Meeting report

ABSTRACT

In October 2007, a joint ANRS-NIH workshop was held on "Mucosal immunity and HIV/AIDS vaccines" in Veyrier-du-Lac, France. Goal of the meeting was to discuss recent developments in the understanding of viral entry and dissemination at mucosal surfaces, rationale for designing vaccines to elicit mucosal immune responses by various routes of immunization, and the types of immune responses elicited. Lessons were drawn from existing vaccines against viral mucosal infections, from the recent failure of the Merck Ad5/HIV vaccine and from attempts at mucosal immunization against SIV. This report summarizes the main concepts and conclusions that came out of the meeting.

Mucosal immunity and HIV/AIDS vaccines
Report of an International Workshop, 28–30 October 2007

1. Introduction

The mucosal immune system, especially in the genital and gastrointestinal tract, is an important target for both the HIV infection and development of HIV vaccines. Excluding parenteral transmission, virtually all HIV-1 infections are acquired across a mucosal surface such as the anorectal mucosa, vaginal mucosa or, less frequently, the oral mucosa. The mucosal immune system, particularly the mucosa-associated lymphoid tissues (MALT) in the genital tract and the gut-associated lymphoid tissues (GALTs), represent the most important target for HIV-1 replication, reservoir establishment and lymphoid tissue destruction [1]. Prospects for designing HIV-1 vaccines to elicit protective mucosal immunity against HIV at site of infection were discussed at the joint ANRS-NIH meeting on 28–30 October 2007 in Veyrier du Lac, France, in light of state-of-the-art knowledge on the mucosal immune system and its specific characteristics, the biology of virus entry and lessons to be learnt from vaccines developed against other human mucosal infections. Participants of this timely meeting were under the impact of the recently announced interruption of the STEP and Phambili trials of the Merck Adenovirus (Ad5)/HIV vaccine, and were eager to discuss concepts and methodological issues in the field of HIV-1 vaccinology.

2. Mucosal immune system

The organization of the mucosa-associated immune system and its early involvement in HIV infection [2] were reviewed in depth by Jan Holmgren (University of Göteborg, Göteborg, Sweden), who pointed out that, contrary to a generally accepted belief, the commonality of the mucosal immune system is at best a gross overestimation. Thus, immunization by the oral route induces mucosal immunity both in the upper part of the intestine and the mammary glands, but not in the lower part of the gut; immunization by the nasal route induces a mucosal immune response both in the respiratory tract and the female genital tract; immunization by the rectal route only induces mucosal immunity in the rectum and lower part of the gut; and immunization by the vaginal route only induces mucosal immunity in the vagina. Holmgren and colleagues have been investigating the potential of sublingual (s.l.) immunization for induction of mucosal immune responses. They have shown that s.l. administration of a prototype antigen together with cholera toxin adjuvant induces a broad range of immune responses in lung tissues and in systemic organs [3]. More recently they have demonstrated responses in the respiratory, gastrointestinal and genital tracts as well as systemically following s.l. immunization.

Several registered vaccines are to be administered by the oral route, such as live oral polio, inactivated oral cholera, live oral typhoid fever, and rotavirus VLPs. Live vaccines must achieve a delicate balance between over-attenuation and under-attenuation to be effective yet safe. In addition, the oral vaccines may be less immunogenic in tropical countries due to a variety of reasons, from higher prevalence of intestinal helminth infections, vitamin A and Zn^{2+} deficiencies impairing gut restoration, to possible immune exhaustion by intestinal flora. Non-live mucosal vaccines require an appropriate adjuvant for eliciting strong immune responses. This is still a major hurdle as Cholera toxin (CT) and *E. coli* heat-labile toxin (LT) are the two most potent mucosal adjuvants that help induce T cell responses, but they remain too toxic to be used as such in human vaccines. Efforts are being made to engineer less toxic forms of these adjuvants. CpG linked to CTB subunit has shown promise and induces good chemokine responses. Fabienne Anjuère (University of Nice, Nice, France) also reported that several approaches are currently in progress to induce mucosal CTL responses against a protein antigen by topical, intravaginal or sublingual administration, using the B subunit of CT (CTB) co-linked to the protein antigen and to a CpG oligodeoxynucleotide [4].

3. Functional architecture of the mucosa-associated immune system

Lymphocyte trafficking patterns, regulated by the selective expression of adhesion molecules in peripheral or mucosal lymphatic tissues, permit the segregation of immunological memory by causing antigen-primed B and T cells to return to specific anatomic

destinations committed to exhibiting peripheral or mucosal immunity. Tissue specificity is under the control of chemokines and homing receptors present in the mucosal tissue. For instance, as reported by Rodrigo Mora (Mass General Hospital, Boston, MA, USA), T cells are instructed to express gut-homing receptors integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 when they are activated by dendritic cells (DCs) from the GALT (gut DCs) but not by DCs from other tissues. The ability of gut DCs to imprint gut-tropism on T cells (and B cells) lies in their ability to produce and store retinoic acid (RA), a vitamin A metabolite [5,6]. Simple stimulation of T cells with an anti-CD3 antibody in the presence of RA can induce the expression of gut-homing receptors. The ability of gut DCs to secrete RA is probably a consequence of their intimate contact with intestinal epithelial cells, which also can metabolize vitamin A into RA. Interestingly, RA also synergizes with IL-6 or IL-5 to induce IgA-ASC. Thus GALT-DC and RA shape gut mucosal immunity.

