MUCOSAL AND SYSTEMIC IMMUNE RESPONSES IN HIV-1 EXPOSED UNINFECTED INDIVIDUALS

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ABSTRACT

Human immunodeficiency virus (HIV) is an infectious agent that, for 26 years, has evaded vigorous attempts to invent either a cure or preventive agent. Even though the immunological understanding of HIV’s pathogenesis has greatly improved, most knowledge has been gained from studies of systemic compartments but less from the mucosal environment, where the majority of all infections occur. Strangely, some individuals, despite their exposure to HIV, seem to be less susceptible to the infection, thus categorized as a HIV Exposed Uninfected (EU) group. These fortunate escapees can be considered nature’s own experiment and, possibly, have lessons to teach us about superior ways to encounter HIV. That knowledge has obvious potential for application to research directed toward formulating a preventive vaccine. In the present thesis, the author has evaluated a cohort of Swedish male homosexual EU subjects with regard to their immune responses to HIV, or lack thereof, in systemic as well as mucosal compartments. Plasma, cells and mucosal samples were collected during a period of two years, while each of these individuals had continuous sexual relations with their HIV-positive partners. Questionnaires described sexual behaviors, and plasma samples as well as clinical records were available for the HIV-positive partners. Low-risk HIV-negative individuals were recruited as controls. The questionnaires revealed that the vast majority of these EU subjects were exposed to HIV through the oral (not anal) route. This is considered a low-risk behavior and is rarely seen as an exclusive behavior if compared to other EU cohorts. We purified the plasma and saliva samples to obtain IgA, an antibody important mostly at mucosal sites. Further, we tested IgA as well as IgA-depleted samples in a PBMC-based neutralization assay, to evaluate whether the EU subjects had any anti-HIV capacity. This was indeed present in salivary IgA, samples of which from 13 of 25 EU subjects could neutralize HIV (in vitro) vs 0 of 22 controls. The neutralizing response was sustained in almost all cases during the study period. Moreover, HIV-neutralizing capacity also detectable in plasma (7 of 25 EU, 0 of 22 controls) was associated with the partner’s HIV-RNA viral load. This suggests that the natural anti-HIV response in these EU men is acquired, not predestined. Also in plasma, the vast majority of neutralization was mediated by IgA; however, the neutralizing antibodies (as in saliva) lacked classical HIV-specificity as measured by ELISA or Western Blot. We then evaluated PBMC from the EU group and found HIV-specific responses in 3 of 25 subjects; this response was detected in both CD4+ and CD8+ subsets of cells as measured by intracellular staining (ICS). Lastly we tested their saliva for innate molecules of known anti-HIV capacity and found significantly higher levels of CC-chemokines than in controls. This outcome was also associated with behavioral aspects of the study subjects, not genetic polymorphisms, which further strengthen the theory of environmental causes for these immune responses.

The conclusions of this work are summarized in the following four points: 1) The HIV exposure was low-grade, since it occurred via the oral route, but could still induce and/or sustain an anti-HIV response mediated by IgA. Therefore, the oral route might be applicable in a vaccine setting. 2) While detectable in >50% of the EU mucosal samples, the HIV-neutralizing capacity also existed to a lesser extent in plasma and, rarely, in systemic T-cells. 3) Amounts of innate soluble molecules were increased in the saliva of EU individuals and were associated with neutralizing capacity. 4) All immune responses against HIV detected in these EU subjects were associated with environmental factors, implying that such responses are acquired. In turn, that supposition favors the possibility that similar or stronger responses can be induced via vaccination.
LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Oral HIV-exposure elicits mucosal HIV-neutralizing antibodies in uninfected men who have sex with men

Klara Hasselrot, Pär Säberg, Taha Hirbod, Johan Söderlund, Mariethe Ehnlund, Göran Bratt, Eric Sandström, Kristina Broliden

AIDS 2009, Jan 28; 23(3):329-333

II. Orally exposed uninfected individuals have systemic anti-HIV responses associating with partners’ viral load

Klara Hasselrot, Göran Bratt, Taha Hirbod, Pär Säberg, Mariethe Ehnlund, Lucia Lopalco, Eric Sandström, Kristina Broliden

AIDS 2009, Sep 23; (E-pub ahead of print)

III. Induction of systemic HIV-1 specific cellular immune responses by oral exposure in the uninfected partner of discordant couples

Carina Perez, Klara Hasselrot, Göran Bratt, Kristina Broliden, Annika C Karlsson

Submitted to AIDS, pending revisions

IV. HIV-1 exposed uninfected individuals have increased levels of mucosal CC-chemokines associated with sexual behavior

Klara Hasselrot, Göran Bratt, Kristina Duvefelt, Taha Hirbod, Eric Sandström, Kristina Broliden

In manuscript

V. The section “Specific introduction” is partly taken from the following review, in its full length presented as follows:

Genital and oral mucosal immune responses against HIV-1 in exposed uninfected individuals

