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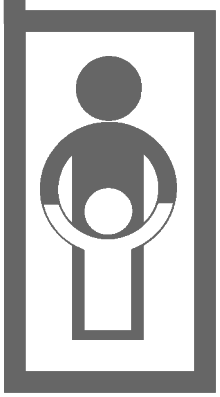
Assessment and harmonization of laboratory diagnostic procedures related to human papillomavirus vaccine research and development

Report of a technical meeting
Heidelberg, 6-7 March 2001



DEPARTMENT OF VACCINES AND BIOLOGICALS

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Contents

Abbreviations

CIN	cervical intraepithelial neoplasia
CMI	cell mediated immunity
CN	cervical neoplasia
cRIA	competitive radioimmunoassay
CRPV	cottontail rabbit papillomavirus
CTL	cytotoxic T-cell
CVLP	chimeric virus-like particle
DMSO	Dimethyl sulphoxide
DTH	delayed-type hypersensitivity
EBV	Epstein Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
IFNg	gamma interferon
IgG	immunoglobulin G
LPA	lymphoproliferation assay
mAb	monoclonal antibody
NAT	nucleic acid amplification technique
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
sIgA	secretory immunoglobulin A
TH	t-helper
VLP	virus-like particle
VNA	virus-neutralizing antibody

Executive summary

Cervical cancer continues to be a serious public health problem of global importance. Of the 232 000 women who die of cervical cancer annually, 192 000 live in developing countries. The incidence of cervical cancer is second to that of breast cancer. A causal link between human papillomavirus (HPV) infections and the development of cervical cancer has been demonstrated. There is little public awareness of HPV infections, their transmission route and the diseases they cause.

An effective vaccine against oncogenic HPV types could have a tremendous impact on the global cervical cancer burden. Recombinant DNA technology is being used to produce subunit vaccines against the most common oncogenic HPV types. These vaccines are under clinical investigation. WHO intends to strengthen its commitment to both advocacy and research in the primary prevention strategies, to promote the further development of suitable HPV vaccines and to make them available to the people who need them most.

The use of appropriate WHO standard materials and reference preparations is fundamental to the worldwide regulation and control of vaccines. WHO has a constitutional mandate from Member States to advise drug regulatory authorities and health ministries to develop the capacity for regulating novel vaccines.

The present technical consultation, sponsored by WHO and convened by its Department of Vaccines and Biologicals, sought to assess and harmonize the laboratory diagnostic tests currently available for monitoring HPV infections, with a view to facilitating the conduct of clinical trials on HPV vaccines. Experts from many of the laboratories involved in HPV vaccine research and development met to consider the proposed agenda (see annex). Large phase IIb proof-of-principle studies or phase III efficacy studies are about to be conducted on at least three candidate prophylactic vaccines. Each of the current candidate vaccines is based on the use of virus-like particles (VLPs), composed of viral capsid proteins, against HPV genotypes 16 and/or 18, which cause approximately 70% of cervical cancer cases. There is some geographical variability in the percentages of cancers caused by these types.

The standardization of assays for assessing viral diagnosis and viral load measurements, as well as immune responses, will facilitate the conduct of trials and comparisons between candidate vaccines. The assessment and harmonization of reagents and assays has been undertaken in order to develop reference reagents for global use and, eventually, to create international standards for work in the HPV field.

Introduction

Cervical cancer continues to be a serious public health problem of global importance. Nearly 470 000 new cases occur annually, about 80% of them in developing countries. Over half of the latter cases occur in Asia. The mortality associated with cervical cancer is the most telling indicator of its impact on women and their families and communities. Of the 232 000 women who die of cervical cancer every year, 192 000 live in developing countries. Cervical cancer is second to breast cancer in respect of global cancer incidence in women.

Human papillomaviruses (HPVs) are associated with a wide spectrum of different mucocutaneous infections, including common skin warts and genital warts, as well as cervical cancer. Genital HPV infections are among the commonest sexually transmitted viral infections diagnosed in clinical sexual health practice, while a minority of women with persistent HPV infections develop premalignant cervical lesions and ultimately cervical cancer. In fact, a causal link between HPV infections and the development of cervical cancer has been demonstrated (Walboomers et al., 1999, *Journal of Pathology*, 189:12). Over 99% of cervical cancer cases contain HPV DNA, and five specific oncogenic HPV types (HPV 16, 18, 31, 33 and 45) cause at least 80% of diagnosed cervical cancers. There is some geographical variability in the percentages of cancers caused by these types. On the basis of studies conducted in Japan and Spain the International Agency for Research on Cancer (IARC) estimates the prevalence of chronic persistent infections in developed countries to be approximately 7%. The corresponding figure for developing countries is estimated at about 15% on the basis of work undertaken in Brazil, Colombia, Morocco, the Philippines, and Thailand. Researchers estimate the global prevalence of clinically premalignant HPV infections to be in the range 28-40 million.

Given the long record of viral vaccines as providing a cost-effective approach to the prevention of infections and the diseases they cause, an effective vaccine against oncogenic HPV types could have a tremendous impact on the global cervical cancer burden. This is particularly true for developing countries, where other diagnostic and therapeutic options are often not affordable or are unavailable. In developed countries such a vaccine could reduce the incidence of preneoplastic and other frequent HPV-related genital lesions.

The presence of oncogenic proteins encoded by HPVs poses theoretical safety concerns in relation to the development of a prophylactic vaccine composed of an inactivated pathogen that carries the entire viral genome. Efforts have therefore been aimed at developing subunit vaccines consisting of only the viral proteins as antigenic material or of DNA lacking the viral oncogenes. Recombinant DNA technology is currently being used to produce protein-based subunit vaccines against

the most common oncogenic HPV types, 16 and 18. L1 capsid protein from HPV, when expressed by recombinant genetic engineering, has the useful property of self-assembling into VLPs. These particles contain no viral DNA and are therefore non-infectious. More importantly, they resemble the virus capsid and when inoculated parenterally stimulate the production of antibodies that bind and neutralize the infectious virus. The encouraging preclinical results obtained by in vivo testing of HPV VLPs as candidate vaccines have prompted both commercial and public institutions to pursue the clinical development of these products.

The results of three phase I human trials with L1-VLPs derived from HPV 18, 16, 11 and 6 have been encouraging, showing excellent tolerability and high immunogenicity (Harro et al., 2001, *Journal of the National Cancer Institute*, 93. 284). Phase II studies are in progress. Polypeptides of non-structural viral proteins are being added to L1 and L2 minor capsid protein in the hope of enhancing protection and conferring therapeutic potential.

Objectives

Vaccines are unique in that they are usually administered to very large numbers of healthy people, mostly infants, in national immunization programmes. Consequently, their safety and quality are of paramount importance. Vaccines differ from therapeutic medicines because of the biological, i.e. inherently variable, nature of the products, the raw materials used in their production, and the biological methods used to test them. Special expertise and procedures are therefore needed for their production, evaluation and regulation.

WHO has a constitutional mandate from its Member States to advise drug regulatory authorities and health ministries to develop the capacity to regulate novel vaccines. The use of appropriate WHO standard materials and reference preparations, where they exist, is fundamental to the regulation and control of vaccines.

The Heidelberg meeting focused on reviewing the three categories of assays currently used to monitor vaccine biological responses, as follows:

- HPV DNA detection assays;
- antibody assays;
- cell-mediated immunity assays.

The objective was to reach agreement on the need and usefulness of developing reference reagents to be qualified and validated in an international collaborative study. These reagents, once validated, would be made available for the evaluation of immune responses to HPV candidate vaccines in different parts of the world and at different times, in a clinical research context. If endorsed by the Expert Committee on Standards and Biologicals they could eventually serve as international standards for HPV diagnostics.

Opening remarks

Dr H. zur Hausen welcomed the participants and mentioned that the successful development of HPV vaccines represented an historical landmark in the control of cancer mortality in developing countries. He acknowledged the support of the German Health Ministry and the initiatives of many organizations that had become active in this field, and encouraged pursuing the recommendations delineated by the expert panel, which met two years ago under his chairmanship, in Geneva, to discuss the HPV vaccine development.

Dr T. Aguado outlined a comprehensive plan of activities, to be conducted in parallel, aimed at accelerating vaccine development for developing countries. From the public health perspective they were likely to have an impact on the control of the global disease burden. The areas of activity were:

- epidemiological studies to prepare field trials and evaluate vaccine effectiveness;
- standardization of laboratory diagnostic tests related to HPV infections;
- development and evaluation of novel multivalent vaccines and novel formulations;
- clinical studies to evaluate the efficacy of VLP candidate vaccines;
- generation of guidelines for vaccine production, exploring its economic impact in developing countries on the basis of technology transfer.

