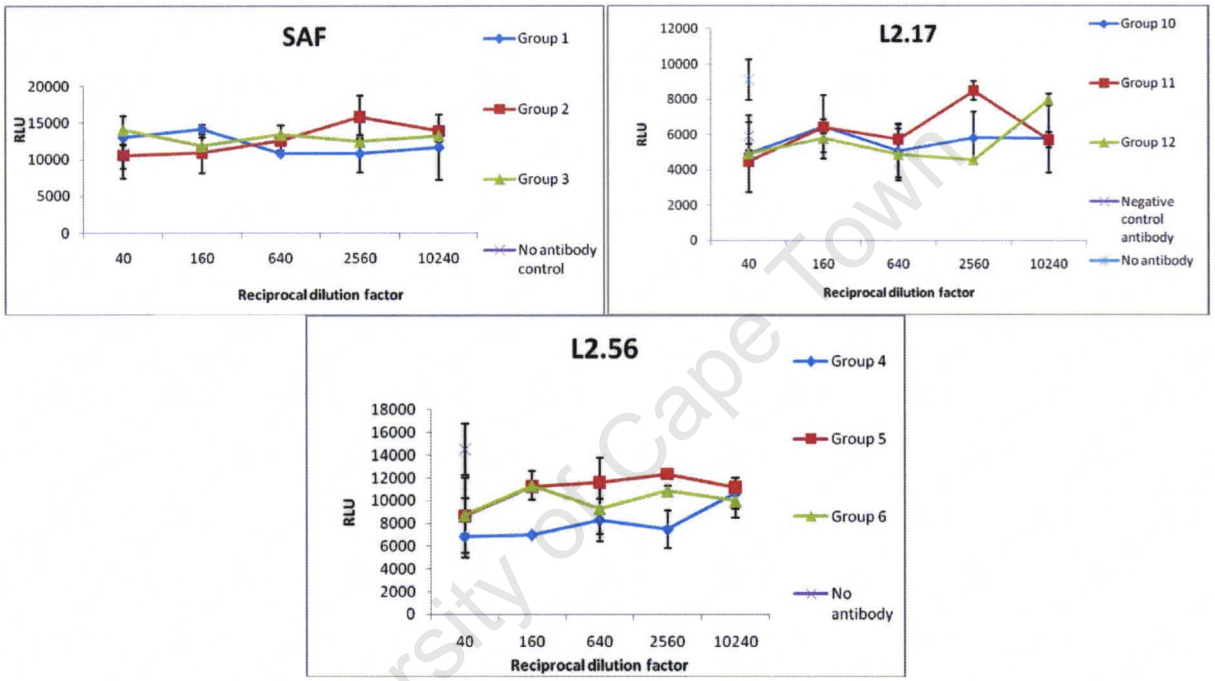


Figure 3.4.(A) *Neutralisation of HPV-45 pseudovirions by the monoclonal antibody H45.N5 at different dilutions. The error bars indicate standard deviation between 4 replicates.* ***(B)*** *Neutralisation of HPV-45 pseudovirions by sera from mice vaccinated with SAF, L2.17 and L2.56. Three groups of mice were vaccinated on Day 0, Day 14, Day 42 and Day 70 and bled 7-10 days after the last vaccination. The negative control is wild-type baculovirus-infected insect cell lysate. Sera from each group of mice vaccinated were pooled and assayed in triplicate. The "no antibody" control wells contained only pseudovirions, but no antibodies. The error bars indicate standard deviation between triplicates. RLU=relative light units*

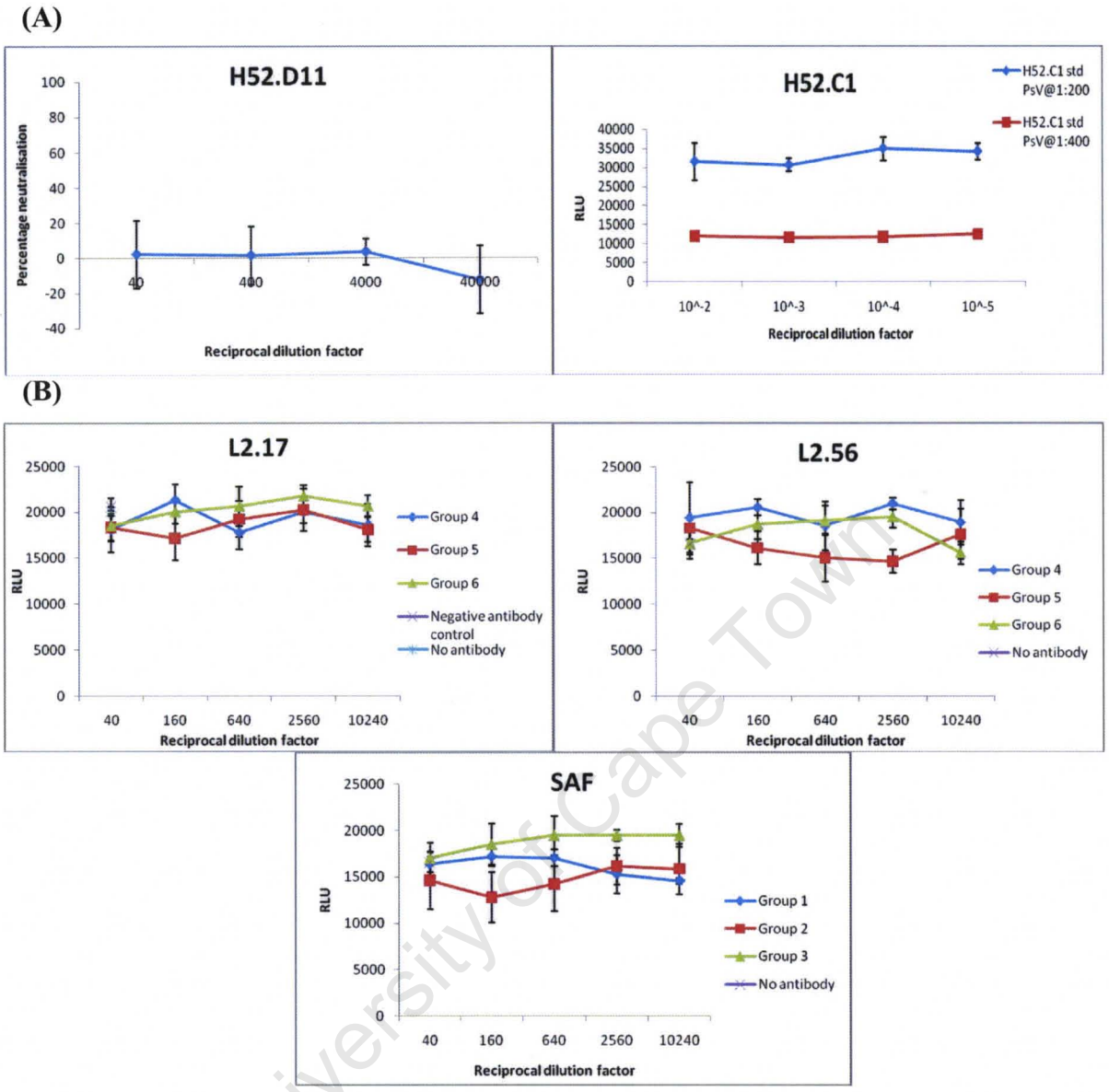


Figure 3.5.(A) *Neutralisation of HPV-52 pseudovirions by the monoclonal antibodies H52.D11 and H52.C1 over various dilutions of the antibody. Shown for H52.D11, whereas for H52.C1, the relative light units (RLU) are shown for two different dilutions of pseudovirions. (PsV) Std=standard curve.* ***(B)*** *Neutralisation of HPV-52 pseudovirions by sera from mice vaccinated with SAF, L2.17 and L2.56. Three groups of mice were vaccinated on Day 0, Day 14, Day 42 and Day 70 and bled 7-10 days after the last vaccination. The negative control is wild-type baculovirus-infected insect cell lysate. Sera from each group of mice vaccinated were pooled and assayed in triplicate. The "no antibody" control wells contained only pseudovirions, but no antibodies. The error bars indicate standard deviation between triplicates.*

3.3.5 Cellular immune responses against the inserted E7 peptides

3.3.5.1 IFN-γ ELIspot assay

Splenocytes isolated from spleens harvested from mice vaccinated with SAF (as a negative control), E7M, E+E7M and HPV-16E7SH, a DNA vaccine serving as a positive control and known to elicit cellular immune responses (Ohlschläger *et al.*, 2006) were used in an IFN-γ ELISPOT assay to determine the frequency of E7 specific CD8 T cells induced by the vaccines. The splenocytes from all vaccinated groups were capable of responding to the polyclonal positive control, Con A, indicating that the splenocytes were intact and that they were capable of secreting IFN-γ. No responses were seen for the other vaccine groups except HPV-16E7SH, not even when IL-2, a cytokine important for the maintenance of $CD8^+$ T-cells was included in the assay. HPV-16E7SH-vaccinated mice had only low levels of CTL responding to the E7 peptide and the response was not enhanced by the addition of IL-2. The results are shown in Figure 3.6.

3.3.5.2 Flow cytometric bead array assay

The lack of detection of IFN-γ secreting cells in the spleens of vaccinated mice could indicate that E7-specific effector cells were not present above levels of detection or that they simply did not preferentially secrete IFN-γ, but some other cytokine, which in turn may indicate that $CD4^+$ T-cells were present and not $CD8^+$ T-cells. Therefore a flow cytometric bead array (CBA) assay, which detects multiple cytokines was performed. For this, splenocytes were stimulated with the E7 peptide and then the content of various cytokines detected in the extracellular supernatant. The nature of cytokines in the supernatant also indicates whether the response to the vaccine is of a Th1 or Th2 type (Garcia-Pineres *et al.*, 2007; Janeway *et al.*, 1997c). Release of IL-6 and IL-10 during stimulation indicate a Th2 response, which favours a humoral immune response (antibody development), whereas IFN-γ, tumour necrosis factor (TNF) and IL-12 indicate a Th1 response, or cell-mediated immune response. The CBA assay used also detected the chemokine, macrophage chemoattractant and activating factor (MCP-1), which is involved in inflammatory responses.