Maria Rescigno, from the European Institute of Oncology in Milan (Italy), emphasized the importance of cross talk between DC and epithelial cells for the mucosal homeostasis. The intestinal epithelial cells are tightly integrated into the mucosal immune system and most DCs in mucosal tissues are educated by the surrounding epithelial cells to suppress inflammation and promote immunological tolerance. The uneducated DCs, which are recruited from Peyer's patches and blood in response to a pathogen's attack, initiate inflammation and trigger an active immune response to the invader.

The mucosal immune system therefore has the difficult task of maintaining the fragile balance between the activation of specific immune responses to fight harmful pathogens and their suppression to avoid undesired immune reactions to self or to harmless antigens such as those usually present in the intestinal lumen [7].

Dominique Kaiserlian (CERVI, Lyon, France) further discussed how mucosal and skin DC induce T cell immunity or tolerance and can be different in their functional properties based upon lineage and tissue localization. Contrary to general belief, resident epithelial tissue DCs, including LCs, are not responsive for T cell priming and in fact induce peripheral tolerance. Only DCs newly recruited into muco-cutaneous tissues by danger signals provided by pathogens or adjuvants directly induce priming and differentiation of CD8^{+} T cells into cytolytic effector T cells *in vivo*. Recent advancements in knowledge of the dynamics and function of epithelial tissue DCs have provided the basis for the screening of novel T cell adjuvants like DNFB, the measles virus NP protein, CpG oligodeoxynucleotides, flagellin and cholera toxin, that can elicit CD8^{+} T cell cross-priming in the absence of CD4^{+} T cell help by increasing CCL20 production in epithelial cells and promote CCR6-dependent recruitment of DC into epithelia [8].

Per Brandtzaeg from the University of Oslo (Oslo, Norway), who has extensively studied mucosal immunity in various immunodeficiencies, described how IgAs are translocated into the gut lumen and onto mucosal surfaces of the trachea, bronchi and genital tract by an IgA pump, whereas IgG leaks out from blood vessels but stays inside tissue parenchyma, including the lungs [9]. Knockout mice that lack both secretory IgA (SIgA) and SIgM exhibit decreased resistance to cholera toxin and pathogen colonization in the gut, but show a compensatory increase in the number and cytotoxic activity of intraepithelial lymphocytes (IELs), which are the cellular arm of mucosal immunity. IELs are not only involved in anti-microbial defence but also are major effectors of oral tolerance [10,11] through the secretion of IL-10 and TGF-β that maintain the state of tolerance. Of note is the fact that mucosal CD4^{+} CD25^{high} Foxp3^{+} T$_{reg}$ cells are significantly increased in number in the intestinal mucosa of HIV-1-infected patients, who also show elevated intestinal IgA levels [12]. Both parameters levels become normalized after initiation of antiretroviral therapy. As discussed by Thomas Schneider (La

Charité, Berlin, Germany), this probably reflects the involvement of mucosal homeostatic mechanisms that operate in the gut of AIDS patients in response to opportunistic infections.

4. The early events in HIV infection

Mucosal transmission of HIV-1 infection is mediated by exposure to infectious virus particles and probably also to infected cells within mucosal secretions [13]. Transmission is critically dependent upon efficient transfer of infectious virus particles across the mucosal epithelium, providing access for the virus to reach intra- or sub-epithelial DCs, macrophages and/or T cells that express the CD4 and CCR5 viral co-receptors.

Multiple mechanisms for mucosal HIV-1 transmission have been proposed including direct HIV-1 infection of epithelial cells; transcytosis of HIV-1 through epithelial cells and/or specialized M cells [14–17]; epithelial transmigration of HIV-1-infected donor cells; uptake of HIV-1 by intra-epithelial Langerhans cells (LCs) [18] and dendritic cells (DCs); or entry via epithelial microabrasions or ulcerations. The architecture of the mucosal epithelium actually dictates the mechanisms of HIV transmission. M cell transfer and transcytosis are the main routes of HIV penetration across the simple columnar epithelium that lines the endocervix, rectum and gastro-intestinal tract [14–17]. Importantly, mucosal epithelial cell transcytosis and tissue entry are substantially inhibited by antibodies such as 2F5, underscoring the potential for vaccine-induced inhibition of mucosal HIV-1 infection, as reported at the meeting by Philip Smith (University of Alabama, Birmingham, AL, USA).

HIV penetrates the first layers of stratified squamous epithelium (oral cavity, oesophagus, anus, vagina and exo-cervix) through breaks in the epithelial barrier. It is then taken up and transported by DCs/LCs through the lower layers of the epithelium. Although male circumcision was reported to reduce male acquisition of HIV by >60%, the mechanisms of HIV transmission via the pluristratified mucosa of the foreskin and associated LCs remain unclear [19,20]. Recent studies suggest active sampling of the luminal virus by LCs, followed by their migration out of the epithelium.

The type of cells initially infected thus depends on the anatomical site of viral entry, and therefore it is very important that this be taken into account for HIV vaccine development. Following cervicovaginal infection, endocervical, ectocervical and vaginal subepithelial CD83^{+} DCs rapidly take up HIV-1 and migrate to regional T-cell areas where they infect CD4^{+} T lymphocytes. Genital macrophages expressing CD4 and CCR5 also rapidly take up HIV, support its replication, and amplify infection of genital CD4^{+} T cells. At the same time, replication of the virus initiates a local innate immune response from the resident DCs, which secrete IFN-α and -β and MIP-1β that are meant to control viral replication. However, that also triggers an inflammatory response which brings in CCR5^{+} CD4^{+} T cells and fuels the expansion and spread of infection both locally and distally.