Dr S. Franceschi emphasized that the speed and efficiency of HPV vaccine trials would depend on the right choices concerning the type of vaccine to be studied, the design of the trials, and the biological end-point for the evaluation of prophylactic efficacy. She suggested that additional efforts to evaluate available candidate vaccines in different settings might accelerate vaccine development and availability. A factorial design and randomization procedure by villages might be of interest. The inclusion of males in the study to evaluate HPV transmission might strengthen vaccine efficacy. Dr Franceschi proposed that an end-point other than HPV infection should be considered, such as high-grade cervical lesions.

Dr J. Robertson drew attention to the need for biological standardization. He stated that a biological standard is the yardstick against which the potency or concentration of the effective constituent of a biological substance is measured. The primary biological standard is the WHO International Standard. Such materials facilitate the standardization of vaccines and other biologicals from different manufacturers, and allow comparisons between different laboratories. Other useful materials include

reference reagents, which might not have the status of an International Standard but are extremely useful for the determination of interlaboratory variation in sensitivity and specificity. International standards and reference materials are established through internationally conducted collaborative studies.

Dr A. Hildelsheim reported on progress related to the vaccine trial pursued by the United States National Cancer Institute. Randomized clinical studies of HPV 16 candidate vaccines are to be conducted in the province of Guanacaste, Costa Rica, where the incidence of cervical cancer is high and reliable baseline data has been collected in the last few years. The goal is to evaluate the prophylactic efficacy of HPV subunit candidate vaccines in a trial involving about 15 000 women. Dr Hildelsheim discussed the clinical outcomes, the virological outcomes, immunological surrogates and modifiers of effects. The evaluation of clinical disease status will be performed by carefully reviewed liquid-based cytology, primarily for low-grade and high-grade lesions. The HPV infection rate will be measured by type-specific HPV determination and the use of quantitative techniques. Immunological responses will be measured by ELISA and neutralization assays for both circulating and cervical levels of antibodies, and T-cell responses will be evaluated by other validated assays.

Session 1: HPV DNA detection assays

Constant progress has been made over the past few years on DNA detection based on the amplification of nucleic acid or signals and specific detection of the amplification products. Various nucleic acid amplification techniques (NATs) have been developed by users, as either in-house systems or commercial test systems, by in vitro diagnostics manufacturers. However, NAT systems may differ in their intrinsic features. NAT standardization under the umbrella of WHO has already made rapid progress in respect of viral targets, such as HIV, HBV and HCV. The experience gained might be helpful in the field of HPV DNA detection, which still lacks methodological harmonization. Basic assay features, such as sensitivity, specificity and the accuracy of typing are crucial aspects of HPV vaccine research and development.

The objective of this workshop section was to obtain an overview of existing HPV DNA detection systems, their basic features and current testing experience, and to agree on the next steps towards the harmonization and standardization of the molecular tests.

HPV DNA detection and typing assays rely on various methods, such as the amplification of target DNA, variants of the polymerase chain reaction (PCR), e.g. one-step PCRs and nested PCRs, line blot assays, and direct DNA detection assays, e.g. the hybrid capture assay.

Dr C. Meijer pointed out that the PCR was the most common technology used for HPV DNA detection and typing. The large-scale use of type-specific PCRs is unfeasible with the large spectrum of HPV types. Broad-spectrum PCRs with primers in the most conserved regions, usually E1 or L1, can therefore be combined with subsequent analysis of the underlying HPV type. The typing of PCR products can be performed by hybridization of the amplification products to specific probes, by sequence analysis or by a second PCR with type-specific primers. The detection of multiple HPV types and the correlation with clinical outcome is another field of current research. Further PCR applications include the specific amplification of groups of HPV types (low-risk versus high-risk groups) or the amplification of short fragments in order to increase sensitivity, followed by non-radioactive hybridization. A high sensitivity is especially important for the analysis of formalin-fixed materials and/or biological material bearing low copy numbers of viruses.

Dr R. Sahli presented a probe array hybridization system, which allows the simultaneous typing of 28 different HPV types. After the amplification of a conserved region of the viral genome the biotinylated amplicons are hybridized to type-specific probes, which are covalently bound to a nylon membrane. The filters can be reused

at least eight and up to 10 times without detriment to sensitivity, and this contributed strongly towards reducing the cost per sample of the assay. This was of particular interest in developing countries where cost could be a major consideration. The hybridization products are detected by means of the electrohemiluminescence reaction. Cross-hybridization was only found in restricted combinations (e.g. HPV 6 with probes 26 and 31), while real mixed infections were found in up to 37% of cases. HPV 53 (low-risk type) was as prevalent as HPV 16 (high-risk type) in the population studies (ASCUS- atypical squamous cells of undetermined significance – or low grade squamous intrepithelial lesions). The sequences of hybridization-negative amplicons always corresponded to HPV types not represented in the hybridization system. On the basis of the prevalence of individual types, as determined by PCR and reverse blotting, Dr Sahli estimated that false-positive samples for high-risk types were negligible in comparison with the results obtained by means of other techniques.

Dr L. Villa said that it was important to consider the source of DNA to be used for diagnostic analysis, its quality, and the amount of HPV DNA sequences present in specimens. Ideally, PCR amplifications should be performed in a single tube so as to avoid the risk of contamination, particularly at the nesting step. The performance of such reactions in a closed tube system with cycle-by-cycle monitoring of product accumulation in real time means that rapid analysis of large numbers of samples was possible. There was the additional advantage that information on viral load can be provided. Dr Villa concluded that reference samples, validated reagents and standardized protocols are needed in order to increase the reliability of HPV detection in both large epidemiological studies and vaccine trials.

The reverse line blot assay, developed by Roche Molecular Systems, was presented by Dr J. Kornegay. It allows discrimination between 27 anogenital HPV types, representing both the high-risk and low-risk groups for cancer development, and easy detection of infections with multiple types. After amplification of a 452 bp moiety of the HPV L1 gene, the denatured amplicons are hybridized to a nitrocellulose strip containing the type-specific probes. Coamplification and hybridization of the human beta-globin serves to provide an internal control for successful amplification as well as a marker for signal intensity. Striking differences in HPV type-specific prevalence between symptomatic women from Taipei (China) and from Mozambique were found when these assays were used. HPV 52 was the most prevalent type in Taipei, followed by HPV 16 and HPV 58. In rural Mozambique, however, HPV 35 had the highest prevalence and was followed by HPV 16 and HPV 18. These data suggest that the use of a combination vaccine would probably be the most efficient strategy for preventing HPV infections and, ultimately, cervical cancer. The oncogenic prevalence was similar for 161 women in the USA (HPV 16 > HPV 39 > HPV 52) and 163 Mexican women (HPV 16 > HPV 59 > HPV 52).

Dr W. Quint outlined the SPF10-LIPA HPV detection and typing system. It consists of a broad-spectrum HPV PCR (SPF10 PCR) followed by generic detection of amplification products (65 bp) by hybridization on a microtitre plate (DNA-EIA). HPV-positive samples are subsequently typed by hybridization with one or several of 28 probes fixed on a nylon strip (line probe assay) allowing discrimination between 25 anogenital HPV types. An investigation of 208 HIV-infected women in Brazil showed detectable HPV DNA in 98% of the cases, with high prevalence of low-risk types and multiple infections. Multiple HPV types decreased and high-risk types

increased with the severity of disease (dysplasia). Other studies were performed on 396 randomly recruited women from a family planning centre in Nairobi and a New York population attending a gynaecology department for routine screening. In both populations this assay detected a high prevalence of multiple infections that would not have been found by means of less sensitive techniques. While the trend of decreasing multiple infections with increasing severity of disease was confirmed for the HIV-positive members of the populations, the opposite trend was observed for immunocompetent women, i.e. an increase in multiple infections with increasing severity of disease. –Epidemiological studies on more than 3000 subjects in Brazil and the USA revealed overall prevalence of detectable HPV DNA in healthy women aged 15-25 of 35% and 28% respectively. There were multiple infections in more than 30% of the positive cases. The overall distributions of HPV types in the two populations were similar: 68% of the HPV types belonged to the high-risk group; HPV 16 and HPV 18 represented 23% of the high-risk types found.