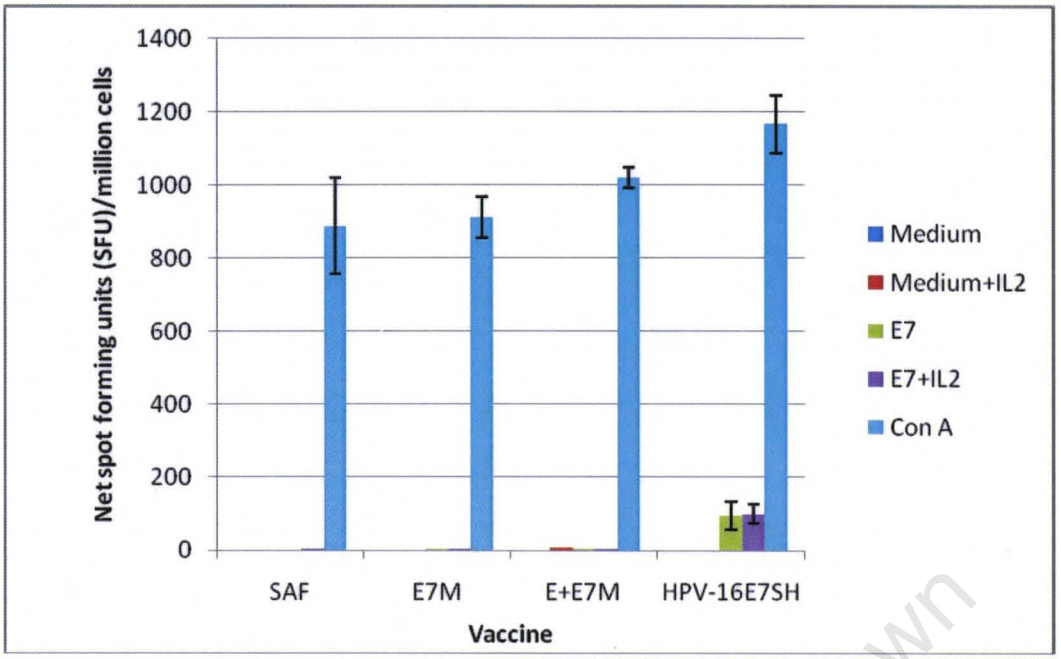


Figure 3.6. *IFN-γ ELIspot responses, indicating the presence of E7 peptide (RAHYNIVTF) specific $CD8^+$T cells generated by the indicated vaccines. HPV-16E7SH was the positive control, SAF the negative control, E7M and E+E7M the test vaccines. Mice were vaccinated twice at 2 week intervals and sacrificed 7 days after the last vaccination. Spleens were harvested and the spleens in each group pooled. An aliquot of the splenocytes from each group was then used in the IFN-γ ELIspot assay. The cells were stimulated with medium only, medium+IL-2, RAHYNIVTF only, RAHYNIVTF+IL-2 or the polyclonal stimulus, Concanavalin A.(Con A)in triplicate for 24 hours. The number of cells secreting IFN-γ was then counted in each well. Shown in the figure are the mean number of spot forming units per million cells for each vaccine. The error bars indicate standard error of the mean.(N=3)*

3.3.5.2.1 Cytokine release as measured by the CBA assay after stimulation with E7 peptide for 48 hours

When splenocytes were stimulated for 48 hours with the E7 peptide, only splenocytes from mice vaccinated with HPV-16E7SH responded and only IFN-γ could be detected above background levels (Background levels are cytokines released when stimulated with medium only). These results correspond to the results of the ELIspot. The splenocytes were capable of secreting all cytokines in response to the positive control, the polyclonal stimulus Con A. The response to Con A once again indicates that splenocytes were intact and healthy and capable of secreting cytokines. However, no IL-12 secretion in response to stimulation with Con A was detected suggesting the level secreted by antigen presenting cells in the splenocyte populations is very low. The results are shown in Table 3.6.

Table 3.6. Cytokine release in response to vaccination with L1/E7 chimaeras and stimulation with various media.

Vaccine	Stimulant	IL-6 (pg/ml)	IL-10 (pg/ml)	MCP-1 (pg/ml)	IFN-γ (pg/ml)	TNF (pg/ml)	IL-12 (pg/ml)
SAF (negative control)	Medium	42.0	12.8	182.3	0.0	19.7	0.0
	Medium + IL-2	40.0	16.3	183.5	0.6	12.9	0.0
	E7 peptide	64.5	12.1	198.4	0.3	12.6	0.0
	E7 peptide +IL-2	46.0	17.1	164.8	1.5	22.2	6.8
	Con A	**357.1**	**69.6**	**777.0**	**393.9**	**82.9**	0.0
E7M	Medium	34.6	22.1	140.1	0.3	22.2	5.4
	Medium + IL-2	37.1	14.3	145.3	0.3	19.0	0.0
	E7 peptide	34.1	22.6	112.1	4.0	18.7	0.0
	E7 peptide +IL-2	39.7	16.7	156.0	0.3	19.8	0.0
	Con A	**284.2**	**79.9**	**460.3**	**509.7**	**116.6**	0.0
E+E7M	Medium	97.2	26.2	357.2	0.6	49.7	5.1
	Medium + IL-2	43.1	27.6	205.5	0.9	36.6	0.0
	E7 peptide	53.0	20.8	203.6	1.4	43.6	0.0
	E7 peptide +IL-2	36.3	18.3	199.1	1.3	33.6	0.0
	Con A	**331.0**	**88.6**	**600.1**	**1088.0**	**154.6**	0.0
HPV-16 E7SH	Medium	298.1	36.1	481.0	313.8	165.8	0.0
	Medium + IL-2	411.3	38.3	612.8	192.0	135.8	1.8
	E7 peptide	340.6	45.0	350.0	**722.7**	144.9	0.0
	E7 peptide +IL-2	303.9	52.2	267.3	**1099.2**	154.6	0.0
	Con A	**694.8**	**139.9**	378.7	**2615.6**	**405.1**	3.8

Bold=positive levels of cytokine. Concentrations of cytokine were accepted as positive when they were at least twice that of the background (when stimulated with medium only)

3.3.5.2.2 Cytokine release after stimulation with E7 peptide for 6 days

The lack of cytokine detection after 48 hours could indicate that the cytokines were below levels of detection and that a longer time period of stimulation with E7 peptide is necessary for clonal expansion of memory CD8 T-cells before E7 peptide specific CD8 T cells can be detected. Therefore splenocytes were stimulated with RAHYNIVTF, but not IL-2, for 6 days. After 6 days, the lymphocytes were harvested and the generated effectors were stimulated with E7 peptide in the presence of EL-4 antigen-presenting cells. Cultures were stopped at 4 hours, 8 hours and 24 hours respectively, and the cytokine content determined using the CBA assay. Because cells in this experiment were not stimulated with medium only, the baseline values were provided by splenocytes from mice vaccinated with the negative control, SAF. SAF does not contain an E7 peptide and therefore the effectors from mice vaccinated with SAF should not respond to antigen-presenting cells presenting the E7 peptide.

As seen in Figure 3.7, the nature of cytokines released indicated the vaccine E7M induced a Th2 response with IL-6 and IL-10 levels 1.4 fold higher than that for SAF at the 24h time point. For E+E7M: only at the 4 hour time point were IL-10 levels 3.9 fold higher than baseline. There was no increase in IL-10 production above baseline with time. On the other hand, IFN-γ, which would indicate a Th1 response, was not secreted above baseline for any of the test vaccines. E+E7M vaccination, however, resulted in about 2-fold secretion of TNF-α above baseline at all time points. MCP-1 levels above background were secreted in response to E7M vaccination only at the 24h time point. No MCP-1 levels above background were seen for vaccination with E+E7M or the DNA vaccine. Very low levels of IL-12 were secreted but this was not a positive response. Vaccination with the DNA vaccine, even though it served as a positive control, did not lead to cytokine release above the non-specific levels of SAF, except at 4 hour time points for IL-10 and IFN-γ. IL-10 and IFN-γ secretion would favour antibody as well as CTL responses.

To summarise all the results for cellular immune responses elicited by E7M and E+E7M: Splenocytes required a six-day stimulation period with E7 peptide to effect clonal expansion of E7-specific memory CD8 T cells above levels of detection. E7M vaccination led to Th2 responses, whereas E+E7M vaccination mostly led to Th1 responses.

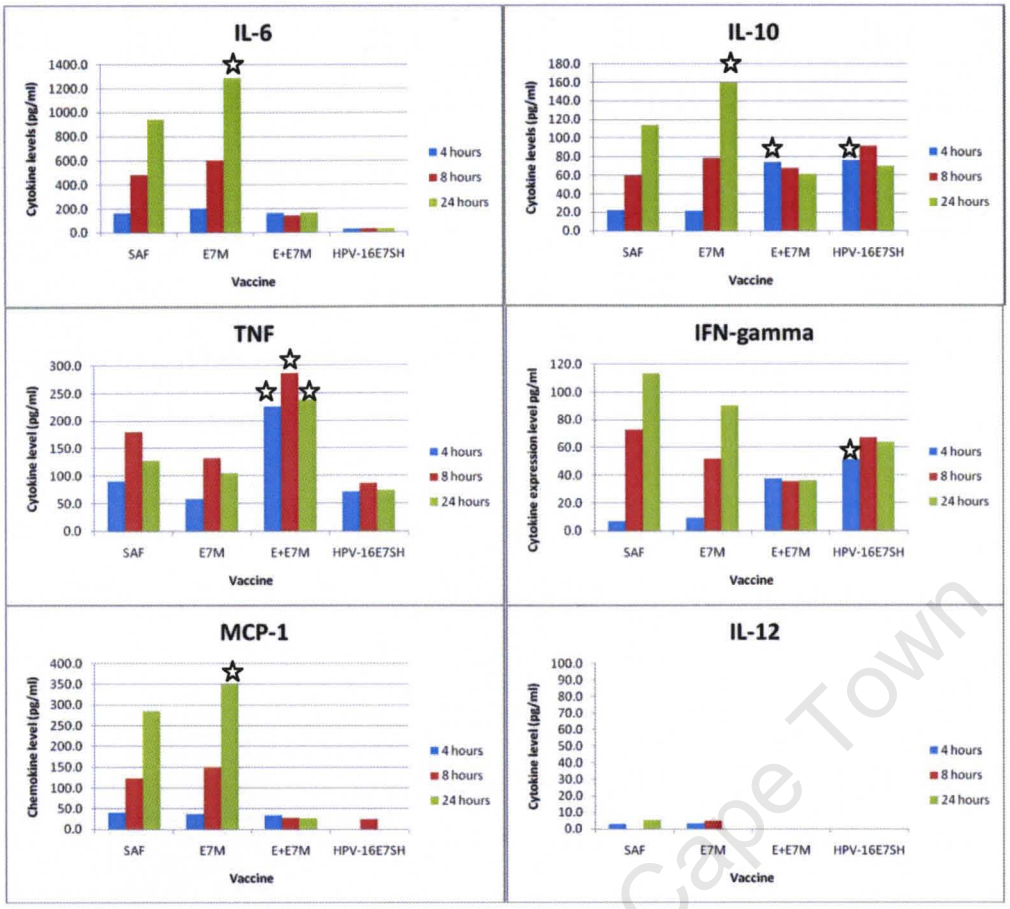


Figure 3.7. *Differential cytokine release in response to vaccination with L1/E7 chimaeras. Mice were vaccinated twice at 2 week intervals with SAF as the negative control, HPV-16-E7SH as the positive control, E7M and E+E7M and sacrificed 7 days after the last vaccination. Splenocytes from 10 mice per vaccine were pooled and cultured for 6 days in the presence of RAHYNIVTF to generate effector cells. On day 6, lymphocytes were isolated and further cultured in the presence of EL-4 cells and RAHYNIVTF. Aliquots of the supernatant were taken at 4 hour, 8 hour and 24 hour time points and used in a CBA assay to determine cytokine content. A ☆ above the bar indicates a positive response above baseline levels, which are provided by the negative control.*