Philip Smith (University of Alabama, Birmingham, AL, USA) discussed the details of time course of early events during viral entry in the gut and genital tissues. They have established an elegant mucosal explant model to dissect out the sequence of events and the cell types involved. Intestinal mucosa, ECs and DCs seem to take up the virus as early as 15 min after inoculation. He also summarized the differences in tissue architecture and receptor expression between various mucosal sites suggesting different mechanisms of entry at these sites. The intestine, rectum, colon and endocervix are lined with monostratifed columnar epithelial cells, whereas the vagina, ectocervix and anus are lined with squamous epithelium. Intestinal macrophages, contrary to vaginal macrophages, express CD4 but not CCR5 or CXCR4 and do not support infection by or

replication of R5 (or X4) virus. Inability to replicate HIV-1 in these cells is not due exclusively to the lack of second receptor expression [21]. In contrast, CD4$^+$ T lymphocytes in the gut lamina propria that express CCR5 and CXCR4, are the earliest and major targets of HIV (or SIV) infection. This results in a rapid and extensive depletion of memory T cells in the GALT [1,22–24]. Furthermore, analysis of lymphoid and non-lymphoid organs of SIV infected Chinese rhesus macaques infected by SIV for more than 5 years has shown that the mesenteric lymph nodes constitute the main virus reservoir [25].

HIV-1 infection has been shown to rapidly induce enteropathy with bowel inflammation and increased gut permeability. It has been proposed that the disruption of the gut mucosal barrier functions allows for the translocation of microbes and microbial products such as bacterial lipopolysaccharide (LPS) into the body interior, resulting in a chronic systemic immune activation/T cell exhaustion that fuels HIV disease progression [1,26]. Interestingly, as reported by Guido Silvestri (University of Pennsylvania, Philadelphia, PA, USA), SIV infection in its natural hosts (sooty mangabeys, African green monkeys) does not result in systemic CD4$^+$ T-cell depletion in spite of high levels of persistent virus replication. The natural non-pathogenic state also is associated with a rapid depletion of CD4$^+$ T cells in the gut and in the respiratory tract that is similar in magnitude to that observed in pathogenic HIV/SIV infections [27,28]. However, the depletion occurs in the context of limited enteric pathology and does not lead to systemic immune activation. The early depletion of gut CD4$^+$ T cells either recuperates with recruitment of more CD4$^+$ T cells, or achieves a homeostatic balance with a very low level of immune stimulation to maintain a non-pathogenic status in these natural hosts. Of relevance may be the observation that SIV-infected sooty mangabeys keep an intact gut Th17$^+$ CD4$^+$ T cell population, which is known to play a protective role against infections, whereas SIV-infection of rhesus macaques and HIV-1 infection of humans results in depletion of Th17$^+$ CD4$^+$ cells in the GALT. The loss of Th17$^+$ CD4$^+$ T cells in the gut might be the key to the chronic immune activation seen in SIV and HIV infection.

The fact that the non-pathogenic state in natural hosts is reached in the absence of apparent immunological control of virus replication [29] emphasizes the tremendous challenge of artificially inducing, with an AIDS vaccine, a type of protective immunity that has not been selected for despite thousands of years of evolutionary pressure from lentiviruses on the primate immune system [28].

Thomas Hope's (Northwestern University, Chicago, IL, USA) presented studies to elucidate mechanisms for sexual transmission of HIV/SIV in human and simian genital tissue explants by visualizing the movement of photoactivable GFP-labelled virus. These studies demonstrate that the first barrier to virus penetration is cervico-vaginal mucus, which impairs diffusion of the virus into the crypts of the endocervix. Penetrating virions could be observed within 4 h in interstitial spaces between differentiated squamous epithelial cells as well as in the sub-basal milieu of the columnar endocervical epithelium where they co-localized with both LCs and CD4$^+$ T cells. Viral replication in macaques infected by intravaginal inoculation of SIV was also studied by Ashley Haase (University of Minnesota, Minneapolis, MN, USA), who has developed a vaginal infection model to study early events and systemic dissemination of virus and how it exploits the local innate response that brings in susceptible host cells to fuel the expansion and spread of infection locally and distally. He suggested that the mucosal barrier was very effective in limiting initial infection that occurred because of a breach in this protective barrier and was initially limited to only a very small number of local foci in the submucosa. This triggered a local inflammatory response characterized by an influx of CD123$^+$ pDCs that secrete IFN-α, IFN-β and MIP-1β which subsequently signals the influx of CCR5$^+$ T cells which fuel the progressive

expansion and spread of infection. The infection remains localized from a few hours up to 2 days before spreading to draining internal iliac lymph nodes by days 3–5, and to the spleen and other lymph nodes by days 8–12. In rectal infections this may even be shorter because of disrupted epithelial surfaces. Thus, to interrupt HIV infection and expansion, vaccine strategies need to be effective at the earliest stages of mucosal infection and the immune system must be capable of responding rapidly to curtail the establishment of a self-propagating infection [30,31].

