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Conference Report

Vaccinating women against premature death Summary of an International Workshop, Helsinki, Finland, 10.01.2000

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At the beginning of the new millenium (Jan 10, 2000) possibilities for preventing human papillomavirus (HPV) related cancer morbidity and mortality, particularly among women, were evaluated in a one day workshop in Helsinki, Finland. The workshop comprised an introductary keynote address and four sessions: **1.** Epidemiology of life-threatening sexually transmitted infections, **2.** Prerequisites for HPV vaccination, **3.** Strategic initiatives in HPV vaccination studies and **4.** Structured discussion.

First, *Professor Harald zur Hausen* (German Cancer Research Center, Heidelberg, Germany) emphasized the ubiquity of most human tumor viruses (e.g. HPV, hepatitis B and C viruses, HBV and HCV, and Epstein-Barr virus, EBV). Following a period of latency up to 30–60 years, a small percentage of infected individuals present with monoclonal tumors harbouring the viral DNA. Ten to 25% of all cancers in affluent and developing countries, respectively, can be linked to infections.

A survey of HPV pathobiology at five stages of cervical lesions highlighted: (A) High multiplicity of input virus required for the establishment of subclinical infection in the basal cells; (B) Cell-growth stimulation following up-regulation of viral gene expression/amplification of HPV DNA, which takes place in squamous intraepithelial lesions (SIL); (C) Inefficient immunological removal of the immortalized cells that results in persistent SIL; (D) Modification of various host cell functions (e.g. anti-oncogenes, cytokines) by the viral oncoproteins E6 and E7 often following integration of high risk (hr) HPV DNA, which in itself, however, is not a necessary event for development of invasive cervical cancer (ICC); and (E) Genomic instability, originally due to uncontrolled expression of the viral oncogenes (and the binding of E6 to p53), that leads eventually to metastatic cancer.

HPV DNA is found in 95%, 70% and 50% of cervical, anal, and vulvar/vaginal cancers. Skin cancer, basal cell carcinoma, oropharyngeal and esophageal cancer also show 70–80%, 50%, 20% and 10–20% HPV DNA prevalences, respectively. In addition, emerging new viral types are expected to increase the number of HPVs beyond the 85 that are presently identified. Elaboration of the association between cutaneous HPVs and the different skin cancers will be of special importance.

Opening the *1st session Dr. King Holmes* (University of Washington, Seattle, USA) considered lessons learned from the human immunodeficiency virus (HIV) epidemic. The fact that HIV mutates 100 million times faster than the human genome set the stage both for the epidemic spread of the virus and the difficulty in developing preventive measures. Worldwide cumulative morbidity and mortality by HIV/AIDS are 33.6 million and

Abbreviations: CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T cell; CVLP, chimeric virus like particle; DC, dendritic cell; E, early; GMT, geometric mean titre; hrHPV, high risk human papillomavirus; ICC, invasive cervical cancer; IL, interleukin; L, late; LCR, long control region; OR, odds ratio; Ro, basic reproductive number; Rt, effective reproductive number; SCC, squamous cell carcinoma; SIL, squamous intraepithelial lesion; STD, sexually transmitted disease; Th cell, T helper cell; TNF, tumor necrosis factor; VE, vaccine efficacy; VIN, vulvar intraepithelial neoplasia; VLP, virus like particle.

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16 million people, respectively. In Africa, since 1980, HIV prevalence has increased from very low to 20% following a wave-like evolution from high risk groups through bridge populations to the general population and to infants.

A paradigm shift from assessing attributable risk fractions of causes for non-communicable diseases, to mathematical modelling of communicable disease dynamics has increased use of community-randomized trials. For example STD interventions (characterized by reduction of unprotected sex from 50 to 37% and increase of condom use from 30 to 47%) resulted in 40% reduction in HIV seroconversion rate in Mwanza, Kenya. Even though early interventions among the infected and the uninfected have an impact on HIV prevalence, they are not widely adopted. This is due to the fact that: (A) Information relating to possibilities for therapeutic intervention is more attractive than that for prevention; (B) Immediate gains from therapeutic advances are preferred to delayed gains in prevention; (C) The benefit of sexual counselling is brief; and (D) The horizons for prevention are too limited. With regard to HPV transmission, the impacts of stage of the disease, anatomical factors (circumcision or cervical ectopy), barrier contraception, presence of other STDs, and the required inoculum size are open. An effective vaccine seems a very desirable means to prevent cancers caused by HPVs.