Klara Hasselrot

Critical Reviews in Immunology 2009, in press
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Anti-retroviral therapy</td>
</tr>
<tr>
<td>CCL(ex CCL5)</td>
<td>Cysteine-Cysteine motif chemokine ligand</td>
</tr>
<tr>
<td>CCR(ex CCR5)</td>
<td>Cysteine-Cysteine motif chemokine receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>(HIV-1) Envelope glycoproteins</td>
</tr>
<tr>
<td>EU/EUI</td>
<td>Exposed uninfected /Exposed uninfected Individuals</td>
</tr>
<tr>
<td>Gp</td>
<td>Glycosylated protein</td>
</tr>
<tr>
<td>HEPS</td>
<td>Highly exposed persistently seronegative</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>MIP-1 alpha/beta</td>
<td>Macrophage inflammatory protein 1 alfa and beta</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STD/STI</td>
<td>Sexually transmitted diseases or infections</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Fifty percent tissue culture infectious dose</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations program on HIV/AIDS</td>
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<tr>
<td>VL</td>
<td>Viral load</td>
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<tr>
<td>WB</td>
<td>Western Blot assay</td>
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<td>WHO</td>
<td>World Health Organization</td>
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1 AIM

The overall aim of this thesis project was to identify immune mechanisms linked with reduced susceptibility to HIV-1 infection in a currently available cohort of EU men who have sex with men. For this purpose, we had to begin by determining the route of HIV-1 exposure in these individuals and the clinical status of their respective HIV-positive partners. Knowing this, we could investigate whether neutralizing responses against the virus existed and, subsequently, whether the underlying cause was predisposing factors (i.e., genetic) or exposure to HIV-1 (acquired/behavioral). The ultimate aim of this project is to identify mechanisms of naturally superior anti-HIV responses as well as the physiology of their induction and dissemination to aid the future construction of prophylactic HIV-vaccines or microbicides.

Paper I: Determine the route of HIV-1 exposure in the EU individuals surveyed here. Since such exposure occurs mainly via the oral route, investigate whether this is sufficient to elicit HIV-neutralizing capacity in the oral mucosa and whether that capacity persists over time.

Paper II: Establish whether oral exposure can create systemic HIV-neutralizing responses. Assess the breadth of neutralizing responses against several primary HIV-1 isolates. Seek an association with the level of exposure by measuring the viral load in HIV-positive partners.

Paper III: Since we have identified systemic antibodies with anti-HIV capacity in these EU subjects, the systemic cellular immune responses of CD4+ and CD8+ T cells against HIV-1 is investigated, with regards to phenotype and durability.

Paper IV: The anti-HIV properties of certain soluble innate factors, for example, CC-chemokines and defensins, are known but rarely investigated in mucosal secretions. Therefore, we determine whether the EU subjects have elevated levels of these factors in saliva and if such elevations are associated with HIV-neutralizing capacity of IgA1-depleted saliva. Further exploration concerns the presence of genetic polymorphisms related to the CC-chemokines as well as the possible effects of sexual behavior on the induction of this innate immune response.
2. GENERAL INTRODUCTION

2.1 The HIV/AIDS epidemic

It has passed 27 years since the discovery of the Human Immunodeficiency Virus (HIV) as the cause of Acquired Immunodeficiency Syndrome (AIDS), but still the virus infects approximately 2.7 million individuals each year (1). In spite of a scientific and economic effort that no other disease in mankind has prompted, there is no cure and no vaccine available. Antiretroviral treatment against the subsequent development of AIDS is effective, but only reaches approximately 42% of all HIV-infected individuals, due to political and economic injustice (1). Clearly many viruses (smallpox, polio, and measles among others) have stroked mankind in history and several of those have been successfully hindered by fairly quickly developed vaccines – what makes HIV so special? Using war terminology, the main answer is that HIV immediately strikes behind enemy lines and put out of action the mastermind of the human immune response; its main target is the CD4+ T-cell. Being described as “the conductor of the immune response” (2), i.e. making all aspects of the immune system acting in concert with each other, these cells are disastrous to lose at a high quantity, and that is exactly what happens early in HIV infection. Instead of conducting a multifaceted defense, it is destructed either by the viral lysis or by apoptosis, leaving all the diverse divisions of the immune system to its leaderless fate.

HIV started infecting humans in the first decades of the 20th century; first HIV-positive blood sample was detected retrospectively in Kinshasa, Democratic Republic of Congo, and dated 1959 (3). Despite conspiracy theories of various kinds (4), the scientific community is now convinced that the infection most certainly originates from Simian Immunodeficiency Virus (SIV), a similar virus infecting African primates but in wild not causing any severe disease (5). Still HIV cannot be regarded as a zoonosis, since phylogenetic evidence suggests that fairly few cross-species transmissions have occurred (6-8). While not causing disease in its natural host the Sooty Mangabey monkey, SIV infects and causes AIDS if transmitted to another primate; the Rhesus Macaque monkey of Asian origin. Subsequently this has been used as an animal model in research settings for studying the pathogenesis, immune responses and possible treatments of SIV/HIV.
The global HIV epidemic however did not start until the 1980’s; the delay possibly explained by urbanization and increased traveling among other factors. Since then, 25 million individuals have died due to the infection, and only in Sub-Saharan Africa 12 million children have turned into orphans (1). The infection is transmitted mainly (>90%) via mucosal membranes, such as sexual contact or by delivery (9). Intravenous transmission occurs if sharing syringes or being a recipient of contaminated blood products. The high-risk groups vary depending on geography; while heterosexual intercourse causes the majority of all HIV-infections in Sub-Saharan Africa and disproportionally affects women, men who have sex with men (MSM) and intravenous drug users (IVDU) are considered the high-risk individuals in Western Europe and in North and South America (1).