5. Preventing mucosal HIV infection and expansion

There was general agreement at the meeting that in order to provide an effective immune protection at relevant mucosal surfaces, preventive HIV vaccines will most certainly need to elicit the production of antibodies such as IgAs at genital mucosal surfaces and in the gut; IgGs that can transudate from the systemic compartment into mucosal tissues; together with cytotoxic T cells that can clear the mucosal infection and prevent systemic virus spread.

5.1. IgA

The adaptive humoral immune defence at mucosal surfaces is, to a large extent, mediated by secretory IgA (SIgA), the predominant immunoglobulin class in human external secretions [9]. It was recognized that neutralizing intestinal SIgA antibodies are a correlate of immunity for enteric virus infections. As reported by Morgane Bomsel (Institut Cochin, Paris, France), mucosal IgA antibodies specific for the HIV-1 envelope have been shown to block mucosal HIV entry *in vitro*. Highly exposed, IgG-seronegative (HESN) women, who have repeated unprotected sex with HIV$^+$ partners but remain seronegative, have been shown to harbour SIgAs specific for conserved determinants of HIV envelope gp41 in their cervicovaginal secretions. These SIgAs can block CD4$^+$ T cell infection *in vitro* as well as transcytosis of the virus through epithelial barriers. A library of IgA monoclonal antibodies was derived from cervical B cells of a HESN woman donor. Screening of the resulting clones provided some monoclonal antibodies that were targeted to the gp41 heptad repeats while others were targeted to the 35 C-terminal amino acid peptide (P1) in the gp41 membrane proximal external region (MPER). Both categories of monoclonal antibodies were able to block HIV-1 transcytosis in a cross-clade fashion, and IgA Fab fragments neutralized R5 clade B HIV-1 isolates in macrophage as well as CD4$^+$ T cell assays. Interestingly, their conversion into IgG Fab was accompanied by loss of neutralizing activity. Conversely, the conversion of the 2F5 IgG monoclonal antibody [32] into an IgA Fab increased its neutralizing potency and conferred the antibody with potent transcytosis-blocking activity.

Although HIV vaccine candidates so far have not elicited mucosal IgA responses because they were either poorly immunogenic, administered parenterally without an appropriate adjuvant, or specifically designed to induce cytotoxic T lymphocytes, it was recommended that measurement of IgA be included in future vaccine/challenge studies in macaque models. However, there are several challenges to obtaining reliable quantitation of mucosal IgAs, as discussed by Pamela Kozlowski (Louisiana State University, New Orleans, LA, USA). IgA measurements often give high background due to high glycosylsation of IgA, which makes these molecules "sticky." The quantification of IgA antibody-secreting cells (ASCs) in biopsy samples of mucosal tissue would be optimal, but is often unpractical. Measuring the proportion of IgA antibody-secreting cells in blood is easier, but does not determine the number of IgA lymphoblasts destined for a specific mucosal site. The analysis of antibody in secretions collected from mucosal surfaces at

which viral exposure most often occurs is more informative. However, collection methods for mucosal secretions clearly need to be standardized. Collection of secretions with an absorbent Weck-Cel sponge seems to be far superior to collection by lavage/wash [33]. There is also a problem with the lack of appropriate SIgA reference standards.

Conventional chromogenic ELISA with recombinant envelope proteins or whole viral lysates can be used successfully for the measurement of virus-specific IgA antibodies in cervicovaginal or rectal secretions of adult macaques and humans, provided an ELISA measurement of total IgA is also included in the analysis so as to determine with certainty whether a secretion contains significant levels of specific SIgAs by expressing results as ng specific IgA per μg total IgA in the sample. These issues need to be considered when designing studies to analyze mucosal IgA responses.

5.2. T lymphocytes

The role of T lymphocytes was discussed in depth throughout the meeting, given the fact that the Adenovirus (Ad5)/HIV Merck vaccine, designed to elicit a strong T cell response, had just been proven ineffective. The nature of protective anti-SIVmac T cell immunity in the genital tract of monkeys immunized with SHIV89.6 was discussed by Meritxell Genescà (University of California, Davis, CA, USA). Vaccination of monkeys with live, attenuated SHIV89.6 provided 60% protection from uncontrolled viral replication after vaginal challenge with pathogenic SIVmac. The protected animals showed little if any IgG or IgA in vaginal secretions but showed polyfunctional Gag-specific CD8$^+$ CTLs in their genital tract.

However, the mere presence of HIV-specific mucosal T cells does not ensure protection against HIV-1 infection and disease. Mucosal T cells were detected *ex vivo* in the semen and in rectal or cervical biopsies from volunteers vaccinated with the Merck Adenovirus (Ad5)/HIV vaccine tested by the HVTN. Even though the vaccine-induced HIV-specific cellular immune responses [34] (as well as SIV-specific cellular responses in monkeys vaccinated with and Ad5/SIV vaccine [35]), it was not effective at preventing HIV infection in high risk volunteers who became infected in spite of vaccination [36]. As discussed by Julie McElrath (University of Washington, Seattle, WA, USA), it still is unclear if the vaccine just failed to provide the quality of T-cell responses necessary to efficiently control virus replication.