3.3.6 Tumour regression

We also tested the effect that vaccination with the L1/E7 chimaeras would have on existing tumours in mice. The DNA vaccine, HPV-16E7SH, previously shown to effect tumour regression (Ohlschläger *et al.*, 2006) was used as a positive control. The negative control was wild-type infected insect cell lysate as for the other experiments. We also included SAF, for the sake of interest, because L1 protein alone has previously been shown to effect tumour

regression in mice when C3 cells, the same cells we used in our study, were used to induce tumours (Ohlschläger *et al.*, 2003; De Bruijn *et al.*, 1998).

3.3.6.1 Growth rate of tumours

In order to deal with differences between average initial tumour sizes for the different vaccine groups, the average growth rate was analysed for each group. This analysis was done over the first 36 days since vaccination for E7M, E+E7M and the negative control, and the first 39 days since vaccination for SAF and the positive control. These time periods were chosen, since, after this time, mice had to be culled due to tumour size exceeding the upper limit. SAF and the positive control were monitored after 39 days, since these two vaccine groups were not done concurrently with the others, and so the tumours were not measured on exactly the same days as for the other three groups. The 39-day time period was the closest one to 36 days. The results of this analysis is shown in Figure 3.8.

Table 3.7 lists the P-values associated with whether the means are significantly different. Only the negative and positive control differed significantly with respect to growth rate, and so did E+E7M and the positive control. The test vaccines (SAF, E7M and E+E7M) therefore did not show a clear trend in slowing the growth rate of tumours, since their growth rates were not significantly different to the negative control. The test vaccines also did not differ significantly from each other in their ability to slow the growth rate of tumours.

In terms of regression, 6 out of the 9 mice in the positive control group showed complete eradication of tumours, whereas only 1 out of nine mice in the E7M and SAF groups, and none in the E+E7M and negative control group, showed complete eradication of tumours (data not shown) confirming that none of the test vaccines had an effect on the growth of tumours in mice.

3.3.6.2 Effect on tumour volume after administration of second boost

The remaining mice in the negative control and test vaccine groups that had not been culled, were given another boost on Day 39. The boost was given to see if the lack of tumour growth inhibition was perhaps due to the number of doses given. In an attempt to include this data post-day 39, a survival curve was constructed to see how long it took for the tumours in each

group to reach a size of 250 mm^3. This arbitrary cut-off was chosen, because a tumour of that size was unlikely to regress. The curve is shown in Figure 3.9. The positive control is significantly different from the test vaccines, but not the negative control, although the P-value for this analysis was 0.07, yielding a 93% chance that they are different. E7M and E+E7M therefore did not have an effect on tumour size under these experimental conditions and there was no difference in their effect on the tumours. Despite the second boost given on day 39, the remaining tumours still increased in size, therefore the negative results do not seem to be a dose-dependent effect.

In summary, SAF-, E7M- and E+E7M-vaccination did not have any effect on the size of existing tumours in mice.

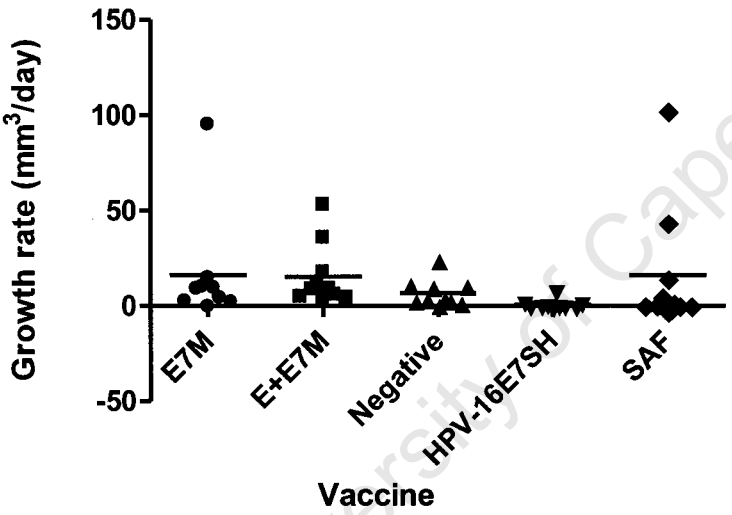


Figure 3.8. *Average growth rate of tumours after vaccination with L1/E7 chimaeras and SAF. Mice were injected once subcutaneously with $0.5x10^6$C3 cells. When tumours reached a diameter of 2-3mm, mice were vaccinated on Day 0 and 14 with the three test vaccines, wild-type baculovirus-infected insect cell lysate as negative control and a DNA vaccine expressing the shuffled E7 oncogene as a positive control. Tumour size was monitored until the first mouse had to be culled 36 days after the first vaccination, due to tumour size, for the L1/E7 chimaeras and the negative control; and 39 days for SAF and the positive control. Growth rate was calculated as ((endpoint tumour volume-initial tumour volume)/number of days).The horizontal bars indicate the mean growth for the group of mice and each point indicates the growth for an individual mouse.*

Table 3.7. P-values for two-tailed, unpaired t-tests to determine significance of difference between mean growth rates of different vaccine groups in the tumour regression experiment

	HPV-16E7SH	Negative control	E7M	E+E7M	SAF
HPV-16E7SH		**0.0186**	0.1123	**0.0108**	0.1526
Negative control	**0.0186**		0.3358	0.1433	0.3877
E7M	0.1123	0.3358		0.9278	0.9931
E+E7M	**0.0108**	0.1433	0.9278		0.9413
SAF	0.1526	0.3877	0.9931	0.9413	

Bold=Significant P-values<0.05

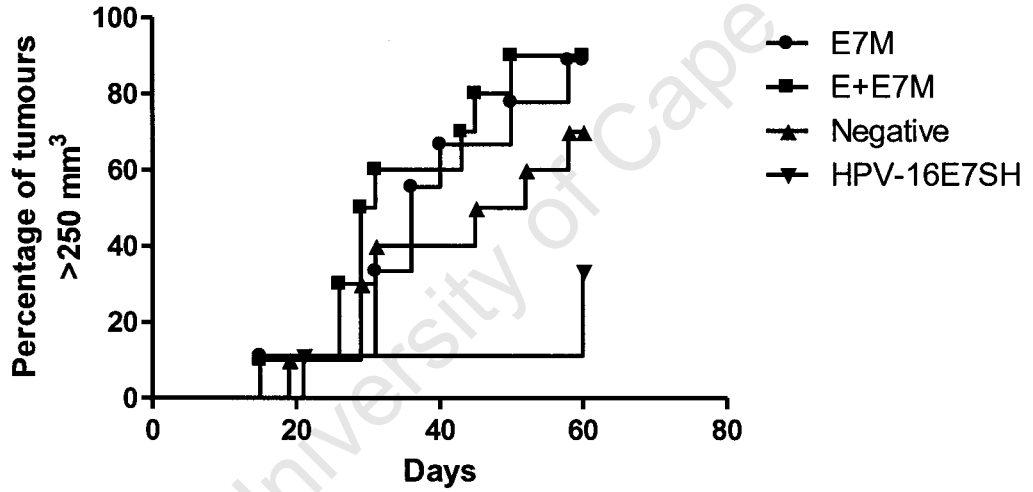


Figure 3.9. *Percentage of tumours that have reached a tumour volume larger than 250 mm^3. Mice were injected once subcutaneously with $0.5x10^6$C3 cells. When tumours reached a diameter of 2-3mm, mice were vaccinated on Day 0, 14 and 39 with the two test vaccine and wild-type baculovirus-infected insect cell lysate as negative control. A DNA vaccine expressing the shuffled E7 oncogene was given on Day 0 and 14 as a positive control. Tumour volume was calculated as $0.5xwidth^2xlength$ and monitored over 60 days.*

3.3.7 Electron microscopy analysis

The ability of L1 protein to assemble into higher order structures, especially capsomers, has been positively correlated with its ability to elicit high levels of antibodies (Schädlich *et al.*, 2009; Kuck *et al.*, 2006). The formation of a repetitive array of epitopes displayed on the

surface of the virus particle has been implied in the acute activation of dendritic cells, the most important antigen-presenting cells (APCs) of the immune system. The interaction of dendritic cells with VLPs, not capsomers, has been shown to be essential for their activation (Rudolf *et al.*, 2001a; Lenz *et al.*, 2001). For this reason, it was important to analyse the structure of the chimaeric vaccines, because their ability to assemble into higher-order structures may explain differences in immunogenicity observed. The preparations captured onto electron microscopy grids were treated the same way as the vaccines were treated, since freeze-thaw cycles, as well as incubation at 37°C may have an effect on assembly. All vaccine preparations were stored at -80°C, defrosted prior to vaccination and incubated at 37°C to enhance emulsification with Freund's adjuvant.

Important controls were included in the electron microscopy analysis. Wild-type baculovirus-infected insect cell lysate as used in vaccination was used as a negative control to avoid false positives. Humanised L1 was also expressed in insect cells and prepared the same way as for the vaccine proteins in order to determine whether the preparation conditions had any effect on the assembly of L1. In an effort to select for the proteins in pentamer or VLP conformation, protein preparations were captured onto the grids using V5 antibody, which binds only to conformation-dependent sites on L1 pentamers or VLPs.