The use of the hybrid capture II assay was presented by Dr C. Clavel. This method is based on the use of two detection probes containing synthetic RNA cocktails that are specific for high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and low-risk HPV types (HPV 6, 11, 42, 43, 44). After denaturation, the viral DNA is allowed to hybridize to the RNA probes, and the RNA/DNA hybrids are detectable in a semiquantitative manner by signal amplification. In France, a study on 7932 women attending for routine examinations investigated the correlation between age and the detection of high-risk HPV types by using this assay in comparison with cytological methods. The results showed that the frequency of positive high-risk HPV DNA increased from 20.1 % for women aged under 20 to a peak of 23.6 % for the age group 21-30 and then decreased for women aged over 30 years. Persistent high-risk HPV infections clearly represented a risk factor for developing a high-grade lesion. The high sensitivity of high-risk HPV DNA screening showed a significant improvement over cytological screening. The analytical sensitivity of the hybrid capture assay might be slightly lower compared to PCR methods, but its clinical sensitivity was between 90% and 100% for high-grade lesions, whereas the corresponding values for liquid cytology and conventional cytology were 88% and 69% respectively. Potential drawbacks are a lower clinical specificity and relatively high costs.

Discussion

External quality assessment for HPV DNA detection

Current experience with proficiency studies, e.g. for detection of HCV-RNA, shows a remarkable improvement in overall test quality with time. Participation in internationally organized proficiency studies is strongly encouraged for all laboratories performing HPV-DNA diagnosis. Proficiency studies are very important in promoting mutual recognition of data provided by different laboratories, improving internal quality procedures and obtaining greater certainty in assays.

HPV DNA detection in connection with vaccine trials

The harmonization and standardization of HPV DNA detection and typing assays are prerequisites for clinical trials to be performed in the near future for prophylactic vaccines against human papillomavirus. HPV DNA detection and typing methods are important tools for estimating the epidemiological situation of HPV infections, the screening of precancerous and cancerous cervical lesions, the further analysis of cytological and histological findings, the control of follow-ups after treatments and the evaluation of preclinical and clinical trials. The optimal features of such assays are high sensitivity, robustness and the potential for high throughput of samples. The relative efficacies of different candidate vaccines can be compared on the basis of harmonized and standardized NAT assays.

Analytical and clinical validation of the different HPV DNA detection assays should be performed by a small number of laboratories and should be the responsibility of the individual laboratories. Suitable common test panels and test materials are to be defined by a working group in collaboration with the WHO international laboratory for international standards, National Institute for Biologicals Standards and Control (NIBSC) or Central Laboratory of the Red Cross Blood Transfusion Service (CLB), in order to standardize the assays. Suitable assays may then be selected on the basis of the data obtained.

The HPV DNA investigations in direct connection with clinical vaccine trials should only be performed by a very small number of the most competent laboratories under well-controlled conditions. These laboratories should be selected on the basis of proficiency and their current experience with HPV DNA analysis. This approach is necessary to ensure the comparability of data, the recognition of data provided by different laboratories and the acceptability of the data by regulatory agencies.

Session 2: Antibody assays

Anti-papillomavirus systemic antibodies in animal papillomavirus models have been shown to confer protection against infections, and the extent to which this is true for anti-HPV antibodies in humans can only be demonstrated by vaccine trials. Currently, several prophylactic HPV candidate vaccines based on VLPs with the viral L1 or L1/L2 capsid protein as the immunogen are under clinical development. The principal method for determining the outcome of the vaccine trials involves assessing the protection induced against HPV infection and associated disease. However, an effective serological response is expected to be required for protection.

Prophylactic HPV vaccines are likely to be most efficacious in uninfected individuals, and so there is a requirement for sensitive assays to detect exposure to HPV before vaccination and to assess specific vaccine responses. Current assays for anti-HPV antibodies are generally based on direct or capture ELISA using HPVL1 VLPs as the antigen.

Dr K. Jansen described a competitive radioimmunoassay (cRIA) that had been developed for these purposes. In this cRIA, human or animal test sera compete with HPV type-specific and neutralizing monoclonal antibodies (mAbs) for limited binding sites on HPV VLPs. Whereas the ELISA might have a high background and could be influenced by impurities and the quality of the antigen, the cRIA generally has a low background, allowing the test to be performed with the use of undiluted sera. It could be more sensitive and less influenced by impurities. Furthermore, the cRIA is not species-specific, unlike the ELISA. Assay validation is important for correct and precise measurements, and an in-house standard has been prepared to quantify the serum antibody. However, an international standard is needed for comparison between different assays. cRIAs has been developed for HPV 16 and HPV 18, the two types most commonly associated with cervical cancer. Although the assays are not currently being used by all the centres involved in HPV vaccine trials, they are transferable. Known positive and negative non-human primate sera were used in order to validate the assays. In addition, children's sera were used as negatives to validate the assay cut-offs, on the assumption that there were no maternal antibodies and that there was limited infection of children with genital HPV other than at birth.

The cRIA was compared with the ELISA in assessing HPV 16 seroprevalence in more than 2000 females aged 16-23 and was shown to be the more sensitive assay, detecting more HPV infection: 12.7% of the sera were positive according to cRIA whereas only 5.3% were positive by ELISA.

Virus neutralization assays have also been investigated, using pseudovirions instead of live virus. The assays involved the uptake of L1/L2 VLPs coupled to a beta-lactamase reporter construct (pseudovirions). Upon uptake of the pseudovirions the enzyme is expressed inside the cells and can be visualized with a fluorescent substrate. In the absence of neutralizing antibodies the majority of cells exhibit a blue fluorescence. In the presence of such antibodies the pseudovirions are not taken up by the targeT-cells, beta-lactamase is not expressed, and the cells exhibited a green fluorescence. Cross-reactivity was tested between immune sera positive for HPV 16, 18, 6 and 11 from non-human primates. Only HPV 6 and 11 were strongly cross-reactive at the 1:100 serum dilution. There was a good correlation with the nude mouse neutralization assay in comparison with the pseudoneutralization assay as tested for HPV 11, and there was a reasonable correlation between cRIA titres and neutralization.

Dr J. Dillner discussed the application and use of serological assays for HPV antibodies in epidemiology. Studies on HPV serology have been usefully applied to assess attack rates, trends of disease, risks of cancer among exposed subjects and infection at non-cervical sites. Following HPV infection, human serum antibodies against HPV have neutralizing action, although there is little or no correlation with regression of disease or progression to cancer.

Assays for antibodies are based on an ELISA using L1 VLPs, employed directly, or by capture assay with the use of a type-specific mAb, or by competition ELISA, again using a type-specific mAb. The antibodies recognized by L1 VLPs were type-specific, and most can be blocked by a single neutralizing mAb. In contrast, disrupted or incorrectly folded VLPs exposed novel epitopes with limited type-specificity. The loss of their type-specific epitopes make disrupted VLPs useful as a control antigen. High-quality VLPs hav little or no exposure of cross-reactive epitopes, which could be tested using type-specific mAbs. Quality control of ELISA depends on maintaining intact VLPs of good quality and a quality assurance system. The definition of an antibody unit also has to receive careful consideration and becomes highly important in vaccine studies.

The induction of antibodies is a slow process, in some cases occurring several years after infection, and is commonly delayed for several months after detection of HPV DNA. The sensitivity of detection of infection markers varies with the quality of the assay. DNA detection is more sensitive than antibody detection, although most comparative studies report that over 50% of DNA-positive samples are positive for antibodies. As antibody assays become more sensitive the number of seroconverters is likely to increase. The percentage of women with antibodies to a particular type increases with increasing numbers of partners over their lifetime, i.e. seroprevalence is strongly related to the number of partners a woman had had and not to recent contacts. Antibody levels have been found to be stable, and in a five-year study of 1656 women there was no significant change in antibody levels. In some instances, persistence has been documented for more than 15 years. The specificity of antibody detection was greater than 98%. The IgG response is dominated by IgG_1 but there is also an abundant type-specific serum IgA response, and IgA in cervical mucosa have been shown to be related to the type-specific DNA.

Dr P. Coursaget presented data on cross-reactivity in serological and neutralization assays. Many HPV-infected patients are positive for multiple HPV types, and increases in antibody titres in some patients arose simultaneously against different types. This raises the question of cross-reactivity in serological assays. Many studies had found that seroreactivity to any given type was associated with elevated seroreactivity to other types. Cross-reactivity has been investigated by immunizing mice with VLPs from a variety of high-risk HPV types and testing the induced sera by ELISA with the different VLPs. Homologous titres were generally high whereas heterologous titres were low. There was evidence of some cross-reactivity between types 18 and 45 and between types 16 and 31.