Dr. Aaron Halpern (Univ. of New Mexico, Los Alamos, USA) compared evolutionary potentials of HPV and HIV. Differences in mutation rates (HPV:10^{-9}/site/y/HIV:10^{-2}/site/y); intrapatient evolution (HPV:undetectable/HIV:yes); genotype (HPV:conserved/HIV:no); and zoonotic origin (HPV:5×10^6 years ago/HIV:recent) defy generalizations. Diversity is large in both virus families, which have experienced periods of explosive diversification and show mixing of host species in their virus trees.

In contrast, the low (2%) worldwide diversity of HPV subtypes suggests a population bottleneck. Small size (10,000) of total human population 100,000 years ago would have increased competition of similar viruses leaving but one representative of each type. This would implicate relative immunological independence of different HPV types, but the corresponding mutation rate of 10^{-7}/site/y in the increasing population suggests a possible change from intertype to between-type substitution during the last 100 000 years. Most, if not all, evolution within HPV subtypes is due to point mutations. There are no clear cut data for indles or recombination for HPV, for gain/loss of genes, or for rearrangements. This reveals low potential for virus escape from immune control. Vaccination against protective HPV types could facilitate emergence of hrHPVs if vaccine protects more narrowly than does natural immunity. This possibility should be looked for in field trials.

Dr. Joakim Dillner (Karolinska Institute, Stockholm, Sweden, and University of Tampere, Finland) assessed the correlation between HPV infections and cervical neoplasia incidence trends. Finland has been most successful in reducing cervical cancer morbidity from 15 to 2.8×10^5 women, and mortality from 6 to 1.6×10^5 women, by organized screening between 1963–90. During 1991–95, however, the incidence of cervical cancer doubled among fertile-aged Finnish women. Though there have been few changes in screening coverage or compliance, alterations of risk and screening practices might explain the increase. Smoking, which is presently considered an independent risk factor for cervical cancer, increased among young Finnish females during the early 1980's but not after the late 1980's. Between 1968–1991 up to 10-fold increase of HPV16 seroprevalence was observed in middle-aged Finnish women. Both in Finland and Sweden, the bulk of the increase in HPV16 (and in herpes simplex virus type 2, HSV-2) seroprevalence (a measure of cumulative exposure) had already taken place during the 1970's. Corresponding increases in cervical cancer precursor (moderate/severe dysplasia) and carcinoma in situ incidences have been ongoing since the 1970s among the youngest age-groups (25–34 years). Finally, replacement of cotton swabs by the cytobrush in intracervical sampling may coincide with the increase in the determination of ICC incidence. In summary, the more than 10 years previous increase in background prevalence of cervical cancer risk factors has, partially at least, contributed to the rapid increase of ICC in Finland.

Opening the *2nd session Dr. Peter Stern* (Paterson Institute for Cancer Research, Manchester, UK) reviewed what is known about immune response to HPV vaccines, and prospects for clinical trials. Of the two models based on the interplay of the innate and adaptive immune systems, i.e. self: non-self and response to danger signals, the latter is the more relevant to HPV infection, since it occurs in the absence of any apparent damage. Following recognition of alterations in the host at the epithelial borders, cells of the innate immune system (monocytes/macrophages, dendritic (DC) and NK cells) produce effector molecules (e.g. interferon (IFN)-$\alpha\beta$, IFNγ, TNFα, and the interleukins-1,-6,-10,-12,-15), the signalling pathways of which may be blocked (IFNα) or downregulated (TNFα) by HPV E6/E7 oncoproteins, or otherwise utilized (IL-10) by the virus. In the adaptive immune response, activated antigen presenting cells (APC) express costimulatory molecules (CD80/B7) necessary for activation of T cells. In the draining lymph nodes, polarization of T helper (Th1/Th2) cell response may be skewed (by the first experiences of tissue DC of the oncogenic HPVs)