The negative control showed high levels of protein as expected, as well as structures of similar size to capsomers and "small" VLPs, which could be confused with those, as can be seen in Figure 3.10(C). However, no spherical structures with a diameter of 50 to 70 nm were observed in the negative control. VLPs are about 55 nm in diameter.

Because the structures observed in the negative control images could be confused with capsomers and "small" VLPs, the presence of structures with a diameter in the range of 50 to 60 nm was used to determine assembly-competence of chimaeric L1 proteins. This could be done, since occasional VLPs are often seen in preparations only expected to form capsomers.

Figure 3.10(A) shows VLPs purified by caesium chloride gradient ultracentrifugation, showing staining of the cylindrical cavity. This staining of the cylindrical cavity was not seen in the spherical structures seen in the negative control, and this cylindrical staining provided another way of distinguishing between false and real positives.

Figure 3.10(B) shows the presence of these structures in insect cell lysate containing wild-type L1. These structures indicate that, under the given experimental conditions, L1 protein was capable of assembly into higher-order structures.

The other chimaeric vaccines were analysed for the presence of VLPs with staining of the cavity, structures with a diameter in the range of 50 to 60 nm, as well as for any structures that are significantly different from that seen in the negative control. As can be seen in Figure 3.11(A), SAF formed putative VLPs about 50 nm in size. Similar structures could be seen for L2.17 (Figure 3.11(B)), but it looks as if it may be in a state of partial assembly. In both cases, these particles were only seen rarely.

Significantly different spherical particles with an appearance differing from protein aggregates seen in the negative control, were observed for L2.56, as seen in Figure 3.11(C). These ranged in size from 30-50 nm and occurred frequently. The particles may be "small" and full-size VLPs.

In the case of BPV, the background is covered with ring-like structures, with an appearance unlike the background seen in the negative control. The size of each particle is between 10 and 20 nm and therefore it is proposed that these are aggregates of pentamers. There may also be some VLPs in a partial state of assembly of about 50 nm. This can be seen in Figure 3.11(D).

As Figure 3.12 shows, E7M and E+E7M also formed putative VLPs, which were more abundant in the E7M preparations than in the E+E7M preparations.

In summary, all the chimaeric proteins showed the potential to assemble into tertiary structures. However, these structures are most likely to be pentamers, since VLP-like structures were not abundant in the images. The exception is L2.56, which showed the highest potential to assemble into higher-order structures, most likely "small" VLPs.

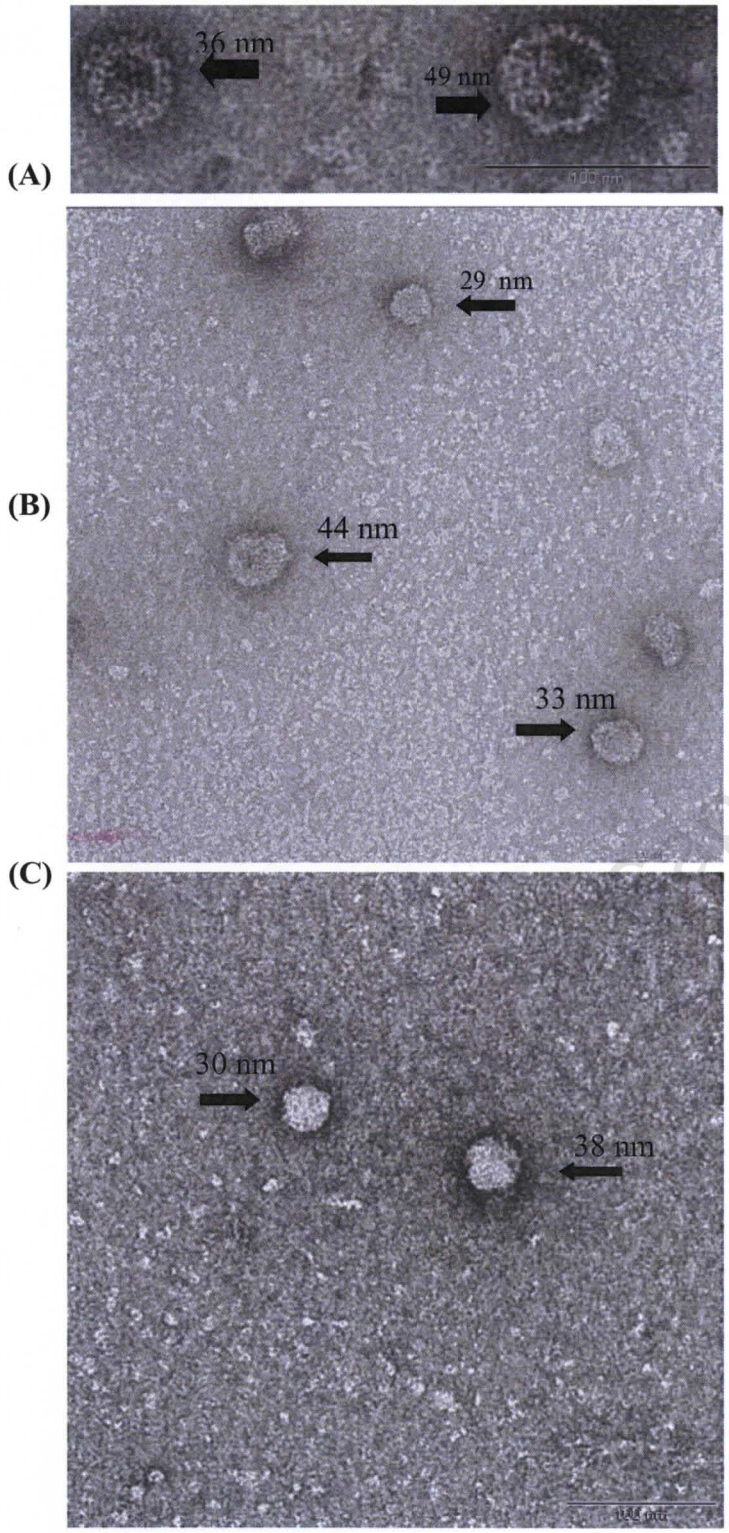


Figure 3.10. *Transmission electron micrographs of (A) wild-type L1 purified by caesium chloride gradient ultracentrifugation, (B) insect cell lysate infected with wild-type L1 recombinant baculovirus, (C) wild-type baculovirus infected insect cell lysate. Protein preparations were captured onto carbon-coated copper grids with V5 antibody, negatively stained with 2% uranyl acetate and viewed under 50,000xmagnification. The diameter of particles are indicated.*

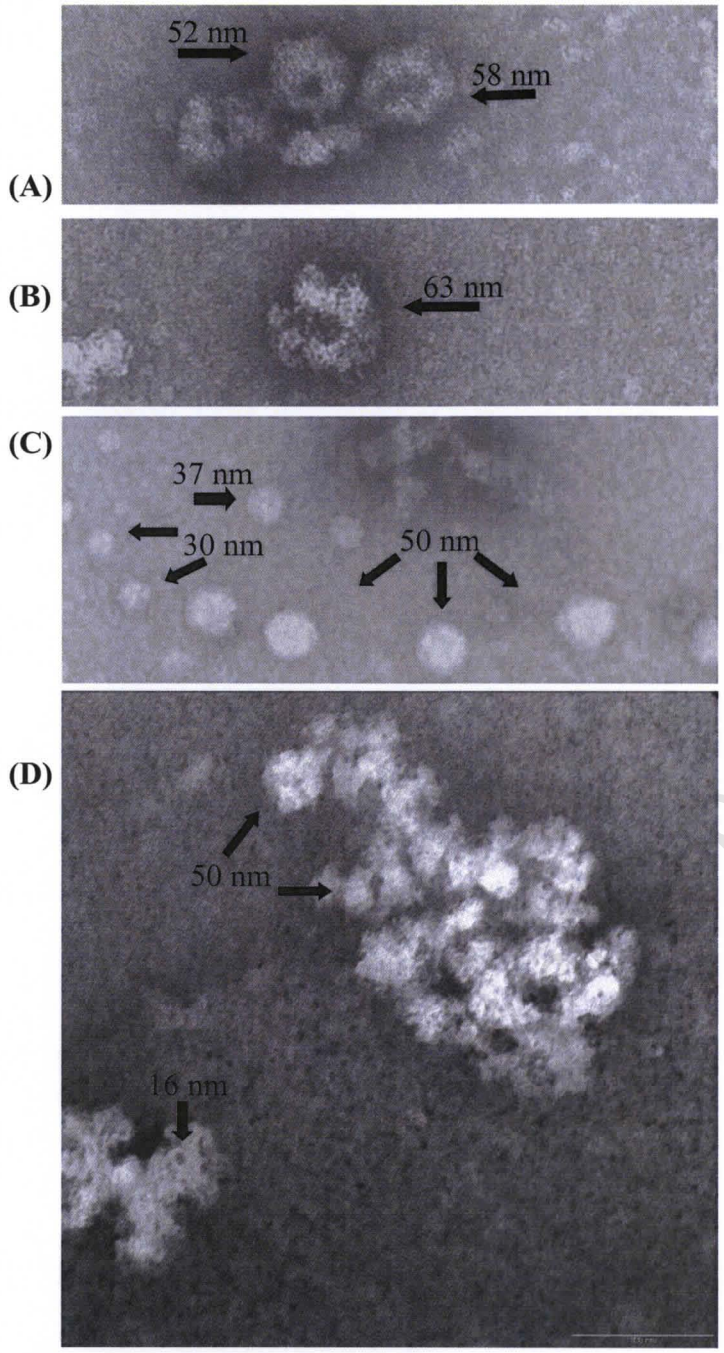


Figure 3.11. *Transmission electron micrographs of insect cell lysate containing (A) SAF, (B) L2.17, (C) L2.56, (D) BPV. Protein preparations were captured onto carbon-coated copper grids with V5 antibody, negatively stained with 2% uranyl acetate and viewed under 50,000xmagnification. The diameter of particles are indicated.*

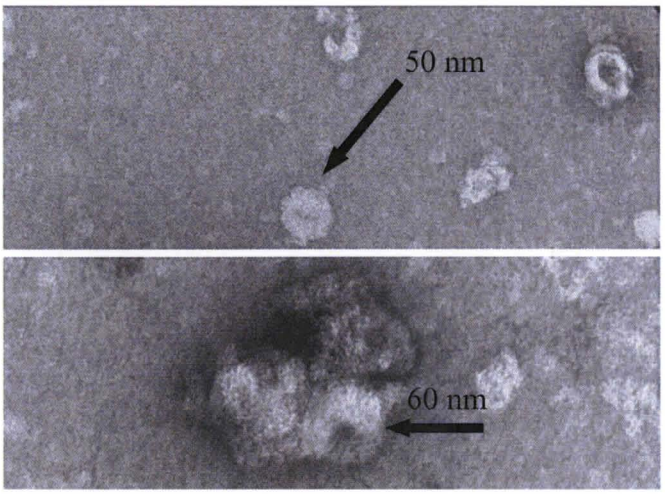


Figure 3.12. *Transmission electron micrographs of insect cell lysate containing (A) E7M and (B) E+E7M. Protein preparations were captured onto carbon-coated copper grids with V5 antibody, negatively stained with 2% uranyl acetate and viewed under 50,000xmagnification. The diameter of particles are indicated.*

3.4 Discussion

The first aim of this project was to select vaccine candidates for purposes of creating a monovalent vaccine capable of simultaneous protection against infection with multiple types of HPV. The second aim was to test the idea of a vaccine capable of protecting against infection with HPV, while at the same time treating existing infections.