Virus-neutralizing antibodies (VNAs) would probably provide an even more relevant marker for assessing vaccine potency, although testing for these was hampered by the absence of a suitable laboratory system for virus production and infectivity assay. To overcome this difficulty, an alternative method has been developed. It involves the generation of complexes between a DNA plasmid encoding luciferase and capsids formed from viral L1 protein, thus forming an artificial virus or pseudovirion. The level of neutralizing antibodies was determined by the level of inhibition of luciferase gene expression in targeT-cells (Cos-7) after preincubation of the pseudovirions with the test serum. The cross-reactivity of VNAs has to be considered in assessing multivalent vaccines and might also show that a monovalent vaccine could protect against additional types. The presence of cross-reactive VNAs against HPV types 16 and 31 was investigated by immunizing mice with a variety of HPV types and assessing antibodies by using pseudovirions formed with type 16 or 31 L1 protein. The results indicated that anti-VLP type 16 antibodies could partially neutralize type 31 pseudovirions and that type 31 Abs could partially neutralize type 16 pseudovirions. In addition, type 18 Abs partially neutralized type 45 pseudovirions and type 33 Abs partially neutralized type 16 and type 58 pseudovirions.

The levels of VNAs in human sera were also investigated. No VNAs were detected in sera derived from infants. However, if plasma was tested rather than serum the samples became 100% positive. This was attributable to the use of heparin as an anticoagulant, inhibiting the process of VLP transfection and resulting in false positives. It was therefore clearly desirable to avoid the use of heparin. In HPV-infected adults, VNAs were detected against types 16 and 31 in 20% and 19% of samples respectively. VNAs to types 16 and 31 were detected in an even greater proportion of cervical cancer patients. The VNA level increased with the progression of the disease. However, the detection of VNAs did not correlate with the detection of anti-VLP antibodies as determined by ELISA.

Dr A-L. Williamson reported on the prevalence of anti-type-16 VLP antibodies in oral fluids and cervical mucosa in South Africa. There were two aspects to the studies reported: firstly, the levels of secretory and serum antibodies in women with cervical neoplasia (CN); secondly, the levels of anti-type-16 antibodies in HIV-positive versus HIV-negative female sex workers. In South Africa the rate of cervical cancer is 32/100 000 population overall but it is much higher for black and other non-white women. In non-white women under 30 years of age the prevalence of high-risk HPV types is 25-35%.

There is a need for non-invasive techniques for assessing infection because of the large numbers of HIV-positive women. In the first study, therefore, oral fluid, cervical samples and sera were collected from women with CN who attended a colposcopy clinic, in order to assess antibody to type 16 VLPs. Control oral fluids were obtained from healthy volunteer laboratory workers. Among the women with CN, DNA was detected in 44% of cervical samples and 10% of oral samples. Of the oral samples from CN patients, 55% were positive for anti-VLP IgA, whereas the corresponding value for controls was only 8%; the respective values for samples positive for IgG were 45% and 13%. For the CN patients there was no correlation between the levels or prevalence of Abs from a particular sample and the presence of HPV type 16 DNA. There was a good correlation between the prevalence of cervical mucosa IgG and serum IgG. The detection of antibody in oral fluid was less sensitive than in serum. There was some correlation between serum and oral antibodies but it was not very significant. Nevertheless, detection in oral fluid might have a role as an epidemiological tool for determining HPV status.

Currently, HIV-positive women constituted over 22% of those attending antenatal clinics, and in the second part of the study the impact of HIV1 status on HPV prevalence and antibody levels was assessed. The study population consisted of HIV1- positive and HIV1-negative sex workers in KwaZulu Natal. None had AIDS but some had quite low CD4+ counts. Their mean age was 26 years. Most of the HIV-positive women were also HPV DNA-positive (85%), while fewer than half of the HIV-negative women were HPV DNA-positive (42%). A multiple regression analysis was performed to determine the impact of various factors, such as years as a sex worker, number of partners per day, condom usage. The only significant variable associated with HPV infection was HIV-1 seropositivity. An analysis of IgG and IgA prevalence in these women revealed that serum IgA was considerably more prevalent in HIV-negative women. This was possibly indicative of a greater ability of the HIV-negative sex workers to cope with exposure to HPV 16. In contrast, the local mucosal antibody response did not appear to have a bearing on the control of HPV infection in HIV-negative women, since the prevalence of cervical IgA was lower and cervical IgG levels were significantly lower.

Dr D. Nardelli-Haefliger presented data on the development of vaccine-induced antibodies at the mucosal surface of the female genital tract. These antibodies could be critical to the prevention of infection and disease since this site is where HPV-induced neoplasia occurrs. Generally, vaccines inoculated parenterally induces systemic IgG, which can transudate to mucosal sites but do not produce secretory immunoglobulin A (sIgA), whereas mucosal administration of a vaccine can induce both IgG and sIgA in mucosal secretions. Cytotoxic T-cells lysis (CTLs) can be induced by both types of vaccination although only mucosal vaccination can induce long-term immunity.

Intranasal instillation of type 16 HPV VLPs induced both neutralizing IgG and sIgA in the female genital tracts of mice. The levels of IgG and sIgA varied inversely during the estrous cycle. After parenteral vaccination the level of virus-neutralizing IgG fell below detectable levels during estrus whereas VNAs were produced throughout the cycle after intranasal inoculation, giving rise to both genital tract IgG and sIgA.

Similar studies have been performed with human female volunteers who have received two intramuscular doses of HPV VLPs (type 16) at four-week intervals. Samples of serum and cervical secretions were obtained from one group receiving oral contraception and another not receiving it. In the women on oral contraception there was less variation in the cervical antibody titre during the cycle. In the group not on oral contraception, however, there was a pronounced decrease of up to 2 log in the cervical ELISA titre at the time of ovulation. IgA titres, as expected, were much lower, but followed a similar trend.

The effect of HPV VLP vaccine administered by the nasal route or by aerosol was also assessed in female volunteers. Doses in the range 2-250 mg were administered and sampling was conducted eight weeks after the first dose or six weeks after the second dose. Nasal administration was not efficient even at 250 mg, while aerosol administration of 50 mg achieved similar IgG serum levels to those obtained by intramuscular inoculation in three of six volunteers. Serum IgA levels were lower than IgG levels, and aerosol administration gave comparable results to intramuscular administration. assays for the detection of IgA-secreting cells suggested that aerosol administration induced an IgA mucosal response. Levels of IgG and IgA in cervical secretions after aerosol administration were as good as or better than those obtained after intramuscular inoculation, most of the IgA being of the secretory type.

Dr J. Schiller discussed neutralization assays, which are considered the gold standard for evaluating protective antibody responses after vaccination. However, they were rendered difficult because of the problem of obtaining infectious HPV virions, and an alternative type of assay has been developed which involves the use of pseudovirions consisting of capsids formed in vitro by the expression of viral L1 or L1/L2 protein from vaccinia, Semliki Forest Virus, baculovirus or yeast vectors. The capsids are complexed with plasmids or other DNA-carrying marker genes, such as green fluorescent protein, beta-galactosidase or luciferase. The inhibition of expression of the marker gene after preincubation of the pseudovirions with the antibody sample is used as a measure of neutralization activity. Neutralization assays are considerably more complex than the ELISA, which is therefore likely to continue to be the most widely used assay in assessing vaccine trials. Some virus-neutralizing assays are about 20 times less sensitive than the ELISA. Nevertheless, there was a good correlation with ELISA data, even within individual samples, but this holds true only if well-formed VLPs are used as the immunogen against which the antibodies were raised. Because its complexity, the need for quality control and the expense of VLP production, the virus-neutralizing assay was unlikely to be used routinely in vaccine trial assessment. Its primary use was to validate VLPs manufactured as immunogens. It was also used in analyses of abnormal vaccinee responses and breakthrough infections. In addition, it would be used as a surrogate marker for a protective immune response.

Standardization is required of the variables that exist within the assay, such as pseudovirion production and the mode of assay, and of their effects on sensitivity, specificity, reproducibility and transferability. No single type of virus neutralization assay is perfect. However, the most useful one with respect to reproducibility and transferability is likely to be that involving L1 combined with in vitro assembly of the pseudovirion. Although the VLP/pseudovirion system is not a true viral system for assessing VNAs, its usefulness could be demonstrated in a small study against authentic HPV virus. In order to do this it would be advantageous to have a panel of mAbs that neutralized real virus but this is not yet available.