In Sweden the epidemiological statistics on HIV started in the late 1980’s; in total approx 8500 individuals have been reported HIV-positive and 5000 are living with HIV today (10). For a long time the incidence was fairly stable with approximately 300 new infections each year, however since a few years back this number has increased to 400 infections/year. This increase was anticipated, since a massive increase in diagnosed Chlamydia infections due to low condom use was evident a few years before (10). The largest increase in domestic HIV-incidence comes from the MSM group (Fig 1).

Figure 1. Incidence of (domestic) HIV-infections in Sweden 2002-2009

among men who have sex with men

Source: Smittskyddsinstitutet.se
2.2 The Human Immunodeficiency virus

HIV belongs to the Retrovirus family of the Lentivirus genus. The virion consists of double-stranded RNA, which is translated into DNA in the infected host cell by the virus own enzyme Reverse Transcriptase. HIV’s RT has an inefficient proof-reading, and since many mutations might be deleterious they contribute to a relatively high percentage of non-infectious virions being produced. On the other hand, the high recombination rate can also result in unique viral variants which enable the virus to either evade the immune system or to develop resistance towards many treatment regimens(11), making the construction of a vaccine more difficult. The HIV genome contains 9 genes (that in turn encodes 15 proteins): the structural Env, Gag and Pol, the regulatory Rev and Tat and the accessory Vif, Vpu, Nef and Vpr. The HIV-RNA together with viral proteins are contained within a capsid comprised of the gag p24 proteins. Surrounding the capsid is a matrix of viral p17 proteins, and the envelope (the outer shell) consists of the host-cells lipid-bilayered membrane (12). Embedded in the bilayer are several of the host-cells proteins, as well as the virally derived spike proteins (envelope glycoproteins) named gp41 (transmembrane) and gp120 (outer surface).

HIV infects the human body by binding the envelope trimeric protein gp120 to CD4-receptors in the host, found mainly on CD4+ T lymphocytes but also present on Dendritic cells, Langerhans cells and Monocytes/Macrophages. It is still not known whether infection is mediated foremost by cell-bound virus or by free virions. When binding to CD4 a conformational change occurs, this allows further binding to the CCR5 or CXCR4 co-receptors, hence the descriptive term “R5” or “X4” HIV-strains. In general only R5 strains are transmitted via mucosal membranes while intravenously both R5 and X4 strains are transmitted (the mechanisms why X4 strains are unable to infect the musocae are not fully known(13)). Within the infected individual, R5 virus can mutate into X4 viruses, often coinciding with a more severe clinical status and the development of AIDS(14).
HIV is in fact two separate viruses, HIV-1 and HIV-2. HIV-2 is somewhat less pathogenic and has not caused severe epidemics; the incidence is relatively stable and originates from western Africa (15). HIV-1 is the major concern globally and consists of groups M (Major), N, O and possibly P. The M group is responsible for >90% of all HIV/AIDS cases, and can in turn be divided into different subtypes or clades, with different geographical distributions. Subtype A and C are most prevalent in Africa, clade B is most prevalent in North and South America and Europe, while the recombinant clade CRF_AE, clade B and clade C is common in Asia (16).

2.3 Clinical course of HIV infection

When HIV is transmitted, typically mucosally through a squamous or columnar epithelium, the infection propagates in the submucosa and subsequent reaches the draining lymphnodes (17). When viremia is reached (most likely within 2-4 weeks), the clinical manifestation with very diverse symptoms (fever, rashes, tonsillitis, diarrhea, vomiting) is called Primary HIV infection (PHI)(18). PHI can however be asymptomatic in 50% of all cases. The diffuse and sometimes absent symptoms of
PHI certainly facilitates the spread of the epidemic, when newly infected individuals do not realize their infected (and infectious) status. Early diagnosis has been shown to alter the sexual behavior and reduce onwards HIV-1 transmissions (19). Laboratory tests show a dramatic decrease of CD4-count in peripheral blood during PHI, followed by a reconstitution although never reaching the levels before infection occurred. The viral decrease and (partly) T-cell reconstitution have in clinical and laboratory studies been suggested to rely both upon the cellular and the adaptive immune system (20, 21). In the mucosa however, the depletion of CD4+ cells is more profound and probably does not regenerate to the same extent (22). The viral load declines after PHI, to a viral set point (latency phase) often diagnostic for future disease progress. Without ART, the natural course of HIV is to develop AIDS after a median of 8 years. AIDS is defined by peripheral blood CD4+ count < 200x10^6/l, or an AIDS-defining event such as for example an opportunistic infection, Kaposi sarcoma or B-cells lymphoma.