3.4.1 BPV is not a vaccine candidate

The BPV construct did not show potential as a vaccine candidate. Antibodies against L1 could not be detected in sera of mice vaccinated with this construct, as can be seen in Figure 3.1 and Table 3.4. In the same way, antibodies against HPV-16 L2 could not be detected (Figure 3.2). It is unlikely that the lack of anti-L2 antibodies is due to the fact that the inserted epitope is from a divergent papillomavirus, BPV-1, since Pastrana *et al. (Pastrana et al., 2005)* showed that polyclonal antisera against BPV-1 L2 bound to purified HPV-16 L2 protein at a similar titre than to purified BPV-1 L2. This cross-reaction is most probably due to amino acids 1-88 of BPV-1 L2, since antisera to this peptide could also cross-neutralise HPV-16 pseudovirions at similar titres than to BPV-1 pseudovirions. Due to the lack of

antibodies seen, the BPV construct was not tested further in pseudovirion neutralisation assays.

There are various explanations for the lower immunogenicity of BPV. It was injected at a lower total dose than for the others. However, at a total dose of 1.8 μg, some immune response may be expected, since, according to Öhlschläger *et al.* (Ohlschläger *et al.*, 2003) a humoral response was elicited by at least 1 μg of capsomers. E+E7M was administered at twice the dose, but only twice, yet an immune response could be detected in the ELISA (Figure 3.1).

Another explanation for the lack of immune response induced by BPV could be the destruction of important anti-L1 neutralising antibody sites by the replacement and thus deletion of 88 amino acids from the C-terminal of HPV-16 L1. A deletion and point mutation study of HPV-16 L1 (Varsani *et al.*, 2006) showed that the deletion of amino acids 428-483 of HPV-16 L1 abolishes critical sites for the binding of known conformation-specific monoclonal antibodies. This region is deleted in the BPV construct and it is therefore conceivable that the deletion lowers this construct's ability to elicit conformation-specific antibodies that would bind to HPV-16 L1 in an ELISA. However, the deletion of amino acids 428-483 may not be the reason, since amino acids 414-439 and 414-433, which overlap with this region are deleted in the L2.56 and L2.17 constructs and these were capable of eliciting an anti-L1 response in our study.

Other studies have shown that capsomer formation is not tolerated when more than 32 (Kuck *et al.*, 2006) or 34 (Schädlich *et al.*, 2009) amino acids are deleted from the C-terminal of L1. Assembly of monomers into capsomers is very important for the induction of high titres of conformation-specific antibodies (Kuck *et al.*, 2006). In the BPV construct, 88 amino acids are deleted. However, in our study, after electron microscopy analysis, we detected possible pentamer aggregates, as seen in Figure 3.11(D). It may be that these were baculovirus or insect cell proteins, since the lack of antibodies elicited by BPV seems to indicate that capsomers did not assemble.

The inferior expression levels of the BPV construct in insect cells (see Table 2.4) as well as in plants, as seen in other work done in our research group, also does not make BPV a feasible vaccine candidate. Therefore the BPV construct does not show promise as a vaccine candidate.

3.4.2 L2.56 is the best vaccine candidate

SAF, L2.17 and L2.56 all show potential as prophylactic vaccines against HPV-16, the homologous type. All three constructs elicited anti-L1 antibodies, that were also capable of neutralising HPV-16 *in vitro,* as seen in Figures 3.1 and 3.3, and Table 3.5. Sera from one of the groups immunised with SAF did not show an average inhibition of alkaline phosphatase activity, although the standard deviation for the particular group was quite large. The standard error of the mean is also high for SAF in the ELISA, suggesting that one of the immunised groups did not react to the vaccine. For this reason the antibody titres for SAF could be artificially lowered. The most likely explanation for the lack of reaction to SAF by one of the mouse groups is technical. It may be that this group of mice was vaccinated last and that the vaccine emulsion in Freund's adjuvant had started to become destabilised, so that the concentration of antigen left in the syringe was not as high as for the other two groups. Because a high volume of vaccine was injected per mouse, it may be that for this particular group, not all of the vaccine was injected. A repeat of these mouse experiments will confirm whether the lack of response to SAF was due to technical error.

No comparison could be made between the antibody titres elicited by these three constructs in the pseudovirion neutralisation assays, since the sera were not assayed to their endpoints in all cases, due to the expense of the assays. However, the ELISA indicates that L2.56 elicited significantly higher levels of antibodies than L2.17 and SAF.

The antigen used in ELISA was plant-produced SAF. SAF has been shown by other members in our laboratory to assemble into capsomers in plants. Therefore this antigen could be utilised to test for conformation-specific antibodies, since antibodies against capsomers can still neutralise live virus, (Rizk *et al.*, 2008; Thönes *et al.*, 2008) and important binding sites for known monoclonal conformation-specific antibodies are not destroyed in SAF (Varsani *et al.*, 2003). It is conceivable that if sera elicited by SAF is bound to SAF protein in an ELISA, antibodies that were elicited against amino acids 108-120 in the chimaeric protein would also bind to these amino acids in the protein bound to the ELISA well. Not only anti-L1 antibodies will therefore bind to the SAF protein in the ELISA. In the case of SAF, an ELISA utilising SAF as an antigen does not assay only for anti-L1 antibodies. In our study, using SAF as antigen was not considered to be a problem, because SAF did not induce detectable anti-L2 antibodies above background as seen in Figure 3.2. Sera from mice vaccinated with

SAF is therefore unlikely to show an artificially higher anti-L1 immune response than the other constructs in the ELISA.

The highest titre observed in the ELISA was 800 (Table 3.4). This is a low titre obtained in an ELISA when compared to titres obtained after vaccination with L1 VLPs. Maclean *et al. (Maclean et al., 2007)* obtained titres of the order of 40 000 after two vaccinations with 10 μg of insect cell-derived VLPs without adjuvant. It should be considered that pentamers do not elicit as high titres of antibodies as VLPs (Thönes *et al.*, 2008). Even so, when Schädlich *et al.* administered two doses of 5 μg of *E. coli*-derived pentamers to mice, they still obtained titres of the order of 20 000 to 50 000, even when adminstering constructs where the helix 4 was deleted, which in their study showed even lower immunogenicity than pentamers where only the first 10 amino acids were deleted from the N-terminal. In our study the helix 4 was effectively deleted for all of the constructs. Constructs where both the helix 4 was deleted as well as 29 amino acids from the C-terminal, which is comparable with the extensively modified L1 chimaera, BPV, in our study, elicited titres of about 1600. However, in other studies where capsomers were administered at a similar dose, the titres were of the same order as in our study (Ohlschläger *et al.*, 2003; Thönes *et al.*, 2008) The low titres we obtained in our study may be due to many factors: firstly, it could not be confirmed what proportion of each vaccine preparation assembled into pentamers, due to the high quantity of other insect cell and baculovirus proteins (see Figure 3.10 (C)) and assembly into at least pentamers has been found to be crucial for eliciting antibodies (Schellenbacher *et al.*, 2009). Secondly, the high quantities of protein other than L1 in the vaccine preparations could have masked the immune response to L1, thus lowering it (Cleveland *et al.*, 2000a). Thirdly, as observed from Thönes *et al.* (Thönes *et al.*, 2008) and Ohlschläger *et al. (Ohlschläger et al., 2003)* a higher dose may be necessary in order to achieve titres of an order of 10 000. Varsani *et al.* (Varsani *et al.*, 2003) immunised mice with 100 μg of Chi-F (similar in amino acid sequence to SAF in our study) and obtained titres of 20 000. Therefore, in order to increase the titres obtained by the chimaeric L1 proteins in our study, purification of pentamers and/or VLPs may be necessary, as well as immunisation with a higher dose.

The presence of anti-L2 antibodies above background, as seen in Figure 3.2, for L2.56 and L2.17 may indicate their ability to elicit cross-neutralising antibodies, because neutralising antibodies against L1 is highly type-specific (Breitburd *et al.*, 1995), and anti-L2 antibodies have been shown to mediate cross-neutralisation (Roden *et al.*, 2000). The presence of anti-

L2 antibodies for the two constructs suggests that L2 is sufficiently displayed on the surface of chimaeric L1 protein. However, the sera from mice immunised with these constructs could not neutralise the heterologous HPV-45 and HPV-52 pseudovirions, as seen in Figures 3.4(B) and 3.5(B). This lack of neutralisation could be because the levels of anti-L2 antibodies were not high enough to mediate neutralisation, which could be improved by increased dosage, as well as selectively purifying capsomers and VLPs by a purification method that selects for these, such as sucrose gradient ultracentrifugation. By selecting for capsomers and VLPs, the display of L2 in repetitive arrays on the surface of the particle is ensured, which would increase humoral immunity against the displayed epitope.