Discussion

Standardized reagents

There was agreement that it would be useful to have a pool or pools of monotypic serum of high titre. Such a pool would probably be derived from a vaccine trial, i.e. the serum would have been raised against VLPs. A serum derived from natural infection, i.e. from real virus, would be preferable, although its titre could be expected to be lower. The identification of such sera might take more time and effort than for a vaccine-derived serum. Standard sera of genotypes other than that used as vaccine would also be of value for assessing the cross-reactivity of a vaccinee's response. However, this type of serum would probably have to be derived from a natural viral infection.

The need was discussed for other standard reagents used in assays, such as VLPs and mAbs. For example, the production of VLPs to be used as antigens might require validation. This could best be achieved with appropriate mAbs, and these would have to be freely available. It was felt that the source of VLPs being used as both immunogens for vaccination and antigens for ELISAs was important, since impurities in VLPs could be highly immunogenic and their presence might result in false-positive results.

Enzyme-linked immunosorbent assays

It was agreed that ELISAs were likely to be the main antibody-dependent assays. They are easier to carry out than neutralization assays and easier to replicate in various laboratories. It is therefore likely that the standards would be characterized primarily in ELISAs. It is desirable for the sources of VLPs as immunogens and antigens to be distinct. There were ways around this issue, however, for instance the use of a cRIA or the careful design of the ELISA, incorporating appropriate controls such as a totally denatured VLP preparation as antigen. The use of a serum derived from real virus infection and the use of one derived from VLP induction both have advantages and disadvantages, and both approaches are feasible in practice. It was therefore proposed to proceed with both.

Virus neutralization assays

The use of standard reagents in these assays would be beneficial, although current neutralization assays were felt to be impractical on a large scale. In any case it was considered that virus neutralization of any standard serum should be assessed in a small study. Particles containing L1 and L2 are much more efficient in infecting cells and they mimic the natural virus more closely. Consequently, their use as an antigen in ELISAs seemed preferable.

On the basis of current knowledge and foreseeable problems it was suggested that a study of a pool of a few sera derived from a vaccine trial be initiated as soon as possible. This would be used in a collaborative study and would provide comparative information on different ELISA or cRIA methods. It could also be used as a run control (internal standard) with representative assays and eventually as a means of establishing the potency of a reference serum. An initial collaborative study with such a pool could provide much useful information.

Session 3: Cell-mediated immunity assays

Dr C. Melief presented an overview of the delicate balancing act involved in T-cell immunity against tumours. Apparently only those patients with insufficient HPV immunity and thus with persistent HPV infections develop HPV-induced cervical lesions and cervical cancer. Eventually, however, these patients with HPV-induced tumours develop a T-cell response naturally, although it is not maximal and could well be boosted, e.g. by vaccination or by adoptive transfer with tumour-specific T-cells. Among women with HPV-related cervical disease a T-helper response to HPV 16 E7 is seen with the progression of the disease in those with persistent cervical intraepithelial neoplasia (CIN). Furthermore, a memory cytotoxic T-cell (CTL) response against HPV 16 E7 was seen in those with high-grade dysplasia and invasive cervical cancer. With disease progression, however, immune competence was decreased and/or lost. It is thought that immunity largely develops as a consequence of increasing antigen burden in the tumour, but that it might be too late to be effective.

In animals the mode of delivery of tumour virus epitope strongly determined the outcome. During natural infection in humans, tumour viruses often caused insufficient antigen-presenting cell (dendritic cell) activation to induce vigorous T-helper cell and CTL responses. Indeed, the failure of a sufficient inflammatory response might be associated with inappropriate dendritic cell activation and thus with specific CTL tolerance. In HPV infection there seemed to be a delicate balance between immunity and tolerance: many women spontaneously cleared HPV infections, whereas persistent infection was established in many others. The presentation of the same viral epitopes in a more stimulatory context, e.g. in a viral vector such as an adenovirus, could be expected to cause strong antiviral and antitumour protection in a much higher percentage of individuals.

Following HPV inoculation, the virus affects suprabasal cells with the expression of E6, E7, E1, E2, E4 and E5. Ultimately, the expression of L1 and L2 occurrs, linked to the differentiation of the squamous cell epithelium from the basal to the superficial layers. It is therefore necessary for vaccines to target any one of these steps. For prophylactic vaccines it is necessary to obtain a cellular response to the HPV early proteins, as well as a neutralizing antibody response. The aim of vaccination is to retain these responses so that when vaccinees come in contact with HPVs they do not become infected. For therapeutic vaccines a T-cell response to E2, E6 and E7 is desired. In naturally regressing warts, CD4 and CD8 cells are involved, and a large gamma interferon response is linked to a good outcome.

In invasive cervical cancer, the Major Histocompatibility Complex class I expression is downregulated leading to escape in immune surveillance. TH1 responses, characterized by HPV-specific T-cells producing gamma interferon, are associated with protection, whereas TH2 responses (IL-4, IL-5 release) are associated with progressive disease.

In order to understand in more detail the natural CD4+ T-helper response against HPV 16, Dr Melief's group investigated the specific T-helper proliferative response to HPV 16 epitopes in patients with high-grade dysplasia and invasive cancer-utilizing 22-mer overlapping peptides of HPV 16 E2, E6 and E7. They identified several naturally processed T-helper epitopes, some of which are also recognized by T-cells of patients infected with HPV 16. They used the assay in which peripheral blood monocytes were stimulated by specific HPV antigen. The resultant cytokine release was captured by anticytokine antibody, and this was followed by washing and counting the spots that were proportionate to T-cell lymphocytes producing gamma interferon. This assay used influenza matrix protein as a positive control. An objective result was obtained by using an automated reader. The short experimental set-up resulted in the specific detection of HPV-specific memory T-cells without any risk for false positives. False positives could occur, however, with recombinant proteins (attributable to contamination by vector-related bacterial components). The delayed-type hypersensitivity (DTH) response for HPV 16 E7 was compared to an with respect to sensitivity and specificity, and a good correlation was found. The measurement of true memory T-cell response requires the collection of peripheral blood lymphocytes (fresh or frozen), which then have to be separated into CD45RA+ cells and CD45RO+ memory cells. The use of CD45RO+ cells allowed longer in vitro stimulation periods without the risk of generating primary T-cell responses in vitro. T-cell cultures could be stimulated for 10 days, after which the HPV-specific response was assessed by or intracellular cytokine staining. This resulted in the detection of low-frequency HPV-specific T-cells. HPV 16 E2, E6 or E7 positive responses had so far been detected in the CD45RO+ memory fraction, indicating past infection.

In the cottontail rabbit papillomavirus (CRPV) model the regression of papillomas correlated with a proliferative response against E2. It had also been noted in healthy human donors that E2 resulted in a high proliferative response. Dr Melief's group investigated whether this was because E2 primed naive T-cells or because an E2-specific memory response was induced. E2 was not only a highly immunogenic region of HPV but was a conserved region. TH responses to one HPV genotype might lead to cross-reactivity to many other HPVs that were very type-specific. Remarkably, a single E2 TH clone led to gamma interferon release to all HPV E2 peptides of many other types, in contrast to responses to E6 and E7.

Dr H. zur Hausen remarked that with the integration of HPVs there is often disruption of E2, which could explain escape of immune surveillance. This response could possibly be driven with vaccine strategies. It is desirable, therefore, that vaccines should not only be directed at E6 and E7 but also at E2.