*Figure 4: Pathophysiology of natural HIV-1 infection*

An individual with PHI or AIDS is considered most contagious due to the higher levels of viral load in blood, mostly corresponding to viral load in genital secretions (23). During chronic infection (latency phase) an infected individual is more contagious the higher viral load he/she has, although the viral load in blood does not necessarily reflects the levels in mucosal secretions. Co-infections such as HSV or other ulcerative diseases (but not bacterial STI’s) have been regarded to increase HIV
susceptibility; this is however questioned by more recent studies and is still a matter of controversy. Case studies have shown infections transmitted from HIV-infected individuals with undetectable levels of HIV-RNA in blood (24) although this is considered a rare event.

2.4 Antiretroviral treatment

Antiretroviral treatment against HIV was first developed in the late 1980’s, through a nucleoside analog (NRTI) that inhibit the virus’ RT. This drug was effective in reducing mother-to-child transmission, but was not alone effective in preventing AIDS due to the fact that HIV readily develop resistance against a single drug. In 1996 however, the protease inhibitors (PI) arrived, and ART is today given as a combination of different antiviral drugs(25). The results are remarkable and many HIV-infected individuals can reverse even an AIDS-diagnosis to keep on living without detectable viral loads in blood. The adverse effects of ART are however many, and cardiovascular morbidity is a major clinical concern after only 12 years in clinical practice. This is why drug therapy preferably is not started in the latency phase until CD4-counts drop, viral load rises or AIDS-defining events appear (26), although the clinical praxis varies between different countries. PHI (when able to diagnose in time) is however always treated to prevent the initial depletion of CD4+ cells. In Sweden the majority of AIDS-diagnoses are due to the fact that they are identified as HIV-positive at the same time as the onset of AIDS (10). Most individuals are treated successfully, however a few does not respond to treatment or is not provided it due to low compliance (as in cases of IVDU).
3. SPECIFIC INTRODUCTION

Certain parts of the following section are taken from a more extended review which is to be published in the journal Critical Reviews in Immunology (Begell house Publishers) with the title “Genital and oral mucosal immune responses against HIV-1 in Exposed Uninfected individuals” Klara Hasselrot, 2009 (in press)

3.1 Importance of mucosal immune responses against HIV-1

The epidemiology regarding mucosal transmission being relatively stable since the beginning of the HIV-1 epidemic, it is unfortunate that the research on mucosal immune responses against HIV-1 for many years have not been in proportion to its importance. Peripheral blood samples are sterile and more readily obtained than mucosal fluids for laboratory purposes, and tissue samples from mucosal compartments requires invasive procedures. To better understand the characteristics of human anti-HIV immune responses such as in vaccinees, long time non progressors (LNTP) or HIV-1 exposed uninfected individuals (EU), both systemic as well as mucosal samples should ideally be investigated simultaneously.

HIV-1 can be transmitted sexually via rectal, genital and oral intercourse, the highest risk of transmission attributed to the rectal route and the lowest risk via the oral route. The mucosal environment in these three areas is different from each other in many aspects; both in terms of immune responses against HIV-1 and also the distribution of target cells for the virus. There is a gender-related difference when talking about genital exposure, while women have a large area of non-keratinized epithelium in the vagina and ectocervix, males have non-keratinized epithelium mainly at the meatus. Women have a single layer columnar epithelium in the endocervix and in the uterus, this is also present in the rectum of both males and females. Oral mucosa consists of non-keratinized squamous epithelium (Fig 5, 6). All areas of the female genital tract are also under the influence of sex hormones, and the vulnerability likely changes with the menstrual cycle. Males have a more keratinized epithelium in their genital tract (except for the meatus), this discrepancy explaining the fact that women are more readily infected by HIV-1 during heterosexual intercourse than males. Male circumcision reduces the permissive area of possible HIV-1 transmission, which is now proven in several studies (meta-analyzed in (27)). The viral burden of the
infected subject (measured as RNA copies/mL) is of course also detrimental regarding the risk of HIV-1 transmission, and hopefully the epidemic will be affected by the fact that access to antiretroviral drugs (ART) is increasing globally, however not as fast as recommended by the World Health Organization (WHO). This further underscores the importance of ongoing research in HIV-1 immunology and vaccinology.

Figure 5, 6: Illustrations showing non-keratinized squamous epithelium where virus enter through a breach or via intraepithelial dendritic cells (above) or single columnar epithelium (below) where virus can transcytose directly to the submucosa (and the target cells available). Copyright of Sam Hirbod, illustrator.
Fortunately the interest in mucosal immunology against HIV-1 has been increasing greatly, and important questions are now highlighted such as the exploration of which are the first target cells for HIV-1 in the genital mucosa (28, 29), and how vaccine candidates can be improved as to recruit host defense to where it is most needed - the mucosal compartments (30). The exploration of genital tract immune responses and the distribution of different cell subsets are investigated not only within vaccine trials or in HIV-1 infected subjects but also among healthy controls, and as such the basic knowledge gained in mucosal immunology is most certainly beneficial also in other research areas.