away from the Th1 response to the Th2 response. First longitudinal studies suggest that cervical intraepithelial neoplasia (CIN) patients can mount E2 and E7 specific cytotoxic T cell (CTL) response and clearance of HPV infected epithelial cells. It is anticipated that studies involving use of TA-HPV, a live recombinant vaccine expressing HPV 16/18 E6/E7 proteins, will show therapeutic effectiveness against vulvar intraepithelial neoplasia (VIN). By contrast, neutralizing antibodies are directed against viral L1 (and L2) capsid proteins, reduce the viral load, and are readily induced by virus-like-particles (VLPs). Chimeric VLPs, containing both L and E antigens, should prevent both the initial infection and induce CTL response against the early proteins to eliminate the infected cells, and are reasonably, the most likely approach to an effective vaccine.

Dr. John Schiller (National Cancer Institute, Bethesda, USA) presented the findings in a placebo-controlled, dose-escalating trial in 72 individuals (mean age 24 years, ≤ 4 life time sex partners, LTSP) immunized at 0, 1 and 4 months with an HPV16 VLP vaccine produced in a baculovirus expression system according to GMP standards. All who received VLP, VLP in Alum or VLP in a proprietary adjuvant MF59 seroconverted 1 month after the last booster was administered. Neutralizing antibodies were measured both by a specially designed HPV16 pseudovirion assay and by the standard VLP ELISA, that is a surrogate for measuring neutralizing antibody. The highest geometric mean titers (GMT, 1/10 000) of neutralizing antibodies were obtained in persons given the largest dose (50 μg) of antigen formulated without an adjuvant. Only small individual variation in antibody response was noted. In animal experiments, comparable serum titers have given protection against a challenge of 10^{10}–10^{11} virions. If the entry of HPV into the basal cervical cells requires cervical microtrauma, then concomitantly exudated serum IgG antibodies probably can neutralize a very high viral load. Reactions to the vaccine were not significant. In an ongoing phase II trial using three 50 μg doses of vaccine without adjuvant, 150 vaccinees displayed no important adverse side effects. Three alternatives to conventional i.m. immunization with plain VLPs are being investigated. These are: (1) Nasal/intrabronchial immunization to generate secretory IgA; (2) Chimeric (C)VLP in which part of truncated HPV16 L2 protein is substituted by mutated (inactivated) E7 and (parts of) E2 proteins for purpose of eliminating infected suprabasal/basal cells expressing these proteins by specific CTLs; (3) Generation of cross-neutralizing antibodies of high-titer against the L2 protein engendered by use of a non-VLP vaccine.

Dr. Lutz Gissmann (German Cancer Research Center, Heidelberg, Germany) described work with HPV16 CVLPs constructed by fusing HPV16 E7 sequences into the C-terminus of the L1 gene. Supplementing hemagglutination inhibition, a versatile assay to measure neutralization of yeast cell-derived pseudovirions carrying a green fluorescence reporter gene has now been developed. This surrogate assay measures neutralizing HPV16 antibodies that are elicited by the HPV16 CVLPs.

Mice immunized with the HPV16 CVLPs generate E7-specific CTLs, which kill E7 expressing or E7 peptide loaded, MHC-expression destabilized RMA-cells, protect against tumour formation by syngeneic HPV transformed cells and they also induce regression of already established tumours. As an alternative to multivalent (e.g. HPV16, 18 and 45) CVLPs replacement of the E7 protein with more broadly cross-reactive E1 protein is being attempted at this time.