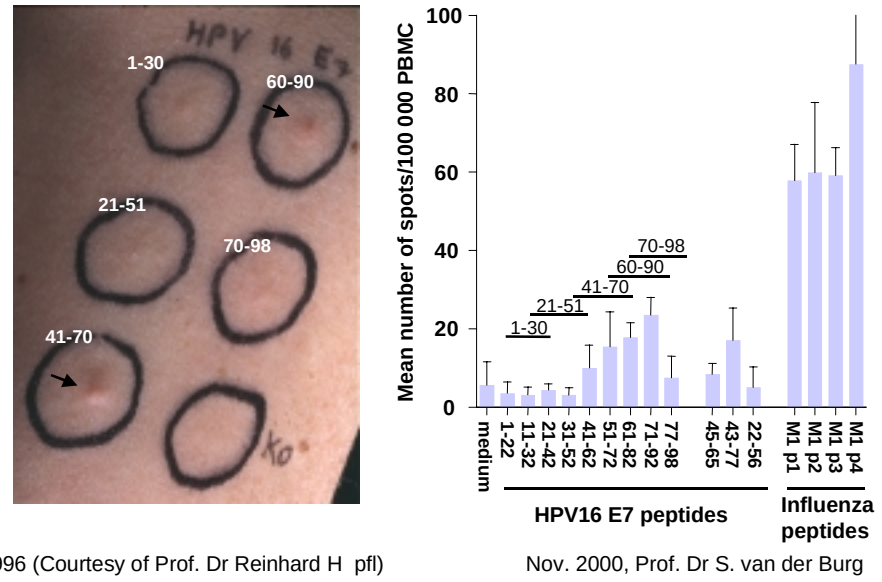
Dr R. Hopfl reported on the DTH assay on the skin of patients. Skin tests (intraepidermal challenge) mimick the interaction of the immune system with intraepithelial pathogens. Specific immune measurements by skin-test challenge included reactions involving DTH, typically starting on the second day after challenge and peaking on the third or fourth day. Skin tests for DTH are, in general, extremely sensitive and specific and are commonly utilized in clinical practice (e.g. the Tine test, the Merieux multitest) in looking at responses to various antigens and as markers of immune suppression. The use of a skin test to assess immunity in the CRPV model had shown that skin test reactivity to intact CRPV was associated with a regression of viral papillomas. Skin test reactivity indicated Th1 helper cell effects. Responses to capsid proteins are a marker of interaction of the immune system with actual or past infection, while reactions to early proteins ware associated with regression of CRPV papillomas.

Dr Hopfl's group has devised a DTH assay as a measure of TH cell-mediated immunity to HPV 16 in a skin test. They have devised a skin test, using a separate intraepidermal injection of HPV 16 E7 synthetic overlapping peptide sequences, covering the complete sequence of HPV 16 E7 protein. Each peptide was approximately 30 amino acids long. The use of separate peptide injections and DNA-free material overcomes any potential hazard of oncogenic activity. In a study of women with HPV-associated disease the DTH response arose within two to six days. Histopathological studies on biopsies of skin reaction showed lymphocytic infiltration, the CD4 response being more marked than the CD8 response. There were positive responses, i.e. skin induration at the site of inoculation (Fig. 1), in 73% of regressers (8 of 11), in 7% of progressers ($P < 0.001$) (2 of 30) and in no patients with invasive cancer. On further analysis, epitope specificity was found by gamma interferon assay (Fig. 1) and this correlated with epitope localization as detected by skin test, although the skin reactivity was much more pronounced in the only patient to date.

The skin test had the further advantage of being extremely sensitive, requiring low amounts of antigen (1–10 ìg peptide per test). Even a 1:100 dilution of antigen gave a positive reaction. As had been shown in the rabbit model, by increasing the number of proteins it was possible to increase sensitivity, and this was therefore a potential strategy in humans. The skin test is simple to perform, economical, and readily adaptable in a clinical setting. In vaccine trials it could be used to monitor cellular immunity and to measure spontaneous immunity to HPV as a marker of low risk of disease progression. The number of patients tested has been small, and further validation studies to compare in vitro and in vivo data are required. On the other hand, DTH testing could have an immunizing effect, because the vaccine antigens are immunogenic without adjuvant. This represents a major drawback to the use of DTH, which could interfere with vaccination schedules and dosing. However, DTH would have a great advantage over other T-cell assays that are expensive and laborious. In addition, cellular immunity (cytotoxic T-cells and T-helper cells) against early antigens is thought to be essential for the regression of disease.

Skin tests for DTH are used in other tumour vaccinations (e.g. for melanoma) and most reports indicate that survival was better in patients who converted their DTH skin test than in those whose DTH test remained negative (Fig. 1).

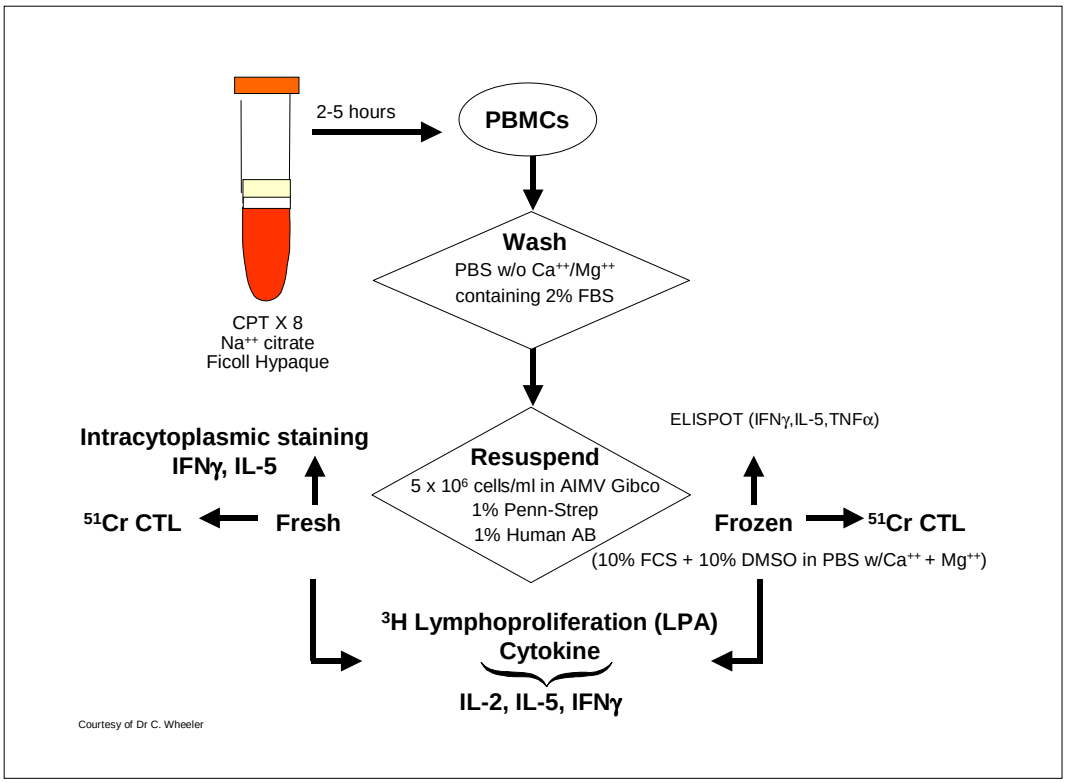
Figure 1: DTH compared to ELISPOT with stored T-cells in a patient with natural immunity to HPV16 E7 and spontaneous clearance of disease showed correlation of reactivity to peptides within the region 41-90



Dr C. Wheeler reported on current methodologies used for monitoring T-cell responses. The types of T-cell assays include: the lymphoproliferation assay (LPA), cytokine detection by ELISA, and cytotoxic T-cell (CTL) assays, and correlates of T-cell measurements, i.e. antibody titre, long-term protection studies. Issues of importance in standardized measurements of antigen-specific T-cell responses in HPV vaccine settings include: mononucelar cell collection; collection medium and transport; whether peripheral blood mononuclear cells (PBMCs) are used fresh or frozen; purity and lot variations of antigenic targets (e.g. sources of VLPs, whether they were from yeast or baculovirus or whether the source was peptides); reagent standardization. Evaluations of human and fetal calf serum lot performance are needed for the longitudinal follow-up of patients. Antigen-specific cell-based controls that perform within a specified range are required, as PHA varied between individuals. In addition, laboratories with excellent performance, proficiency and a high degree of flexibility will be required, and sensitivity, specificity and reproducibility will have to be defined. In general, the costs of achieving these goals are high yet the practical feasibility is low.

For HPV-specific cell-based assays, the collection of PBMCs for either an assay or for LPAs is shown in figure 2. The LPA is complex, require well-trained scientists, laboratories with radioactive facilities (Fig. 3) using ^{3}H thymidine, and the calculation of a stimulation index, i.e. geometric mean of counts per minute of cells with VLP/geometric mean of counts per minute of cells without antigen.