The immediate human immune response in mucosa is obviously in many cases not sufficient against HIV-1 transmission, yet some individuals seem to be less susceptible to the infection than others. There is a great interest in these individuals since they possibly can teach us lessons about a natural yet superior immunologic response to resist infection, information that hopefully can be implied in the clinical setting.

3.2 Different cohorts of Exposed Uninfected individuals

Individuals who are studied regarding reduced susceptibility to HIV-1 infection consist of commercial sex workers, discordant couples (one partner HIV-positive and the other HIV-negative) and uninfected infants born to infected mothers. The first observation of mucosally exposed uninfected individuals with immune responses against HIV-1 took place 20 years ago (31), and since then these type of cohorts have been identified worldwide, however the nomenclature unfortunately differs – Highly exposed persistently seronegative (HEPS), Exposed seronegative (ESN) and Exposed uninfected (EU). There are also a lack of consistency regarding the inclusion criteria for each terminology, thus it is important to declare a detailed description of the actual cohorts exposure to HIV-1 (by route and during which time period) in each study performed. Here, the term Exposed uninfected (EU) has been used and the focus is on sexually exposed individuals thus excluding infants.

As the case with many human physiologic mechanisms, the immune system is very complex and multifactorial. When trying to identify a single mechanism responsible for resistance against HIV-1 only one have been found; the homozygous CCR5Δ32
genotype (32) resulting in a complete absence of the CCR5 co-receptor for HIV-1, needed to establish an infection via mucosal exposure (when HIV-1 is transmitted intravenously it can also use the co-receptor CXCR4). The CCR5Δ32 genotype however, is present ironically enough where it is least needed – the northern hemisphere (33) where the burden of HIV-1 is low. All other hypotheses regarding EU status described here and elsewhere are most probably acting in concert with each other, together creating an immune response less prone to be invaded by HIV-1. It is important to be cautious regarding the terminology describing these individuals, and it is here suggested not to use the word “resistant” – firstly because it is not shown scientifically beyond reasonable doubt, and secondly because these individuals are then at risk of being falsely reassured of being resistant against the infection and subsequently taking greater risks.

3.3 Innate immune responses at the mucosal level

The time interval between virus exposures at mucosal level to detectable viral RNA in the blood is approximately 7-21 days, in ex vivo Non-human primate (NHP) models labeled virions have been shown to reach target cells even within the first few hours of exposure (34). Studies from NHP have also shown that not only activated but also resting CD4+ T-cells are targets for HIV-1 in the mucosa, thereby increasing the number of potential target cells. This was previously not known, since resting CD4+ T-cells were difficult to infect with HIV-1 in vitro, underscoring the importance of in vivo or ex vivo models of mucosal immune responses. The cellular immune response against HIV-1 in genital mucosa is typically delayed as shown in studies on NHP, thus it has in the case of productive infection been described as “too late and too little” (35) – an additional powerful immediate immune response in mucosa and submucosa is critical to hinder the infection. If the virus subsequently reaches the systemic compartments via the lymphatic system, the battle is most certainly lost. Thus, the innate immune system against HIV-1 play two roles – partly acting as the first defense line, partly by recruiting other immune cells to the site of potential infection. There is however a concern that recruiting immune cells to the site of infection can be hazardous; maybe the benefit of an increasing defense is overrun by the fact that more target cells for HIV-1 is attracted to the portal of entry (36).
Innate anti-HIV-1 immunity in the mucosa consists of for example beta-chemokines, which are natural ligands to HIV-1 co-receptor CCR5. Indeed a higher expression of the beta-chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) have been reported in female genital mucosa of EU by two independent groups (37, 38). Alpha-defensins, an antimicrobial peptide with known anti-HIV-activity, have also been found over expressed (by mRNA as well as production by cervicovaginal mononuclear cells) in EU female genital tract (39). High levels of antimicrobial peptides in vaginal secretions, such as defensins and LL-37, have been showed to correlate with HIV-neutralizaing capacity in a prospective cohort of EU sex workers (40). Being able to neutralize HIV-1 did however not protect these subjects from future HIV-1 transmission, the hypothesis in this case being that sexually transmitted diseases (STI’s) correlates with higher levels of antimicrobial peptides and the believed negative effect of STI’s on HIV-1 transmission overrun the capacity of HIV-1 neutralization. Using a proteomics approach in a small study population, both known and novel proteins have been found to be over expressed in the genital mucosa of EU from Kenya (41), and probably there will be additional findings like these due to increasing access to refined techniques.

Oral innate immune responses in mucosa of EU individuals have has also been investigated compared to low-risk controls, even though the oral cavity naturally is a hostile environment to HIV-1, hence lower transmission rates than by genital exposure. Beta-defensins 2 and 3 have been found over expressed in EU oral mucosa compared to low-risk controls by mRNA copy numbers, although this was not the case in the genital tract of the same individuals (42). Our group has also found higher levels of several beta-chemokines in unstimulated saliva of EU individuals compared to controls (unpublished data).