Although the Merck vaccine elicited HIV-specific IFN-γ producing T cells, that measure does not indicate whether those cells are capable of efficiently lysing virus-infected cells. Experimental studies of Ad5-SIV recombinant vaccines administered orally or intranasally to monkeys showed that there was no correlation between protection against a SIVmac intrarectal challenge and IFN-γ ELIspot or T-cell proliferative responses in peripheral blood lymphocytes [37]. In the live attenuated SHIV vaccine/SIV vaginal challenge study reported by Meritxell Genescà (see above), all protected macaques were found to have vaginal Gag-specific polyfunctional CD8$^+$ CTLs that secreted two to four cytokines, as well as vaginal CD4$^+$ T cells with polycytokine responses and cytolytic activity [38].

Barbara Shacklett (University of California, Davis, CA, USA) elaborated on the polyfunctionality of HIV-specific T-cell responses in human rectal mucosal T cells. The elite HIV-1 controllers, defined as individuals with less than 75 copies/mL plasma viral RNA in the absence of antiretroviral therapy, were found to have unusually strong and complex mucosal CD8$^+$ T cell responses in rectal biopsy tissues, with 8–12.8% mucosal CD8$^+$ T cells expressing multiple cytokines, chemokines and Granzyme in response to HIV Gag peptides [39]. These studies suggest that the polyfunctional cells are likely to play a major role in immune surveillance of the gut

mucosa. In addition, a study of a French cohort of HIV controllers also showed that a systemic CD8$^+$ T cell population with a particular phenotype is able to suppress viral production in an HLA restricted manner [40]. Jean François Delfraissy (Kremlin Bicêtre, France) commented that one of the elite controllers in the French cohort was super-infected but was able to control the second infection.

Jay Berzofsky (NIH, Bethesda, MD, USA) presented studies in both mice and macaques highlighting the role of CTL avidity and its compartmentalization. The studies demonstrated that high avidity mucosal CTL correlated with delay in viral dissemination from intrarectal challenge site into the blood stream of mucosally immunized macaques. When they compared functional activity of CTL at sites proximal or distal to the site of immunization, intrarectal vs subcutaneous, both routes of immunization in mice induced tetramer binding T cells in the gut mucosa. However, IR immunization was much better at inducing IFN-gamma-secreting cells at that mucosal site, and more DCs and IL-12 producing DCs were found proximal to the site of immunization. Thus the protection correlated with having high avidity functional CTL and not just tetramer-binding cells at the site of viral replication, and high avidity CTLs were best induced by immunization proximal to the site at which protection was needed [41,42].

Studies by Linda Saif (Ohio State University, Wooster, OH, USA) on mucosal immunity to porcine respiratory coronavirus (PRCV) in pig models similarly revealed that PRCV infection of the respiratory tract failed to induce protection at another mucosal site (intestinal tract), confirming functional compartmentalization within the mucosal immune system [43]. This concept is highly relevant to HIV vaccines, reinforcing the need to understand mucosal trafficking pathways that can be used for optimal prime/boost vaccine strategies to induce both antibodies and CD8$^+$ T cells with high avidity and broad anti-viral activities to target and protect the multiple HIV-susceptible mucosal tissues [44].

5.3. Blocking the CCR5 coreceptor to block B-cell tolerance

Lucia Lopalco (San Raffaele Institute, Milan, Italy) reported that a small number of HESN women elicit mucosal antibodies directed against CCR5, the dominant chemokine receptor used by HIV-1. These antibodies appear to downregulate the expression of CCR5 and may prevent HIV-1 entry into target cells [45]. Although the induction of anti-CCR5 antibodies requires breaking T and B cell tolerance, this could be achieved by means of novel vaccine vectors coupled to suitable adjuvant formulations and an appropriate immunization schedule [46]. The study of biological features and the protective role of anti-CCR5 antibodies generated through suitable immunization strategies could be essential in learning how to break immune tolerance and elicit broad HIV-blocking systemic as well as mucosal immunity.

6. Designing HIV vaccines to elicit mucosal immunity

It is widely assumed that an effective HIV vaccine will need to provide both cellular and humoral immune responses. It is also assumed that prevention from infection will require immunity at the site of exposure which in most cases is at a mucosal site. How does one induce such immunity? What are the most effective antigen delivery vehicles? What is the best route of immunization? Is it necessary to immunize mucosally to achieve mucosal immunity? Will it be necessary to use adjuvants? All of these questions for the most part remain unanswered. Many of these issues were discussed during the workshop, as summarized below.

6.1. Route of immunization and mucosal adjuvants

Several delivery vehicles have been developed for mucosal vaccines, such as liposomes, immunostimulating complexes (ISCOMS) or virus-like particles (VLPs) [2]. Denise Nardelli-Haeflinger (CHUV, Lausanne, Switzerland), reviewed the development of VLPs to efficiently prevent genital infection by human papillomavirus (HPV) types 6 and 11, which cause anogenital warts, and HPV 16 and 18, the leading cause of cancer of the cervix [47–49]. HPVs are strictly epitheliotropic and thus protective immunization requires the induction of neutralizing antibodies in ano-genital secretions. The three VLP intramuscular immunization regimens readily induced an elevated level of anti-HPV neutralizing IgGs at the uterine cervix. The level of antibody however decreased several fold at the time of ovulation and this may theoretically impact on the long-term efficacy of the vaccine. It also confirms the suggestion that an HIV vaccine regimen will need to consider the reproductive hormonal fluctuations in women to obtain protection across mucosal barrier by systemic immunization. SIgA titers, on the other hand, do not decline during ovulation. Although it has proven difficult in practice to stimulate strong SIgA immune responses and protection by mucosal administration of antigens, intranasal immunization is known to induce SIgA antibody responses in the airways and in the female genital tract [49–52]. This prompted an attempt at vaccinating women against genital HPV infection by aerosol administration of the current VLP vaccine [53]. It will be important to study the intranasal route of immunization in the development of HIV vaccines, which will also help elucidate as to whether mucosal immunization is required to achieve sustained protection at mucosal sites of infection.