Utilization of a DNA vaccine in the HPV context, has been hampered by concern for the oncogenic potential of the binding proteins encoded by the E6 and E7 genes that are expressed. However, generation of CTL response is achieved by presentation of epitopes as short peptides (e.g. E7a, E7b, E7c, E7d) in MHC class I molecules, and proteins are not required. Instead, a shuffled E7 protein (c, d, b, a and the breakpoints a/b, b/c, c/d) has now been used succesfully for generating comparable CTL responses in mice. Further studies are needed to validate that the shuffled protein lacks oncogenic activity.

Professor Jorma Paavonen (University of Helsinki, Finland) evaluated the prerequisite knowledge needed for initiation of efficacy trials. A recent meta-analysis of longitudinal studies showed that up to 44% of cervical cancer is caused by HPV16, suggesting that up to 44% of cervical cancer might be prevented by use of such vaccine. Changing risk taking behavior (RTB) in Finnish women from 1972 to 1991 (doubling of LTSPs, 1.6 years decrease in age at first intercourse) has given rise to emergence of new oncogenic HPV types (other than 16, 18 or 33) in 20% of cervical cancer cases, and has led to doubling of cervical cancer incidence in pregnable women during the 1990s.

HPV16 and combined CIN grade 3 and cervical cancer (CIN3 +) attack rate data in a population of 51,971 fertile women in Helsinki (two pregnancies before 25 years of age) showed an annual attack rate for HPV16 (seroconversion confirmed) infection and 0.3% 10-year cumulative rate for CIN3 + lesions. Corresponding sample size estimates ($\alpha = 0.05$, $1 - \beta = 80\%$) for placebo-controlled trials with 70% vaccine efficacy (VE) and 5 and 10 years of follow-up were 898 and 15,000 vaccinees, respectively. Feasibility study in a subsample (233 women) of the same population predicted 82% compliance and 69% retention of the subjects for a one year follow-up.

The 3rd session was opened by *Dr. Geoff Garnett* (University of Oxford, UK), who defined the basic reproductive ratio of an STD: $R_o = \beta Dc$ (where β is the probability of transmission in a sexual partnership, D is duration of infectiousness, and c is the number of new sex partners that the infected person has in a unit of time) which can be used as a measure of the reproductive potential of a micro-organism. The R_o applies to naive populations, and control measures, e.g. of: (β by condom use, D by targeted treatment, and c by counselling. Vaccination which removes people from the susceptible class to the immune class requires definition of the effective reproductive number: $R_t < R_o$. Considering the determinants of the R_t in the vaccination context; as for reduction of the endemic prevalence of any STD, even a high $R_t(\beta)$ requires effective vaccination in relatively low proportions of the general population (and vice versa). Eradication from the STD core group(s) is not possible without complete coverage. Short D and long duration of immunity result in low endemic prevalence of an STD. Persistent/recurring infections prolong the length of time required for eradication of the infection. Heterogeneity of RTB results in high c in a minority, and low c in the majority of the general population. The duration of RTB and desired duration of the vaccine-induced immunity have an inverse relationship.

Vaccinating women will start to affect men when the R_t approaches 1. An additive effect may be expected for vaccination and other STD control measures. The impact of cross-immunity and/or non-immune competition on population sizes of HPV types deserves attention. Removal of one oncogenic type will increase incidence of cervical cancer by other oncogenic types if infections with the different types overlap, and if the removed type had an earlier onset of the disease.

Professor Finn Egil Skjeldestad (University of Trondheim, Norway) started his presentation by describing the organized mass-screening campaign for cervical cancer that was begun in 1995 in Norway. Women aged 25–70 years are being screened at 3 year intervals, and data on morphology, topography, sampling of all smears, biopsies and other pertinent materials identifiable by personal identification number are being collected at the National Mass Screening Unit of the Cancer Registry of Norway. Follow-up of a cohort of 22,900 women born 1970–1974 and with at least one normal Pap smear at the baseline yielded an annual incidence rate of CIN2/CIN3 (cytological diagnosis) of 0.4%, and a cumulative incidence of 1.8% at the end of the follow-up. Effective vaccination would reduce considerably the number of unnecessary referrals since less than 50% of the cytological diagnoses could be histologically confirmed.