3.4 Adaptive immune responses at the mucosal level

Antibody response in the mucosa consists of IgA and IgG subtype antibodies, the ratio between the two varying between different mucosal sites: while the gastrointestinal tract (oral and rectal mucosa) produces IgA>IgG, the female genital tract produces IgG>IgA (43). EU individuals are by definition HIV-specific IgG negative, but some studies (although controversial, reviewed in (43) have found HIV-
specific IgA from mucosal samples of EU (44-46). Explanations for the induction of adaptive immune responses without productive HIV-infection includes 1) extraordinarily low levels of virus replication in the submucosa limited at the site of viral exposure (47), and 2) immune processing of viral (non-infectious) fragments presented to the immune system. The presence of an IgA-response against HIV-1 without detectable HIV-specific IgG is further hypothesized to occur through T-cell independent pathways in extra-follicular environments (48, 49). It is however important to separate HIV-specific IgA from HIV-neutralizing IgA, since those two might not coincide. HIV-neutralizing antibodies of IgA subtype, but with undefined specificity, have been found in EU individuals, both in cervicovaginal lavages (50-52) and saliva (53). The hypothesis behind this neutralization is that the IgA is directed towards other antigens than the common HIV-envelope epitopes (gp41, gp120, gp160 etc), such as for example anti-CCR5 (54) anti-CD4 or possibly against carbohydrates on the HIV-envelope. Other mechanisms by soluble antibodies to hinder virus infections are opsonization, blocking transcytosis through the epithelial membrane (55), activate complement-mediated cell lysis or induce antibody-dependent cell-mediated cytotoxicity (ADCC). The presence of ADCC in CVL has been correlated to better mucosal viral control in HIV-1 infected women (56), but has not yet been investigated in EU women. Oral ADCC by salivary antibodies was shown to correlate with lower HIV-1 plasma viral load in infected subjects (57), but did however not differ between EU and low-risk controls, although the EU investigated in this study lacked any description of HIV-1 exposure.

Reinforcing the antibody immune response at a mucosal level is thus a possible way to better control a HIV-1 exposure, and a tempting mechanism to use in vaccine design. MEC/CCL28 is a chemokine ligand that recruits IgA-secreting plasma cells to mucosal lamina propria, and it has indeed been found augmented in EU individuals (58). These researchers also immunized mice with CCL28, and could see a significantly higher proportion of IgA-secreting plasma cells in gastrointestinal lamina propria compared to control animals. Further studies in NHP or humans would be complementary to explore this finding.
3.5 Tcell-mediated immune responses in the mucosa

The first report of mucosal cellular immunity against HIV-1 in EU came in 2000, when cervical mononuclear cells (CMC) from exposed uninfected sex workers in Kenya showed a HIV-specific CTL using the IFNgamma Elispot assay (59). There was also a report, however on a small number of subjects, showing augmented cytokine profiles by mRNA expression in CMC from EU compared to healthy controls (60), the immune activation in the EU’s being very similar to those of HIV-1 infected individuals. This study went on to show an absence of HIV-specific cellular responses in EU PBMC, but interestingly a presence of HIV-specific responses in EU CMC (comparable with responses found in HIV-1 infected individuals), which was not detectable in healthy controls. Before and after these investigations, many researchers have reported different levels of HIV-specific cellular responses in PBMC from EU (61-66), and although all these findings may have implications in the mucosal environment, there is urgent needs to determine this by continue to perform comparative studies, by sampling both systemically and mucosally, in EU individuals.

3.6 Genetic influence on mucosal immune responses

Besides the already mentioned homozygous CCR5Δ32 genotype which certainly is a gate keeper in sexual transmission of HIV-1, there are many other studies on genetic correlates of reduced susceptibility to HIV-1 (other have investigated genetic variants of enhanced susceptibility to HIV-1, which is not further discussed here). Several studies have also been performed in HIV-1 infected individuals and their respective progression to AIDS, showing correlates of protection against disease in for example long time non progressors (LNTP), and these findings will also not be discussed here. HLA-discordance has been shown to correlate with reduced transmission in mother-to-child transmission (MTCT) in several studies (most of the genetic studies on reduced susceptibility against HIV-1 have been performed in the MTCT setting), but was also found to be protective against transmission between heterosexual discordant couples (67, 68). Certain HLA class II variants have also been shown to be overrepresented in EU individuals from Kenya (69). NK-cells is present in genital
mucosa, and could possibly be of great importance on HIV-1 transmission, and combined genotype of HLA-B57 and high expression of KIR3DL1 as well as homozygosity of KIR3DS1 have indeed been shown to contribute to EU status (70, 71). One study showed that the polymorphism 64I in the CCR2 loci was found augmented in HIV-positive individuals compared to heterosexual EU (72), however this was not confirmed in another study comparing several gene polymorphisms in both EU and HIV-1 positive individuals compared with low-risk controls in India (73). Polymorphism in interferon regulatory factor 1 (IRF-1), a viral transcriptional regulator, was correlated to resistance among Kenyan sex workers (74). The production of mucosal IgA, already discussed being a possible correlate of reduced susceptibility, was shown in an animal study to depend on variations of chromosome 22, and genotypes at this locus were associated with EU status in an Italian cohort (75).