Oral immunization can induce strong SIgA responses in the small intestine, proximal colon and mammary glands but is poorly efficient at eliciting similar responses in the distal segments of the gut and in the respiratory and reproductive tracts. In the rotavirus gnotobiotic piglet model, as discussed by Linda Saif (Ohio State University, Wooster, OH, USA), initial vaccine priming must take place by the oral route with a live attenuated vaccine to be effective. However, subsequent boosting immunizations can efficiently be achieved with a non-replicating vaccine such as VLPs administered by the nasal route or DNA immunization by the intramuscular route. These prime-boost strategies induce high levels of intestinal neutralizing SIgAs and memory B cells which are the two major correlates of protection. In regards to HIV vaccination, the lesson suggests in the rotavirus model suggests that the effective strategy employed will need to optimally prime for an immune response at multiple mucosal sites (genitourinary and rectal). Boosting assists not only in amplifying and inducing a memory response, but multiple boosts were shown to provide protection against genetically divergent rotavirus strains. Finally, the requirement for inducing neutralizing antibodies at the mucosal surfaces will be critically important because HIV-1 has been shown to infect and disseminate faster than the time required to initiate a productive CD8 CTL recall response.

As mentioned above, sublingual immunization also could be a promising route for inducing both antibodies and CD4^{+} and CD8^{+} T-cell responses systematically as well as at multiple mucosal sites including the gastro-intestinal tract and the genital tract [3].

Jorma Hinkula (Karolinska Institutet, Stockholm, Sweden) reported on the use of multigenic *env, gag, pol, tat, rev* and *nef* DNA prime administered to mice with or without cationic N3 adjuvant, GM-CSF or PCPP. This was followed by booster immunizations with HIV-1 peptides, recombinant proteins, live recombinant MVA or VLPs added with a variety of adjuvants such as L3, PCPP, CpG oligodeoxynucleotides or GM-CSF. The cationic lipid adjuvant (N3) enhanced the intranasal DNA prime and, when followed by a het-

erologous VLP boost at the same site with the Eurocrine L3 mucosal adjuvant, resulted in higher ex vivo neutralizing activity against heterologous A and B-clade viral isolates. The most potent and broadest HIV-neutralizing mucosal immune response was obtained when the vaccines were delivered first during the course of an immunization regimen onto a mucosal surface in the presence of mucosal adjuvants (L3,PCPP) [54]. Although the inclusion of GM-CSF during vaccination gave rise to higher avidity antibodies, the addition of the cytokine appeared to be detrimental for eliciting cellular responses. The mechanism was in part due to innate immunity as NK cells appeared to play a role in reducing ELISPOT responses to the vaccine insert.

In a series of comparative immunization experiments, Michael Vajdy (Novartis, Emeryville, CA, USA) administered the vaccine subunit HIV-1 ΔV2gp120 (gp120 deleted of the hypervariable V2 loop) either by the intranasal route in the presence of genetically detoxified LT or by the intramuscular route in the presence of adjuvant MF59. He showed that the optimal regimen to induce vaginal and systemic IgA and IgG responses in mice and nonhuman primates was one intranasal priming immunization followed by a couple of intramuscular booster immunizations. The same was true with a Gag DNA plasmid on PLG particles: the combined intranasal/intramuscular prime-boost regimen was most efficient at inducing a Th1-type response with Gag specific $\alpha4\beta7$ T cells and IgG2a antibodies. It was noted that vaginal immunization provided the best result in terms of protection and this appeared to correlate with higher numbers of *gag*-specific ELISPOT from the draining lymph nodes. Clearly, more data on routes of prime/boost and the evaluation of the responses that are protective need to be pursued further [55].

6.2. Towards gender-specific vaccines

Immune protection at mucosal surfaces may require different vaccination strategies for men and for women, i.e. gender-specific vaccines. Lawrence Stanberry (University of Texas, Galveston, TX, USA), discussed the results of the GSK herpesvirus (HSV-2) glycoprotein gD2-based vaccine clinical trial. The vaccine afforded 73–74% protection against genital herpes disease to HSV-negative women but no protection at all to men [56]. Utilizing a mouse model, it was shown that vaccinated and T-cell depleted animals were protected while B-cell deficient animals were not, suggesting that protection is due to vaccine-induced antibodies. This raises the possibility that the gender difference observed in the human trial may be due to vaccine-induced antibodies present at the site of exposure in women but not men. Does the female genital tract present opportunities for immunity to HSV-2 and in a similar fashion to HIV-1?

6.3. Mucosal immunity to HIV/SIV vaccines

Induction of mucosal immunity and protection against mucosal virus challenge has been tested in nonhuman primate models after immunization with several vaccine candidates (DNA, live vectored or subunit vaccines) with and without adjuvants, followed by various protein boosters administered by various routes.