The lack of cross-neutralisation could also be due to an inherent fault with the pseudovirion neutralisation assay. The positive control monoclonal antibody, developed against HPV-45 virus particles, H45.N5, could only neutralise HPV-45 pseudovirions to a level of 50%, even at high concentrations of the antibody, as seen in Figure 3.4(A). This low level of neutralisation may indicate contamination with another type of pseudovirion, most likely type 52, which was transfected at the same time as type 45. Pseudovirions are highly infectious. Cross-contamination with HPV-45 pseudovirions could also explain why neither H52.D11 nor H52.C1, monoclonal antibodies that neutralise HPV-52 tested, could neutralise HPV-52 pseudovirions, as shown in Figure 3.5(A). Future experiments need to be performed, involving direct binding of H45.N5, H52.C1 and H52.D11 to the pseudovirion preparations in an ELISA, to elucidate whether the antibodies were functional and whether contamination could explain the lack of neutralisation seen in our study.

It is also possible that the concentration of pseudovirions used in the assay was too high and therefore at a level of saturation with respect of infection. It was observed (data not shown), that the RLU increased each week after storage at -80°C, for both HPV-45 and -52, which suggests, however unlikely, a maturation process taking place over time. This apparent maturation process could be why, despite titration of pseudovirions and choosing the concentration such that it would not be at saturation level, saturation level could have been reached. But the RLU observed in subsequent experiments, was still within the linear range for HPV-45, however, for type 52, the RLUs could have been in the region of saturation. Figure 3.5(A) shows that H52.C1 had the same linear titration curve at half the concentration of pseudovirions, at an RLU which would fall into the linear range of the titration curve, which suggests that the concentration of pseudovirions was not the problem. Another

problem could be that crude cell lysate and not purified pseudovirions were employed in the assays. The neutralising antibodies in the antisera to L2.56, L2.17 and SAF may have preferentially cross-reacted with mammalian cell lysate components instead of pseudovirions. This effect was not seen in assays for HPV-16, therefore it is not clear whether crude cell lysates influenced the lack of cross-neutralisation.

The data suggests that L2.56 may be the preferred vaccine candidate. This construct elicited significantly higher levels of antibodies than the other constructs, as seen in Figure 3.1. L2.56 also elicited the highest levels of anti-L2 antibodies, as seen in Figure 3.2, lane 7. Electron microscopy analysis revealed the presence of possible VLPs in a range of sizes. (Figure 3.11(C)) This regular array of particles with their particular appearance was not observed in the negative control (Figure 3.10(C)). The presence of a regular array of particles suggests that of all the chimaeras, L2.56 showed the highest probability of assembling into higher-order structures, which correlates with levels of antibodies elicited. Even though SAF and L2.17 may have assembled into higher order structures as seen in Figure 3.11(A) and (B), these were not observed in such a regular array and as frequently as for L2.56.

3.4.3 Therapeutic vaccine candidates

E7M and E+E7M were constructs created to have a dual function: protection against infection with HPV, while at the same time treating an existing infection. The aim of our work was only to provide the proof of concept for this idea, since the E7M epitope is a CTL epitope for mice and will need to be replaced with an epitope specific to human alleles. Also, in the case of E7M, even if the epitope is replaced with a human CTL epitope, such a vaccine construct will only prevent infection with HPV-16, and is only likely to treat existing HPV-16 infections, due to the insertion of an HPV-16 E7 epitope (Youde *et al.*, 2005; Piersma *et al.*, 2008; Hohn *et al.*, 1999; McCarthy *et al.*, 2006). E+E7M, which contains a cross-neutralising L2 epitope, is therefore a more feasible idea, since it has the potential to protect against infections with types other than type 16, while treating existing HPV-16 infections, which in the majority of cases is the most likely type a person would be infected with.

The L1 in L1/E7 chimaeras could also function as a carrier molecule for the E7 epitope, better presenting the CTL epitope to the immune system on its first encounter with it (Street *et al.*, 1999) and enhancing cellular immunity by interaction of the L1 protein with cells of

the immune system. (Lenz *et al.*, 2003; Da Silva *et al.*, 2001) However, combining L1 with E7 may not always be a good idea, since L1 may dominate the immune response. (Bian *et al.*, 2008) L1/E7 chimaeras have been tested in human clinical trials, but their efficacy in treatment of cervical cancer was not clear (Kaufmann *et al.*, 2007), yet this approach is showing promise, due to the detection of cellular immune responses *in vitro* (Kaufmann *et al.*, 2001).

Our main aim in this study was to test for cellular immune responses and tumour regression induced by vaccination with E7M and E+E7M. In addition the sera were tested for the presence of anti-L1 antibodies. The results were not comparable to those of the L1/L2 chimaeras, since only two vaccinations were administered for the L1/E7 chimaeras, and not four, as for the others. Figure 3.1 shows that E7M and E+E7M elicited a higher average humoral response to L1 than the negative control, although this was only significant for E7M. Table 3.1 also indicates that E+E7M and E7M elicited similar titres of anti-L1 antibodies that were higher than that of the negative control. Electron microscopy analysis also indicated the possible presence of higher-order structures, as seen in Figure 3.12(A) and (B). Taken together, E7M and E+E7M are capable of eliciting antibodies against L1 at similar levels. Sera from mice immunised with E+E7M were also tested for anti-L2 antibodies, but these could not be detected. Neutralisation of HPV-16 *in vitro* could also not be detected, as seen in Table 3.5. Once again, optimisation of dosage and selective purification of higher-order structures may effect the induction of anti-L2 antibodies and anti-L1 antibodies capable of neutralising HPV-16 *in vitro*.

Ex vivo E7 peptide IFN-γ ELISPOT responses using splenocytes from E7M- and E+E7M-vaccinated mice were negative -no E7 specific IFN-γ secreting cells were detected and when the splenocytes were stimulated for 48h with the E7 peptide no cytokines could be detected in the culture supernatant. However a 6-day in vitro stimulation of the splenocytes from mice immunised with E7M and E+E7M with the E7 peptide revealed an interesting cytokine release profile when the generated effectors were stimulated for a further 24h period with the E7 peptide. Restimulated lymphocytes from E7M-vaccinated mice released Th2 type cytokines (IL-6 and IL-10 and MCP-1) while these cells from E+E7M vaccinated mice released Th1 type cytokines (TNF-α). No IFN-γ was detected for either vaccine. These data suggest that E7M induces E7-specific cells that favour antibody responses while E+E7M induces E7-specific cells that favour cellular immune responses with induction of cytotoxic

T-cells (Janeway *et al.*, 1997c). Cell-mediated immune responses are implicated in the combating of tumours by the immune system (Human Papillomaviruses/IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2005). For this reason, E+E7M may be the preferred vaccine candidate over E7M for treatment of HPV-induced tumours.

It could be speculated that the L2 epitope included in the E+E7M construct enhanced the interaction of this construct with cells of the immune system, leading to infection of the cells with VLPs or capsomers. It is known that chimaeric VLPs (cVLPs) are capable of entering dendritic cells in the same way as live virus, thus initiating an MHC class I response, for which intracellular penetration of virus is important. The cVLPs are degraded in the cytoplasm and peptides presented by MHC class I molecules on the surface of the molecules, thus initiating a cellular immune response (Kaufmann *et al.*, 2001). L2 amino acids 108-126, similar to the L2 peptide included in E+E7M, have been shown to interact with different cell lines and to enter the cytoplasm (Kawana *et al.*, 2001). It is therefore plausible that including this peptide in E+E7M enhances the infectivity of the construct, thus enhancing the cellular immune response. However, the cells tested for infectivity with the L2 peptide were all of epithelial origin, so it is not clear whether antigen-presenting cells have appropriate receptors for this peptide to bind to. Also, it is not clear whether capsomers, which we expected to obtain for E+E7M, can infect and penetrate cells to the same extent as VLPs (Lenz *et al.*, 2001).

The weak cellular immune responses induced by the L1/E7 chimaeras did not translate into tumour regression (Figures 3.8 and 3.9 and Table 3.7). The negative control group in fact showed a slower tumour growth rate than any of the test vaccines. Because the negative control started out with significantly smaller tumours than for the positive control and SAF (data not shown), it could also mean that a group with slower-growing tumours were selected for in the negative control group. This could have been circumvented by redistributing the mice when their tumours have grown to the required size before vaccination so that the spread of initial tumour size is similar for the different groups.

However, the results indicate that the test vaccines had no clear effect on tumour growth. It could be that L1 dominated the immune response, weakening the immune response to the E7 epitope. The lack of response to E7 may be rescued by using appropriate adjuvants, which enhance the recruitment and maturation of dendritic cells, such as anti-CD40 and GM-CSF,

and have been shown to enhance the cellular immune response to peptides included in cVLPs (Qian *et al.*, 2006). Another problem could be that a primary immune response to cVLPs cannot be boosted with the same cVLP, due to the induction of neutralising antibodies to the construct (Da Silva *et al.*, 2003). Heterologous boosting may be the solution to this problem (Da Silva *et al.*, 2003; Qian *et al.*, 2006).

3.4.4 Summary

In summary: BPV did not show potential as a prophylactic vaccine candidate. L2.17 and L2.56 showed potential as monovalent prophylactic vaccines protecting against multiple HPV types, because they elicited detectable levels of anti-L2 antibodies. L2.56 showed the greatest potential as a prophylactic vaccine, because it elicited the highest level of anti-L1 antibodies as measured by ELISA, elicited the highest level of anti-L2 antibodies as measured by Western blotting and showed the greatest potential for assembling into more immunogenic higher-order structures. As for therapeutic vaccines, E+E7M may be preferred over E7M, because vaccination with E+E7M led to the activation of the cellular arm of the immune system, which is important for the eradication of tumours, whereas E7M vaccination led to activation of the humoral arm of the immune system. By optimisation of experimental conditions, which would include increasing the dose of test vaccine administered to the animals as well as selectively purifying capsomers and/or VLPs from insect cell extracts, *in vitro* cross-neutralisation as well as tumour regression may be observed, which would verify the conclusions above.

4. CONCLUSIONS

4.1 Expression of chimaeric L1 protein vaccines

All eight chimaeric L1 proteins constructs used in this study could be expressed in insect cells; however, expression levels varied markedly between them. The highest concentration of protein obtained was for L2.17, followed by L2.56, E7M, SAF, BPV and E+E7M in that order. Protein concentrations ranged from 3.7 to 562 μg/ml per 10^6 cells.

We consistently obtained a lower amount of protein for the L1/BPV-L2 chimaera and the L1/L2/E7 chimaera. This difference in expression for some constructs when compared to others highlights an important aspect of recombinant protein production; namely, that it is not always possible to predict whether a particular construct can be expressed to high levels in a given expression system. Currently, the ability to expressa construct in a given expression system is something that needs to be empirically determined for each construct. Expression levels of HPV-derived and other proteins may vary between the same genes with as little as one nucleotide difference between them (Touze *et al.*, 1998); and when constructing chimaeric genes where part of one gene is replaced, the exact position of the replacement or insertion may also have an effect on expression levels of the construct (Murata *et al.*, 2009).