3.7 Systemic immune responses; adaptive and cellular

Although mucosal immunity might be crucial to resist HIV-transmission, undoubtedly systemic immune responses are important as well – they have however gained more attention in the history of HIV research and therefore are not presented here in the same detail. Systemic immune responses, adaptive or cellular, can theoretically act against the virus in two principal ways: either to encounter the virus at the site of infection by transudation (antibodies) or migration (T-cells), or to act in the systemic compartment by reducing the viral burden. The latter alternative is in itself a goal for a so called therapeutic vaccine; i.e. a vaccine that does not prevent the infection but reduces the damage done by a high viral burden and subsequently delays the natural progression to AIDS. In order to obtain sterilizing immunity however, systemic cellular immune responses was for a long time the primary goal of vaccine research, as they were believed to be able eradicate the virus both systemically as in the submucosa. After a large clinical vaccine trial with a T-cell based vaccine (the STEP study) failed completely in 2007 (76, 77), there now seem to be a consensus that antibodies and cellular immune responses are both needed to create an effective vaccine (acting systemically and in the mucosa simultaneously).
4. MATERIAL AND METHODS

4.1 Study population and sample collection

EU MSM were recruited via the HIV-positive partner attending the Gay Men’s Health Clinic (Venhälsan) in Stockholm. The HIV-positive individual, who regularly attended the Gay Men’s Health Clinic due to his infection, was asked whether he had a HIV-negative partner who possibly was interested in participating in the study. If so, the HIV-negative partner himself set up a first visit with the responsible clinical physician of the study (Dr Pär Säberg). Inclusion criteria were: HIV IgG-seronegative, homosexual male who had at least 6 months relationship with a HIV-positive partner. Every individual was physically examined at the first visit by the responsible physician. The uninfected partners were followed at five visits every 6 months. At all visits, the study subject received a questionnaire regarding demographical data, sexual behaviors, attitudes towards condoms and further psychosocial questions and was tested for HIV (regular plasma screening), Chlamydia (throat, urine and rectum) and Gonorrhea (throat, urethra and rectum).

The sampling of the HIV-negative partners at each visit included: plasma, serum and PBMC purified from whole blood (100mL) collected from each subject by venous puncture; samples were stored at -80°C. Whole unstimulated saliva (3-5mL) was collected in a 50mL sterile container with the directions not to eat or drink the preceding two hours (the samples were all collected in the evening). The saliva was divided into aliquots and frozen at -80°C within two hours. Rectal lavages were obtained through inserting and aspirating 3mL (x 5 times, total amount of 15mL) of sterile PBS in the rectum. Semen and 10mL of urine samples (as well as the saliva samples, given instructions to keep cold) were obtained at home by the participant and brought to the clinic for further processing at each visit.

The control subjects were recruited by advertisement at a blood donor clinic. Samplings of all body fluids were collected in the same way as from the EU, however only at one occasion. Since MSM according to Swedish legislation is a group considered at risk of HIV infection and not allowed to become blood donors, these low-risk healthy controls were heterosexual males.
The HIV-positive partners were not personally involved in the study; however, clinical data from their files as well as plasma samples were available for the study in a fashion that did not reveal their identity to the study personnel.

### 4.2 Ethical approval

The study was approved by the ethical committee at Karolinska Institutet. All participants gave their informed consent.

### 4.3 Methods

**Virus isolation and neutralization assay**

Viruses used in the HIV-1 neutralization assays were obtained from the NIH Aids Research and Reference Reagent Program (USA): BZ-167 (X4R5-virus, Brazil), 92/US/727 and QZ- 4589 (R5-virus, USA). Viruses were chosen with the criteria’s of being primary isolates of subtype B (the HIV-positive partners were infected with this subtype) and having R5-tropism (the predominant virus tropism in mucosally transmitted infections). BZ-167 was used as a screening virus, and all neutralizing samples were further tested against one R5-tropic virus for confirmation. The virus was collected from phytohemagglutinin (PHA) and interleukin 2 (IL-2)-stimulated peripheral blood mononuclear cells (PBMC). TCID\textsubscript{50} values were determined for each isolate, and three virus dilutions (ranging from TCID\textsubscript{50} x 5-20) were always tested at each collection time to compensate for PBMC donor variability. Whole plasma samples were diluted 1:20, IgA1-fractions were diluted 1:2 and non-IgA1 fractions were diluted 1:2.5 in RPMI 1640 medium when tested for neutralizing capacity (IgA1 and non-IgA1 fractions already diluted 1:5 and 1:4 in the purification process, respectively). Whole saliva samples were diluted 1:10 in RPMI 1640 medium; IgA1-fractions and IgA1-depleted fractions were assayed undiluted (through the purification process already diluted 1:4 and 1:3, respectively) when tested for neutralization capacity.
The neutralization assays were performed according to previous studies by our group and others (50, 51, 78). In brief, duplicate wells of each virus dilution and each sample fraction were incubated for 1 hour at 37°C, followed by the addition of 1x10^5 PHA-stimulated PBMC pooled from two different donors. The cells were washed at days 1 and 4, respectively, and on day 6 of incubation at 37°C, supernatant from each well was collected and analyzed with an enzyme-linked immunosorbent assay (ELISA) of p24 antigen (Vironostika HIV-1 Antigen Kit, Biomérieux, Netherlands). Neutralization capacity was defined as a >67% reduction in the supernatant as compared with p24-antigen content in samples from low-risk healthy controls, a cut-off shown to be biological relevant in several previous studies (50, 51, 53, 79).