Paul Johnson (New England Primate Research Center, Harvard Medical School, Southborough, MA, USA) compared the efficacy of a multigenic SIV *gag, pol, env, rev, tat, nef* DNA/MVA prime-boost vaccination regimen to that of the live attenuated SIVΔ*nef* vaccine to induce mucosal responses to SIV and to protect female macaques against a low-dose vaginal pathogenic SIVmac challenge. Challenge was repeated twice a day at weekly intervals for up to 17 weeks. The DNA/MVA prime-boost regimen resulted in relatively high levels of Gag-specific $\alpha4\beta7$ T cells at mucosal sites but the levels of

neutralizing IgG antibody were lower in both serum and vaginal secretions as compared to those in the Δnef vaccinated animals. Monkeys with higher titers of SIV specific IgAs required a greater number of challenges to become infected. Upon infection, viral load set point in the DNA/MVA vaccinated animals was 1-2 logs lower than that in control infected monkeys, whereas viral loads in the SIVΔnef vaccinated animals remained as low as 4 logs below controls and often at the limit of detection. Although the Δnef vaccine did not induce as good a peripheral T cell response, compared to the DNA/MVA vaccine, it showed quite a greater protection efficacy raising a note of caution regarding the use of PBMC-based immune response measurements as correlates of protection.

Martha Marthas (University of California, Davis, CA, USA) concentrated on the development of vaccines for neonates with the goal of preventing HIV-1 breast milk transmission in resource-poor countries. Three vaccine regimens were compared in newborn monkeys vaccinated orally at birth. Monkeys were either vaccinated with recombinant MVA-gag pol env SIV vaccine given IM, or with a recombinant vesicular stomatitis virus (VSV)-gag pol env SIV given orally, or with the oral VSV vaccine followed at 2 weeks of age by an MVA vaccine boost IM. No protection was seen in any of the arms against low dose SIVmac251 oral challenge, although the VSV/MVA regimen elicited SIV-specific systemic IgG and IgA as well as CD4+ and CD8+ T cell responses in PBMCs and tonsillar lymphoid tissue. This communication called attention to the specific challenges that a vaccine must overcome to protect against HIV breast milk transmission.

A more promising approach was described by Marjorie Robert-Guroff (NIH, Bethesda, MD, USA), based on the oral administration of wild-type Ad4 and Ad7 viruses via enteric coated capsules, a strategy used for over 25 years to protect US military recruits against acute respiratory diseases. Rhesus monkeys were immunized with a replication-competent, SIV env, rev, gag and nef recombinant Ad5 host range mutant (Ad5hr), administered by the intranasal or oral route, followed by intramuscular boosting with SIV gp120 in monophosphoryl lipid A stable emulsion (MPL-SE). Both vaccination strategies gave equivalent and significant protection against homologous SIVmac251 intrarectal challenge, with 40% of macaques exhibiting either no viremia, clearing viremia, or controlling viremia at the threshold of detection for more than 40 weeks after challenge [57]. A second rectal challenge done at that time on the protected animals showed that 8/11 were durably protected whereas the other three showed decreased viremia compared to naive controls.

Both oral and nasal administration of the Ad5hr-SIV vaccine elicited SIV-specific CD8+ T cells in bronchial alveolar lavages, α4β7 gut-homing receptors on SIV-specific peripheral blood T cells, central and effector memory T cell responses, and SIV-specific IgG and IgA antibodies in mucosal secretions. These antibodies were not neutralizing but could mediate antibody-dependent cellular cytotoxicity (ADCC). Durable protection against infection was associated with the presence of SIV-specific IFN-γ ELISPOTs and T cell proliferative responses. Importantly, depletion of CD8+ T cells lead to re-emergence of viremia in 6/8 animals, which disappeared after re-appearance of CD8+ T cells and restoration of SIV-specific cellular immunity [58]. However, neither IFN-γ ELISPOT nor proliferative T cell assays performed on PBMC prior to the initial challenge were predictive of the protection observed, once again highlighting the lack of predictability of PBMC immunological assays and the need for other, more reliable correlates of protection [37].

Another promising approach was highlighted by Sylvain Fleury (Mymetics, Nyon, Switzerland) who described the use of the HIV-1 gp41 MPER C-terminal peptide P1 [59] grafted onto virosomes and administered by the intranasal, intramuscular or combined intranasal/intramuscular routes to female Chinese rhe-

sus macaques. Although the sera of vaccinated animals contained high levels of MPER specific IgG, these antibodies did not possess HIV inhibitory properties. All routes of immunization induced similar levels of gp41-specific IgA and IgG antibodies in cervico-vaginal secretions, while IgAs could be recovered only from rectal secretions. In contrast to the sera IgG antibodies, the mucosal IgA antibodies were capable of blocking up to 90% in vitro HIV-1 transcytosis of primary clade B or clade C virus isolates. Purified IgAs from vaginal secretions were also found to neutralize primary clade B virus infectivity with a CI$_{90\%}$ of 1–2 µg/mL. An immunization/challenge experiment in rhesus macaques is planned to take place shortly to determine if these antibodies play a role in vivo.

Yves Lévy (Hôpital Créteil, France) reported on the mucosal arm of a recent ANRS-sponsored phase I trial, ANRS VAC14, which involved three administrations by either the intranasal or intravaginal routes of purified HIV-1 subunit gp140 in the presence or absence of the adjuvant DC-Chol [60]. This study, performed in 34 French female volunteers, showed the feasibility of phase I trials designed to assess mucosal responses in nasal and vaginal secretions.