Figure 2: HPV-specific cell-based assays



For cytokine detection an ELISA system for the detection of gamma interferon and IL-5 was used by detection from LPA supernatant replicates, as shown in figure 3. In addition to the ELISA, the IL-2 bioassay was discussed (Fig. 4). CTL assays are difficult, requiring the establishment of autologous lymphoblastoid cell lines with transformation of the PBMCs with EBV in 96-well Microtiter plates and freezing at a concentration of 5 x 10^6 cells per ml. The autologous lymphoblastoid cell lines have to be prepared before CTL effector stimulation began. This methodology is highly dependent on patient compliance. CD8-positive cells were then isolated by using magnetic beads at different target and effector ratios, and ^{51}Cr release was measured.

Figure 3: Lymphocyte proliferation assay (LPA)

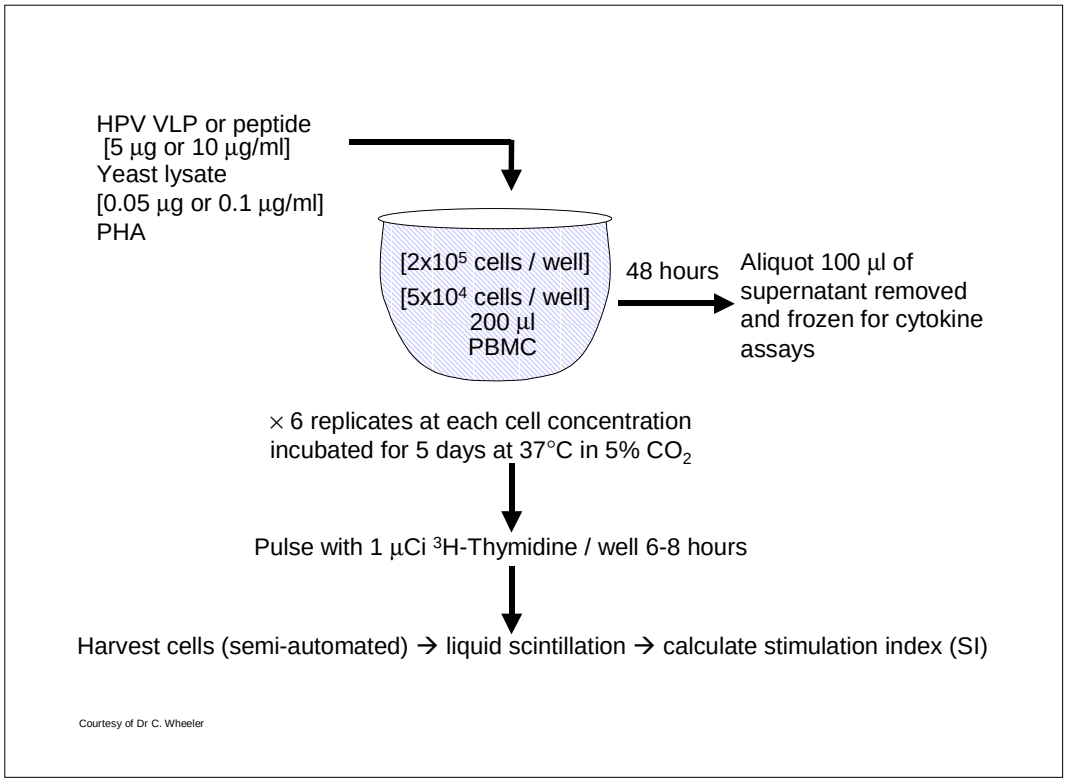
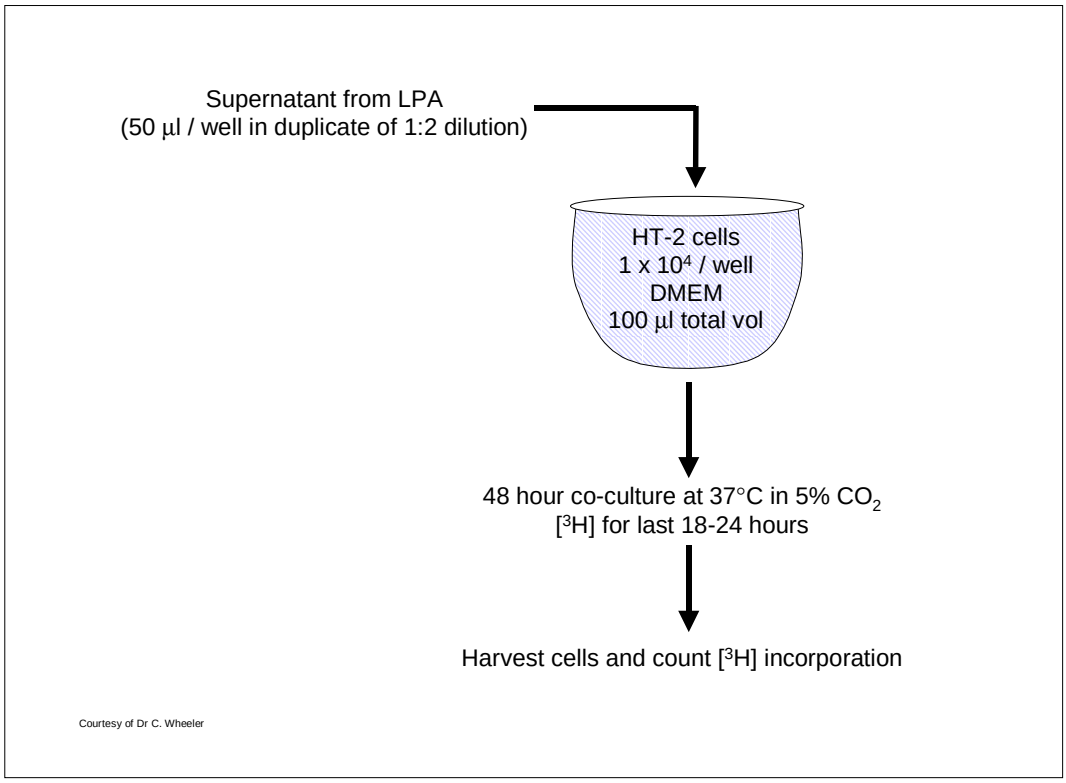


Figure 4: Cytokine detection IL-2 bioassay (HT-2)



These complex assays had shown that cytokine production correlated with antibody and proliferative responses in vaccine recipients. CTL measurements were difficult and were not consistently observed. Whether this reflected inadequate methodology or transient responses still has to be determined. They might provide the best surrogate assay. Collection tubes, whether citrated or heparinized, could modify functional assays (citrate led to the death of cells overnight).

T-cell responses to HPV chimeric virus-like particles (CVLPs) were described by Dr L. Gissman. HPV CVLPs, consisting of truncated L1/E7 fusion proteins, induced L1-specific antibodies that recognized CVLPs and VLPs (without E7 sequences) in preclinical testing. Methods of detection of the immune response to CVLPs included the lymphoproliferation assay, the cytotoxicity assay, the intracellular gamma interferon assay, the assay and tetramer analysis.

In mice the immune response to L1 E7 CVLPs resulted in the induction of neutralizing antibodies, the induction of L1-specific and E7-specific T-cells, the prevention of growth of E7-positive tumours and a therapeutic response of E7-positive tumours. These data suggested that L1/E7 CVLPs are suitable for therapeutic and prophylactic vaccination. In peripheral blood lymphocytes of healthy donors immunized with CVLPs of peripheral blood lymphocytes or dendritic cells, led to the activation of T-cell activation was demonstrated by lymphoproliferation, CTL activity and gamma interferon production (using FACS analysis).

The detection of integrated papillomavirus gene transcripts as a marker for high-grade anogenital dysplasia and emerging cancer cells was reported by Dr M. von Knebel Doeberitz. His group had worked on the basis that in most cervical cancers, HPV genotypes were integrated into the hosT-cell chromosomes. HPV transcripts derived from integrated HPV genomes are therefore a potential marker for both high-grade CIN and invasive cervical cancers. Using a PCR-based system the group has devised a way to detect transcripts that could be identified as episomal or integrated (the amplification of papillomavirus oncogene transcripts assay, or APOT). In a series of 2000 patients, no integrate-derived HPV transcripts were found in cervical swabs or biopsies in lower grade dysplasia or normals. In contrast, there were transcripts of integrated HPV in 88% of cancers and 15% of CIN 3.