Since September 1998 a prospective follow-up of 16–23 year old women has been ongoing at 15 GP-clinics in Trondheim with purpose to provide estimates of incidence of CIN2/3 and type-specific infection. It also serves as a feasibility study for enrollment and follow-up in evaluations of vaccinees. By the end of 1999 more than 400 women (mean age 21 years) had been enrolled. Two-hundred eighty six and 140 women completed 6 and 12 month follow-ups with retention of 95% of subjects at 6 months. Findings of mean age at the first intercourse, 17 years; mean number of LTSP, 5; and number of sexual partners during the last 6 months, <1 all, are consistent with what is known of the Norwegian female population. Overall HPV PCR prevalences at the baseline were HPV6 16%, HPV16 15% and HPV18 7%. Clear-cut correlation of LTSP with PCR positivities was revealed. Altogether the study will enroll 875 women.

Dr. Nubia Munoz (International Agency on Research on Cancer, Lyon, France) reviewed the IARC prevalence/case-control studies conducted in up to 22 countries. Overall, 99.7% of 1000 cases with histologically confirmed ICC were also shown to be HPV DNA positive using the GP5+/GP6+ or E7 primers. Comparisons of the most prevalent HPV types in the IBSCC study and 1622 squamous cell carcinoma (SCC) cases of a multi-centre case-control study (in Brazil, Columbia, Mali, Morocco, Paraguay, Peru, Philippines, Spain, Thailand) showed little difference for HPV16 (53%/59%), 18 (15%/12%), 45 (9%/4%), 31 (6%/4%), 33 (3%/2%). However, there were some geographic variations in positivity for types 31, 33 and 45. In the multi-centre study, with altogether 4000 cases and controls, positivity for any HPV DNA yielded a pooled odds ratio (OR) of 60.The association was equally strong for both squamous cell (OR = 62) and adenocarcinoma (OR = 51). All the following HPV types:16 (OR = 129), 18 (104), 31 (40), 33 (42), 35 (29), 39 (∞), 45 (47), 51 (25), 52 (81), 56 (13), 58 (36), 59 (204) now can be considered as carcinogenic, with HPV as the necessary cause for cervical cancer. Studies of HPV prevalences in random, age-stratified (by 5 years, 15–19 to 65+) subsamples (1100 women) of the general population, measured as two age-peaks (<25 years and >59 years), have been found in some countries (Costa Rica, Mexico, Colombia) but not in all (Argentina). Whether the second peak is due to viral reactivation, variations in screening, or represents a birth-cohort effect remains to be determined. The distribution of the most prevalent HPV types in the general population (HPV16, 18, 45, 31, 58, 33, 35, 6) resembles that for cervical cancer cases.

Dr. Allan Hildesheim (National Cancer Institute, Bethesda, USA) underlined the need to reduce inci-

dence and mortality from cervical cancer as the motivating force for the development of a vaccine against HPV. He evaluated five characteristics of a phase III trial: site, population, method, outcome, and follow-up. Known rates of HPV infections and associated diseases, high ICC rate, and available infrastructure for conduct of the study are important for defining the trial site. Choosing the trial population should maximize both the percentage of unexposed women and the percentage of women at risk, and minimize the time to HPV exposure within a stable population. There is no alternative to a randomized and blinded trial, which, with appropriate design, can also provide head to head comparisons of different vaccines. Loss to follow-up should be minimized in order to avoid post-randomization bias. Since the low grade squamous intraepithelial lesion (LSIL) is a precursor of ICC, it can serve as a reliable clinical surrogate end-point that avoids the ambiguity which a persisting virological end-point gives. The length of active follow-up is determined based on population, outcome, sample size, and ethics.

A tentative plan was presented for a randomized three-arm trial in Guantacaste, Costa Rica among more than 10 000 young women, who are vaccinated with placebo, HPV16 VLP, or CVLP at 0, 1 and 6 months. A preliminary follow up would consist of 6–12-month visits for 4 years, with tests for HPV16+ LSIL and persistence of HPV16. Such study has a 90% power to define a VE of 50%.