7. Lessons learned for preclinical testing of candidate vaccines: post-STEP

An important question that was discussed at the meeting was whether there were any animal models for HIV vaccines that will reliably predict successful induction of protective immunity in humans. As a general rule, potential HIV vaccine candidates are tested in a NHP model before clinical trials are carried out. Differences between rhesus macaques of Indian and Chinese origin, as well as between SHIV and SIV strains used in the various NHP studies, often make direct comparison between studies impossible. As pointed out by David Watkins (AIDS Vaccine Research Laboratories, Madison, WI, USA), the Merck Ad5/HIV vaccine went to clinical trials after it had shown protection in a NHP model. However, all protected macaques in the study were Mamu-A*01 positive, an haplotype related to spontaneous control of SIV infection, and the challenge used a SHIV strain whose HIV sequences exactly matched those in the Ad5 recombinant vaccine. When an Ad5/SIV vaccine was eventually tested in macaques against a SIVmac239 challenge, most of the animals were not protected from infection or disease progression [61,62].

The suggestion was made that future vaccine efficacy tests in NHP models should take into account the genetic origin of the monkeys, and avoid overrepresentation of MHC class I alleles Mamu-*A01, Mamu-B17 and Mamu-B08 in the vaccinated monkeys, as these are associated with spontaneous control of SIV viremia, mimicking HLA alleles associated with the control of HIV-1 replication in human elite controllers [63]. Importantly, vaccines based on SIVmac which do not reduce viral replication by 1.5 logs in such a setting should not go forward to phase IIb trials [64,65]. Also, as an effective HIV vaccine will need to provide protection against globally diverse isolates of HIV-1, demonstrating protection against a heterologous virus challenge becomes a major point to consider.

8. Conclusions

There is a great need for developing new HIV vaccine strategies that would be effective at the earliest stage of mucosal infection and block HIV penetration in both the genital and gut mucosae. A better understanding of the first steps of HIV entry and infection of mucosal immune responses is therefore needed. The mucosal immune system is highly complex, compartmentalised and not entirely deciphered. Contrary to earlier beliefs, a common mucosal

immune system that applies to all mucosal tissues has been shown to be not the case over recent years.

As illustrated by Ashley Haase's (University of Minnesota, Minneapolis, USA) studies [30,31] and discussed in his summary talk by Robin Shattock (St. Georges Hospital, London, UK), there is a very narrow window of opportunity before mucosal SIV- and probably HIV-infection spreads from the site of entry to the systemic lymphoid system. The virus is transported from primary infected DCs to target T cells within 1–4 h after infection, and dissemination of infection to the local lymph nodes takes perhaps 3–4 days or even less, depending on the route of infection. These time periods need to be compared to the known 3–5 days it takes for the reawakening of memory immune responses in a vaccinated individual. Therefore, a strong persistent neutralizing antibody response to prevent HIV infection or at least reduce potential infectious virions at mucosal surfaces is still desperately needed whereas potent specific antiviral mucosal T cell responses will be required to prevent viral dissemination.

It is obvious that appropriate animal models need to be developed, as well as *ex vivo* models that take into consideration the role of co-infections and hormonal variations in the female genital tract. The idea that mucosal transcytosis-blocking and/or neutralizing IgA antibodies may be key to protection against HIV-1 infection must be further documented and confirmed. Would sublingual or intranasal routes of immunization be advantageous in that regard? Would repeated local (vaginal or rectal) booster immunizations be of help? There were varying opinions on whether a mucosal immunization at site of infection was needed to induce and/or boost protective immune responses at that site [42,44,48,55].

Finally, not much progress will be made in the field of mucosal vaccines in the absence of standardized protocols for sample collection and assays for measuring mucosal immune responses. These are critically and urgently needed for evaluating mucosal immunity in an accurate and reliable fashion to compare responses of multiple HIV vaccines on a global scale. Perhaps one of the most frequently heard observations that came out of this meeting was that standard T cell assays such as IFN-γ ELISPOT, ICS and/or proliferation assays, done in PBMC, were not predictors of protective immunity. A recent report by Ron Desrosiers' group well illustrates the point: potent protection against SIV challenge can be provided by live attenuated SIV infection in the absence of strong anti-SIV immune responses in the peripheral blood, whereas, on the opposite, high-magnitude anti-SIV immune responses in peripheral blood provided by DNA prime and recombinant poxvirus or adenovirus boosts provided little or no protection against similar challenge [66]. Similarly, CD8+ T cells responses identified by IFN-γ ELISPOT or IFN-γ/TNF-α intracellular cytokine staining (ICS) assays using a single high concentration of peptides and PBMC-derived T cell populations often failed to predict the recognition of cells infected with escape variant viruses [62].

Nevertheless, many presentations underscored the need for inducing T cell responses to control SIV/HIV infection. The accurate assessment of the antiviral activity of HIV-specific CTLs therefore becomes a crucial step in the evaluation of vaccine potency [67]. Thus, the use of viral suppression assays to test the functionality of T cells, attempts at identifying the T cell populations associated with control of infection in SIV and HIV controllers, as well as the study of T cell transcriptome and proteosome profiles, as advocated by Rafik Sekaly (CANVAC, Montreal, Canada), should be areas for future active investigation [39–42]. It also would be important to study the breadth and avidity of virus-specific IgAs and IgGs and to develop standardized assays to validate their functionality, whether viral transcytosis-blocking or virus-neutralization assays. The identification of reliable correlates of protection against mucosal HIV-1 infection remains at this time more than ever the number one quest in HIV vaccinology.

Acknowledgements

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