Cervical dysplasia is induced by persistent infections of high-risk HPVs. The progression of dysplastic lesions is triggered by increasing expression of viral oncogene proteins E6 and E7, which both interact with various proteins that regulate the cell cycle. For example, E7 binds and inactivates retinoblastoma gene product, which inhibits transcription of the cyclin-dependent kinase inhibitor gene p16 INK4a. The overexpression of p16 in high-grade dysplasia and invasive cervical cancers was used as a further marker of the progression of lesions. In an evaluation of five leading gynaecological pathologists, p16 immunohistochemistry was found to be a valuable and specific biomarker. Variability between observers with p16 was far less pronounced than that of histological diagnosis.

Discussion

Although standardization is not yet possible, a preliminary identification of suitable assays can be performed. Practical recommendations concerning vaccine trials in progress were made on the use of optimally frozen (cryopreserved) PBL samples when possible. A controlled freezing device with 10% DMSO (e.g. haemopoietic stem cells) is essential for quality. The amount of blood required in heparin or sodium citrate is 25-50 ml or more.

Patients and healthy controls (negative), and cryopreserved (known) and donor samples (from leukophoresis) should be used in the same test. Control antigens such as influenza, tetanus toxoid, *Candida*, standardized VLPs and peptides should be used. The exchange of these reagents between laboratories is highly encouraged. Different antigen sources should be made available, such as VLPs from yeast or baculovirus, peptide pools and recombinant viruses.

State-of-the-art descriptions of the assays that are most suitable at present can be obtained from the sources indicated below.

Contact details	Assay type	Reagents	Specific equipment
R. Hoepfl	DTH	Peptides	None
C. Wheeler	Proliferation	VLP H-thymidine	Cell harvester
K. Melief / v.d.Burg	ELISPOT	Gamma interferon IL-4	Plate reader
J. Nieland	Intracytoplasmic fluorescence Double stain CD4/CD8	antibodies IFNg/IL-4	FACS, fluorescence
C. Melief	Tetramers Cytotoxicity	^{51}Cr Europium JAM	gcounter Eu reader ^{3}H-thymidine filters

Conclusions and recommendations

The purpose of the meeting was to assess and discuss the harmonization of diagnostic testing in the context of HPV vaccine trials. It was recognized that there was a need to make a successful vaccine available to developing countries as quickly as possible. A diagnostic HPV-DNA assay is commercially available but it monitors for multiple genotypes at once based on being high-risk or low-risk for causing cervical cancer. There is a need for collaboration among bench scientists and epidemiologists in order to develop specific assays that will facilitate vaccine development. The meeting afforded a novel forum for IARC, WHO, Deutschkrebsforschungszentrum – DKFZ, industry and academia to identify where work is needed on vaccine development and the conduct of vaccine trials.

Conclusions and recommendations from the plenary session on DNA tests

A panel of working materials will be developed in order to compare and assess the variety of DNA tests available. It may be necessary to take different approaches to the standardization of assays in support of vaccine trials in contrast to epidemiological screening. For vaccine trials, assays may be performed in a small number of qualified laboratories. Type-specific assays that can distinguish a broad panel of genotypes may be required to support vaccine trials. This contrasts with clinical screening in diagnostic laboratories. The standardization of methods is important for both. A group headed by Dr M. deVilliers and Dr W. Quint has been formed in order to develop an action plan based on these recommendations.

Conclusions and recommendations from the plenary session on antibody assays

It was concluded that standardized reagents would facilitate the comparison of available assays for the measurement of binding and neutralizing antibodies. Monotypic serum of high titre is needed in order to facilitate vaccine trials. Although sera from natural infection would be useful, it may be easier to derive reagents from recipients of monotypic vaccine. However, sera from non-vaccine types are also desirable. In most assays the source of immunogens (vaccine) and antigens (assays) should be distinct. The use of a cRIA/ELISA or appropriate controls, e.g. disrupted VLPs, may obviate this need. The use of neutralization assays on a large scale is impractical, but it would be beneficial to perform them on subsets in order to make comparisons with ELISA results. A discussion was held on about whether VLPs derived from both L1 and L2 should be used, rather than from L1 alone, in order to mimic real virions more accurately. Collaborative studies should proceed immediately

on identifying suitable vaccine sera in order to facilitate comparison of the available ELISAs and to use them as "run control". An action group coordinated by Dr D. Lowy and Dr J. Robertson was formed to examine these issues in detail.

Conclusions and recommendations from the plenary session on cell-mediates immune assays

It was acknowledged that the standardization of these assays would be premature at the present time. However, it is appropriate to make a preliminary identification of suitable assays. Assays that might be most useful include in vivo assessments of DTH (although there is concern that because the vaccine is immunogenic without an adjuvant, the use of the immunogen as a skin test reagent could be immunizing), LPA, and intracellular cytokine fluorescence (if the laboratories have access to FACS). Tetramer assays need further development. In order to facilitate studies of cell-mediated immunity, it is necessary to ensure that optimally frozen samples of sufficient quality and quantity are used in the conduct of vaccine trials. Consideration should be given to what would serve as appropriate controls, including: reagents generated from healthy HPV-negative individuals; influenza antigens; and/or a "memory mix" of, for example, tetanus, *Candida* and tuberculosis antigens. HPV antigens, whether peptides or recombinant VLPs, should be standardized. It might also be useful to generate T-cell clones or hybridomas against specific genotypes as positive controls. It would be important to exchange antigens and controls for assay comparison. Participants were asked to provide descriptions of current state-of-the-art assays. It was agreed that both arms of immune response should be explored, since no correlate of protection has yet been identified. Furthermore, CMI assays could never be validated as surrogate markers if not measured in the context of vaccine efficacy trials. An inventory and descriptions of available assays would be the first step towards the eventual standardization of CMI assays. An action group headed by Dr C. Melief and Dr S. Garland was formed to consider these matters.

It was recommended that a follow-up meeting be held after an interval of a year in order to examine the advances made in the field of diagnostic tests and to review the efforts undertaken by the action groups formed at the present meeting.

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Annex 2: Agenda

First day

08:30-08:40	Welcome	Dr H. zur Hausen
08:40-08:50	Opening remarks: accelerating HPV vaccine development	Dr T. Aguado Dr S. Franceschi
08:50–09:00	WHO international standards	Dr J.S. Robertson
09:00–09:30	Opening lecture: Lessons from Guanacaste	Dr A. Hildesheim

Session 1: HPV DNA detection assays

09:30-09:50	HPV typing by PCR: an update	Dr C. Meijer
09:50-10:10	Hybrid capture	Dr C. Clavel
10:10-10:30	*Coffee break*	
10:30-10:50	HPV detection in routine diagnosis	Dr R. Sahli
10:50-11:10	One-step PCR	Dr L. Villa
11:10-11:30	Reverse line blot assay	Dr J. Kornegay
11:30-11:50	Accurate HPV genotyping for HPV vaccine research	Dr W. Quint
11:50-13:00	Discussion and drafting of recommendations	Dr E-M. de Villiers and Dr C.M. Nübling
13:00-14:00	*Lunch*	

Session 2: Antibody assays

14:00-14:20	Serological and PCR-based assays to follow HPV vaccine trials	Dr K. Jansen
14:20-14:40	Serological correlates of HPV infections	Dr J. Dillner
14:40-15:00	Cross-reactivity issues in HPV assays	Dr P. Coursaget
15:00-15:20	Oral and cervical mucosa antibodies	Dr A-L. Williamson
15:20-15:50	IgG and IgA levels in mucosal secretions	Dr D. Nardelli
15:50-16:10	*Coffee break*	

16:10-16:30	Neutralization and ELISA assays for HPV vaccine trials	Dr J. Schiller
16:30-18:00	Discussion and drafting of recommendations	Dr D. Lowy and Dr J.S. Robertson
19:30	*Dinner*	

Second day

Session 3: Cell-mediated immunity assays

08:30-08:50	T-cell immunity against tumours, a delicate balancing act	Dr C. Melief
08:50-09:10	Delayed-type hypersensitivity assay	Dr R. Höpfl
09:10-09:30	Monitoring T-cell responses	Dr C. Wheeler
09:30-09:50	Amplification of papillomavirus oncogene transcripts	Dr M. Knebel
09:50-10:10	Immune response to chimeric virus-like particles	Dr L. Gissmann
10:10-10:30	Coffee break	
10:30-11:30	Discussion and drafting of recommendations	Dr C. Melief and Dr S. Garland
11:30-12:00	Closing lecture: Perspectives in HPV vaccines	Dr H. zur Hausen
12:00-13:00	Overall discussion and recommendations	Dr X. Bosch and Dr R. Sheets