Dr. Matti Lehtinen (National Public Health Institute, Helsinki, Finland) considered utilization of the Baltic/Nordic health care infrastructures for phase IV trial(s) employing putatively licensable HPV vaccine(s).

Mass screening for cervical cancer has not been implemented in Estonia (or other Baltic countries), eventhough there is a population-based cancer registry that was established in 1960. Vaccination of three female birth cohorts (e.g. 1986, 88, 89) of Estonia (21 000 HPV hr negative women) by 2005 would suffice for demonstration of 70% VE against cervical cancer in 15 years of passive/registry follow-up ($\alpha = 0.05, \beta - 1 =$ 80%). Future implementation of mass screening would probably shorten the required follow-up time, but the effects of vaccination on spread of the HPVs in the population would need to be monitored.

The country-wide population/health/cancer registration in a stable population of 25 million and comparable mass screening for cervical cancer, makes the Nordic countries a unique venue for evaluation of different cervical cancer control measures. Extension of trials that use surrogate end-points is vital to avoid confounding the data that might result from free supply of licensed preventive or preventive/therapeutic vaccines. Randomization of 16 year old women into the vaccination (vaccine and placebo) and reference cohorts for purpose of extending the clinical trial by registry follow-up gives added assurance for comparability among these cohorts, which undergo similar/conventional Pap screening. Enrollment of $2 \times 6600 + 20\,000$ hrHPV negative women would enable demonstration of 70% VE against ICC in 20 years, and, also, the comparability of new screening methods.

Dr. Franco Buonaguro (National Tumor Institute, Naples, Italy) described Ugandan feasibility studies based on a long lasting collaboration between the National Tumor Institute and the Zambia Hospital in Kampala. Overall, high prevalences of HPV16 of 40 and 45% were found in a sample of HIV-negative and HIV-positive women. Only a modest number of HPV types, other than HPV16, were found among 173 pregnant women, who attended the outpatient clinic of the hospital. HIV-positive women tended, also, to be coinfected with HPV. In a region close to Zaire, where the incidence of penile carcinoma is very high, LCR/E6 sequence analyses of HPV16 types identified a new HPV16 variant (designated Af1U) in 4 of 5 of penile carcinoma cases. The distribution of this sub-type of HPV16 in the Ugandan female populations of different geographic areas is being studied.

The *4th session* was opened by *Dr. Maurice Hilleman* (Merck Research Laboratories, West Point, USA) who defined vaccinology as the science of vaccines, which engages immunology, microbiology and molecular biology. Cells of the innate immune system, e.g. macrophages and NK cells, recognize invariant patterns of microbial substances and evoke direct host defenses. They also activate and condition the adaptive, antigen-specific immune response of B lymphocytes, T helper cells, and cytotoxic T cells which follow. Development of successful vaccines relies on understanding this interplay of immune mechanisms.

Of the 17 licenced viral vaccines in routine use in the USA the only subunit recombinant expressed vaccine is that of HBV. As reported for 1998 by the US National Institutes of Health, 140 of 211 viral vaccine probes that had been taken to clinical testing were of subunit composition comprising recombinant expressed proteins, live recombinant microbial vectors, and DNA plasmid vectors. This may represent an overemphasis of one particular vaccine approach. Targeting of the vectors for uptake by and stimulation of e.g. dendritic cells, and the use of transgenic plants, if they are able to engage the mucosal immune system, are theoretically promising future endeavours. The field of adjuvants and adjuvantation requires focused efforts to generate practical formulations. Knowledge of *what* antigens to present in addition to the *how* to present them needs to be expanded.

Since sterilizing immunity may never be achieved by any vaccine against any virus solely by induction of

neutralizing antibodies to the L1 and L2 proteins may be necessary but may not be sufficient for preventive HPV vaccination. Cytotoxic T cells active against the E6, E7 and other oncoproteins expressed in the basal epithelial layers may be essential to eradication of virus from the host by cell mediated immune mechanisms.

The *structured discussion* and the workshop proceedings will be published in Journal of Clinical Virology.

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