Expression levels can be optimised to some extent by employing the optimum way to modify the L1 sequence, as indicated by previous authors' work. There have been other studies which have shown that deletion of more than 20 amino acids from the C-terminal of L1 affected expression levels negatively (Senger *et al.*, 2009; Maclean *et al.*, 2007) and abolished capsomer assembly of these proteins (Kuck *et al.*, 2006; Schädlich *et al.*, 2009), which may leave the protein more prone to degradation. The data from these studies suggest that deleting more than 20 amino acids from the C-terminal of L1 is not a good approach. These results agree with results from this study, where BPV, the construct where 88 amino acids were replaced on the C-terminal of HPV-16 L1, expressed poorly. Because the L1/L2/E7 and L1/BPV-1 L2 expressed at lower levels than the other chimaeras, and were the only chimaeras with modifications to the C-terminal of amino acid 439 of HPV-16 L1, these data may indicate that replacing amino acids to the C-terminal end of amino acid 439 of HPV-16 L1 may be detrimental to the expression of the protein. These data should be considered when other HPV-16 L1 chimaeras are created. In such a case, where a long

peptide needs to be inserted into a protein, one should consider inserting the peptide without replacing amino acids of L1 or replacing as few as possible amino acids.

The expression levels obtained in this study will need to be optimised before large-scale production of any of these chimaeric constructs will become commercially viable. Optimisation would include adjusting the multiplicity of infection (MOI), cell density or time of infection. Expression in a different cell line, such as *Trichoplasia ni* cells, may also be necessary. Lastly, the MultiBac baculovirus expression system, which has been shown to enhance the yields of insect cell produced proteins (Senger *et al.*, 2009), can be employed.

Large-scale production of these chimaeric vaccines may, however, be more cost-effective and efficient in other production systems, like moulds, yeasts or plants, and attempts should therefore be made to also produce them in these systems. Correctly folded VLPs have previously been expressed in plants (Kohl *et al.*, 2007; Warzecha *et al.*, 2003; Regnard *et al.*, 2010), yeasts (Sasagawa *et al.*, 1995; Carter *et al.*, 1991) and the mold *Pichia* Pistoris (Bazan *et al.*, 2009). The advantages of these systems include high expression levels, easy cultivation, robustness and ability to be scaled up (Rybicki, 2009; Demain & Vaishnav, 2009).

Vaccines that could potentially be administered to humans need to be purified extensively. Purification will therefore need to be optimised for the chimaeric vaccines in this study. In our study, an already published method (Cook *et al.*, 1999), which was also employed in the purification of VLPs in the formulation of Gardasil® (Shi *et al.*, 2007), was attempted for SAF. This method involves cation exchange purification using the HS50 POROS column, which has a polystyrene divinylbenzene matrix. It was found that this method could be applied to the purification of SAF. However, Sepharose columns, which consist of 6% highly cross-linked agarose matrices, did not bind SAF. The difference in matrices was suggested to affect the binding of SAF to the column. Results from our work suggest that cation exchange chromatography, employing the method published by Cook *et al.*, should be considered as a method to purify chimaeric L1 capsomers and/or VLPs, whereas Sepharose columns should not be considered as a method to do so.

Ultracentrifugation is a method often employed in the purification of L1 to a level suitable for administering to animals (Varsani *et al.*, 2003; Kuck *et al.*, 2006; Kondo *et al.*, 2008;

Schellenbacher *et al.*, 2009). In this study, we attempted purification by employing a self-generated 24% Optiprep™ gradient. This method was not shown to effectively purify the insect cell extract. However, the advantages of Optiprep™ over other gradient media (Axis-Shield, 2007), such as caesium chloride or sucrose - which include its lack of osmolarity, and the fact that it can be injected without harm - provides incentive for investigating purifying L1 chimaeric vaccines by this method for animal studies.

4.2 L2 and the design of prophylactic chimaeric vaccines

Research towards second-generation prophylactic HPV vaccines is, amongst other things, currently focusing on approaches including potentially cross-neutralising peptides from L2 (Bosch, 2009). This is because peptides may potentially be produced more cost-effectively in *E. coli*, which is a simpler expression system than the eukaryotic systems currently used for HPV vaccine production. Researchers in the developing world may have more access to such an expression system, which could mean that HPV vaccines could be produced locally in countries where they are most needed. Current HPV vaccines are unaffordable to many in developing countries (Techakehakij & Feldman, 2008).

However, L2 is immunogenically subdominant to L1 (Roden *et al.*, 2000) and thus a higher dose of L2 will need to be administered in order to achieve levels of protection similar to current HPV vaccines based on L1. Peptides in general also display low immunogenicity unless cross-linked to another protein or adjuvanted (Rubio *et al.*, 2009). For these reasons and because L1-based vaccines have already been thoroughly researched, tested and proven to be highly immunogenic and effective (Schiller *et al.*, 2008), cross-linking L2 peptides to L1 may be the best and fastest approach towards putting broadly neutralising second-generation HPV vaccines on the market. Creating a monovalent chimaeric vaccine, as opposed to a polyvalent vaccine including VLPs of many carcinogenic and potentially cutaneous HPV types, will also be more cost-effective (Bosch, 2009).

HPV-16 L1 should be utilised in the creation of chimaeric L1 vaccines, because the overwhelming burden of cervical cancer is due to this type worldwide (Bosch *et al.*, 2008). Such a chimaeric vaccine would generate high titres of protective antibodies against HPV-16,

while also protecting against other types of HPV due to the L2 peptides included in the vaccine.

The L2 peptide showing the most promise in cross-neutralisation of other types according to the literature, consists of amino acids 17-36 (Rubio *et al.*, 2009; Gambhira *et al.*, 2007b). However, in our study, amino acids 56-81, when cross-linked to HPV-16 L1, elicited the highest levels of anti-L2 antibodies and showed great promise as a cross-neutralising sequence. This result reinforces the idea that the region comprising amino acids 56-81 contains multiple epitopes and that shorter peptides from this region may not be as effective in achieving broad cross-neutralisation as the entire region (Rubio *et al.*, 2009). Amino acids 1-88 from BPV-1 have not been extensively tested by other groups and may also show promise. However, such a long peptide may need to be inserted into HPV-16 L1 without replacing too long a stretch of amino acids from L1.

The helix 4 and the DE loop have shown the most promise as region of HPV-16 L1 to be modified with the insertion of L2 peptides. Our study supports the modification of the helix 4, because antibodies against both L1 and L2 could be elicited by two of the chimaeric L1 vaccines: L2.56 and L2.17.

A more meaningful comparison could be made between the immunogenicities of the different constructs if a higher dose were administered, since the titres obtained by ELISA and L2 western were very low. Pentamers, which are formed when the helix 4 has been deleted (Schädlich *et al.*, 2009), have shown a requirement to be administered at higher doses than VLPs to elicit comparable titres (Thönes *et al.*, 2008). This requirement should be considered when further studies are undertaken using chimaeric L1 capsomer vaccines.

Another way of obtaining higher titres of protective antibodies would be to immunise with extracts where capsomers and/or VLPs are selectively purified for, since the assembly of L1 into at least pentamers is a requirement for eliciting high levels of neutralising antibodies against L1 (Schädlich *et al.*, 2009) and the inserted L2 peptides (Schellenbacher *et al.*, 2009). Purification of chimaeric L1 protein would also clarify whether the proteins assemble into capsomers and/or VLPs, since the high level of background proteins in cell lysate samples interfered with the electron microscopy analysis. Further work is also necessary to optimise

pseudovirion neutralisation assays in order to detect cross-neutralisation *in vitro* by sera elicited by L2.56, L2.17 and SAF.

In our study, L2.56 showed the highest potential for a monovalent, prophylactic, broad-spectrum vaccine against HPV, followed by L2.17 and SAF in that order. SAF did not elicit detectable levels of anti-L2 antibodies, but further experimental work in animals, immunising them with a higher dose, may effect this, thus SAF may still be considered a candidate.

BPV did not show potential as a monovalent, prophylactic, broad-spectrum vaccine against HPV, because it did not express to high levels in insect cells in our study or in plants (Personal communication, Cathy Pineo) or elicit detectable levels of antibodies. This construct should therefore not be considered in future work.

4.3 The design of therapeutic chimaeric vaccines

Creating chimaeric L1/E7 vaccines has as its main goal the creation of a dual vaccine possessing both prophylactic and therapeutic properties. A person already infected with HPV, could be treated for the infection in order to clear it, whilst at the same time preventing infection with the same or similar type. It would be more useful if such a dual vaccine protected against infection with types of HPV other than the types the person was infected with. It would therefore be imperative to include an HPV-16 L2 cross-neutralising epitope in such a vaccine. For this reason, the creation of the L1/L2/E7 chimaeras in this study, E+E7M and E+E7H, was a novel and interesting idea that could be further pursued.

In our study, E7M and E+E7M were capable of eliciting similar, but very low levels of anti-L1 antibodies as measured by ELISA. No anti-L2 antibodies above detectable levels were elicited by E+E7M. In both cases, for both ani-L1 antibodies and anti-L2 antibodies, the low levels may be due to the low dose administered and the titres should be enhanced in further work as discussed for the other chimaeric constructs in Section 4.2. E7M and E+E7M therefore show potential to prevent infection by HPV-16, but this should be confirmed in further experiments, since *in vitro* neutralisation of HPV-16 pseudovirions was not seen. Whether E+E7M would be capable of eliciting cross-neutralising antibodies in animals should also be tested further.

The inclusion of the L2 epitope in the E+E7M chimaera seemed to have enhanced the vaccine's ability to elicit cellular immunity, which would be more beneficial to treating cervical cancer, rather than humoral immunity. This effect is something that would have to be investigated further. However, the enhancement of cellular immunity may indicate that E+E7M would be preferred as a therapeutic vaccine candidate over E7M.

No tumour regression was effected in the mouse model by vaccination with E7M or E+E7M. Tumour regression could possibly be effected in further experiments by optimising the dose and purification as discussed above in section 4.2. Because this effect could also be due to immunodominance of L1, the use of adjuvants such as anti-CD40 and GM-CSF (Qian *et al.*, 2006), that would enhance the immune response to the peptide included in the cVLPs, should be considered in future experiments involving L1/E7 chimaeras. Heterologous boosting with a DNA vaccine expressing the E7 peptide or the E7 peptides with adjuvant may also enhance immunity to the E7 peptide by circumventing humoral immunity against chimaeric L1 protein induced by the primary vaccination (Da Silva *et al.*, 2003; Qian *et al.*, 2006).

The concept of an L1/L2/E7 chimaera may, for various reasons listed above, be highly feasible for the creation of a prophylactic and therapeutic HPV vaccine. The concept could also be developed further by creating other chimaeras based on it. For example, the L2 epitope, amino acids 108-120, used in the E+E7M chimaera in our study, could be replaced with an L2 epitope that has shown more promise for cross-neutralisation, such as HPV-16 L2 amino acids 17-36 or 56-81.

CTL epitopes from other early HPV proteins, especially E6 but also others, could also be included in this chimaeric vaccine, since these have also shown to have the potential to mediate tumour regression (Brandsma *et al.*, 2007; Govan & Williamson, 2007; Liu *et al.*, 2007).

The E7 epitope in this chimaera could also be replaced with another E7 epitope that would be specific to alleles other than the HLA-A2 alleles. Peptides specific to other alleles need to be considered, since the HLA-A2 alleles are not necessarily as common in all populations, and women possessing alleles other than HLA-A2 alleles may be at greater risk for cervical cancer (Madeleine *et al.*, 2008; Morishima *et al.*, 2007).

The E7 epitope could also be replaced with an E7 CTL epitope from another carcinogenic type, like HPV-18, which causes a large proportion of cervical cancers worldwide (Bosch *et al.*, 2008). This replacement will be necessary, as E7 epitopes have not been shown to cross-react broadly between types (Youde *et al.*, 2005; Piersma *et al.*, 2008; Hohn *et al.*, 1999; McCarthy *et al.*, 2006). Priorities, as informed by epidemiological studies, will need to be set for which E7 CTL epitopes to be included in such a chimaeric L1/E7 vaccine, since there are indications that HPV types other than -16 and -18 may be responsible for a higher proportion of cervical cancer than previously expected in sub-Saharan Africa (Louie *et al.*, 2009) where there is also a high number of HIV-infected women, a factor which shifts the distribution of HPV types to types other than HPV-16 and -18 (Clifford *et al.*, 2006; Adler *et al.*, 2008). Types that may have to be considered include HPV-35, 45, 33 (Louie *et al.*, 2009), 52 (Banura *et al.*, 2008; Blossom *et al.*, 2007), 58 (Xi *et al.*, 2003), 51 (Moodley *et al.*, 2009), 53 (Sahasrabuddhe *et al.*, 2007) and 31 (Allan *et al.*, 2008).

The inclusion of a $CD4^+$ T-helper epitope is another important aspect to consider in the creation of a chimaeric L1 therapeutic vaccine, since the T-helper epitope has been shown to be important in stimulating the immune system to eradicate tumours (van Driel *et al.*, 1999; Muderspach *et al.*, 2000; Steller *et al.*, 1998; Daftarian *et al.*, 2006; Doan *et al.*, 2005; Vambutas *et al.*, 2005; Tindle *et al.*, 1991; Qin *et al.*, 2005; Xu *et al.*, 2009)

4.4 Conclusions of this study and future work

In conclusion, L2.56 and L2.17 showed potential as broad-spectrum vaccines, because they elicited detectable levels of anti-L2 antibodies. Anti-L2 antibodies could not be detected for SAF or E+E7M, but it is possible that these were below levels of detection and that by improving experimental conditions, anti-L2 antibodies may be detected. Even though *in vitro* cross-neutralisation was not detected for antisera to any of these three constructs, the presence of anti-L2 antibodies suggests that pseudovirion neutralisation assays should be repeated under different conditions. Other types of HPV pseudovirions should be tested, pseudovirions should be purified from cell lysate, pseudovirions should be used at a concentration within the linear range of RLU versus concentration, and positive control monoclonal antibodies should be capable of neutralising the pseudovirions to a high level.

BPV was eliminated as a potential vaccine candidate, due to low expression, and lack of anti-L1 antibodies elicited by this construct.

Weak cellular responses were elicited by E7M and E+E7M, which did not translate into tumour regression, but the responses elicited by vaccination with E+E7M were predominated by responses beneficial to tumour regression, whereas those elicited by E7M were not. Therefore E+E7M may be a better therapeutic vaccine candidate than E7M. Both of these constructs showed potential to protect against HPV-16, because anti-L1 antibodies could be detected by ELISA.

The lack of anti-L2 antibodies observed for SAF, the lack of cross-neutralisation, the lack of neutralisation of HPV-16 pseudovirions by E+E7M and E7M and the lack of tumour regression effected by the latter two constructs could all be improved by administering a higher dose of protein, and by selectively purifying capsomers and/or VLPs. Tumour regression could also be effected by boosting with a DNA vaccine or with peptides or including appropriate adjuvants as discussed above. Purification of chimaeric L1 protein from insect cell lysate will also improve electron microscopy analysis and result in more conclusive results with respect to the ability of our constructs to assemble into higher-order structures.

Chimaeric L1 vaccines which incorporate cross-neutralising L2 epitopes and T-cell epitopes from early HPV proteins for the treatment of cervical cancer, and are produced in more cost-effective production systems, are promising second-generation HPV vaccine candidates. Such vaccines could induce high levels of protective antibodies against HPV, mediate protection against multiple HPV types, treat existing HPV infections and become affordable to the developing world. L2.56, L2.17, SAF and E+E7M should be tested further as vaccine candidates, but other novel chimaeric L1 vaccines could be created, especially ones based on the concept of E+E7M. According to this study, up to 27 amino acids of the region to the C-terminal of the helix 4 of HPV-16 L1 can be replaced in the construction of such chimaeras, without compromising anti-L1 immunogenicity.

Glossary

Adenocarcinoma *in situ* (AIS): A precursor to adenocarcinoma, which is a cancer originating in glandular tissue.

Adjuvant: An adjuvant is mixed with an antigen and used to enhance the immune response to the antigen.

Afferent immune system: The arm of the immune system which receives the foreign material or intruder and sends danger signals to the rest of the immune system, for example by presenting the antigen on antigen-presenting cells.

Antigen-presenting cells (APCs): Cells of the immune system which process antigen and display peptide fragments on their surfaces for recognition by lymphocytes, such as B cells and T cells.

Antisera: The fluid component of clotted blood containing antibodies against the antigen the person or animal was immunised against.

B cells: One of two major classes of lymphocyte. Upon activation by their specific antigen, B cells differentiate into cells producing antibody of the same specificity as their initial receptor.

CD8 T-cells : T cells that differentiate into cytotoxic T lymphocytes. They recognise antigen presented in complex with MHC class I on antigen-presenting cells.

CD4 T-cells : T cells specialised to activate other cells. They are divided into two subsets: Th1 and Th2 cells. They recognise antigen presented in complex with MHC class II molecules.

Cellular immunity (Cell-mediated immune responses): An adaptive immune response involving antigen-specific T-cells. It includes all adaptive immune responses that cannot be transferred to a naive recipient with serum antibody. Adaptive immune responses are divided into humoral and cell-mediated immunity.

Cervical intraepithelial neoplasia grade 1 (CIN1): Abnormal cell growth corresponding to one third of the basal epithelium of the cervix. Equivalent to LSIL.

Cervical intraepithelial neoplasia grade 2: (CIN2): Abnormal cell growth spanning two thirds of the basal epithelium of the cervix.

Cervical intraepithelial neoplasia grade 3 (CIN3): Abnormal cell growth spanning more than two thirds of the basal epithelium of the cervix. Also referred to as cervical carcinoma *in situ.*

CIN2+: Any lesion grade equal to or higher than CIN2. Equivalent to HSIL.

Conformational epitopes : Also called discontinuous epitopes. They are made up of several separate regions in the primary sequence of a protein by folding of the protein. They are not displayed when the protein is denatured.

Cross-neutralisation (cross-protection): When antibodies elicited by a particular antigen bind and neutralise an antigen by which they were not elicited.

Cross-presentation: The ability of certain antigen-presenting cells to take up, process and present antigens to CD8 T cells.

Cytokines: Proteins made by cells that affect the behaviour of other cells, specifically cells of the immune system.

Cytotoxic T-lymphocyte (CTL): T cells that kill other cells, important for killing intracellular cytosolic antigens. They are mostly CD8 T cells.

High-grade squamous intraepithelial lesions (HSILs): Equivalent to CIN2+.

Human leukocyte antigen (HLA): The genetic designation for the human major histocompatibility complex. An example is HLA-A*0201 where the A designates the locus and the numbers designate the alleles.

Humoral immunity: Immunity mediated by antibodies. Adaptive immune responses are divided into humoral and cell-mediated immunity.

Invasive cervical cancer (ICC) : Cancer of the cervix that has spread deeper into the cervix and/or to other parts of the body.

Linear epitopes: Also called continuous epitopes. They are made up of contiguous regions of the primary protein sequence and are displayed even when the protein is denatured.

Low-grade squamous intraepithelial lesions (LSILs): Equivalent to CIN1.

Major histocompatibility (MHC) class I molecules: Proteins that present peptides on antigen presenting cells to CD8 T cells.

MHC class II molecules: Proteins that present peptides on antigen presenting cells to CD4 T cells.

Monoclonal antibodies: Antibodies produced by a single clone of B-lymphocytes, therefore they only bind to one specific epitope on a protein

Pan DR: Relating to T helper epitopes capable of binding to more than one allele of HLA MHC class II molecules.

Pentamers (Capsomers): The HPV capsid consists of 72 pentamers of L1. One pentamer is also called a capsomer.

Polyclonal sera : Antisera containing antibodies against multiple epitopes on the same antigen a person or animal was vaccinated with.

Pseudovirions : An infectious HPV particle, consisting of the capsid, but containing a reporter plasmid instead of the HPV genome.

Restriction elements: Another term for human leukocyte antigens.

Squamous cell carcinoma (SCC): A malignant tumour of squamous epithelium.

T cells: One of two major classes of lymphocyte. They are defined by their development in the thymus.

T helper epitope: An epitope presented in complex with an MHC class II molecule and recognised by a T helper cell. T helper cells provide signals for B cell activation, but also play a role in the activation of cytotoxic T lymphocytes.

Tumour-infiltrating lymphocytes: A T cell that has left the bloodstream and migrated into a tumour.

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