*Figure 8: Schematic illustration of the PBMC-based neutralization assay*
IgA1 purification and quantification

IgA1 was purified from plasma and saliva as follows: plasma was diluted 1:4 and saliva diluted 1:3 in PBS, pH 7.4, and subsequently purified with jacaline-agarose (Vector Labs, Burlingame, C A, USA.) A total of 800 µL was added to 200 µL jacalin/agarose beads and mixed for 2 hours at +4°C followed by centrifugation. The supernatant (i.e, the IgA1-depleted fraction) was collected. The jacalin-agarose beads were washed with PBS, pH 7.4, after which the bound IgA1 was eluted overnight at +4°C by adding 1 mL 0.8 M D-galactose, pH 7.4. The supernatant was subsequently collected; all fractions were stored at -80°C. An in-house ELISA was used for total IgA1 quantification: 96-wells plates (Nunc, Roskilde, Denmark) were coated with monoclonal mouse-anti-human-IgA1 or IgA2 (Nordic Immunology, Netherlands), and alkaline phosphate-conjugated mouse anti-human-IgA1/IgA2 (Becton Dickinson, Stockholm, Sweden) was used as secondary antibody. Pooled normal human IgA (Nordic Immunology, Tilburg, Netherlands) was used as standards and the sensitivity for the IgA1 and IgA2 ELISAs were 1.5 and 1 ng/mL, respectively.

Investigating HIV- specificity of IgA1 antibodies

Plasma samples from 25 EU and their 25 HIV-positive partners were sent blindly to the laboratory of Dr Lucia Lopalco, Milano, Italy. The 22 low-risk controls were labeled to serve as controls. The purification of IgA1 and subsequent binding of IgA1 to recombinant gp41 env proteins is described elsewhere (80). Briefly, IgA1 was purified from plasma using sepharose (Pharmacia, Uppsala, Sweden) coupled with rabbit anti-human IgA (Aldrich, Milan, Italy). 96 µL plasma was incubated on columns containing 2400 µL sepharose-anti-IgA. After washing the columns were eluted with glycine/NaCl. The purified IgA was concentrated on Ultrafree-15 Biomax30 membranes (Millipore, Bedford, Massachusetts, USA).

The samples were then tested in ELISA using microwell plates coated with gp41 recombinant proteins at 0.1µg/well. The plates were saturated with PBS and bovine serum albumin. IgA was added and incubated for 1h at 37°C, and binding was demonstrated with HRP-conjugated rabbit anti-human IgA (Dako, Santa Barbara, California, USA). The reaction was read at 492 nm.
For Western Blot analyses, we used a commercial kit normally used for detecting HIV-specific IgA in urine (Calypte Biomedical HIV-1 WB test, Berkeley, California, USA). This test detects the following HIV-1 proteins (p) or glycoproteins (gp) on a nitrocellulose strip: p17, p24, p31, gp41, p51, p55, p66, gp120, gp160 (the number refers to the apparent molecular mass in kilodaltons). The kit was used according to the manufacturer’s instruction, with the modification of adding IgA1 plasma or saliva samples (purified by jacaline as described above) instead of urine, the conjugate being changed to goat anti-human IgA-HRP (Southern Biotech, Birmingham, Alabama, USA) and the substrate being changed to ECL Advanced WB Detection System (Amersham Biosciences Uppsala, Sweden).

CCR5Δ32 genotyping

CCR5Δ32 genotyping was performed by pyrosequencing essentially, as described elsewhere (81). Briefly, genomic DNA was purified from 200 μL of blood by use of a QiaAmp 96 DNA Blood Kit, in accordance with the manufacturer's instructions (Qiagen, Alameda, California, USA). DNA was eluted into 200 μL of AE buffer (Qiagen) and stored frozen, at −20°C, until analyzed. Ten microliters of patient DNA was used to amplify a 132-bp-long fragment of CCR5, including the deletion with primers 5'-CACCTGCAGCTCTCATTTTCC-3' (forward) and 5'-BIOTIN-GTTTTTAGGATTCCCGAGTAGCA-3' (reverse). Amplified PCR products were then sequenced by pyrosequencing as described elsewhere (81) using the sequencing primer 5'-CAGCTCTCATTTTCCAT-3' and the dispension order GACAGTC (instrument PSQ96 MA, Biotage AB, Uppsala Sweden).

SNP genotyping to investigate possible mutations in genes correlating with levels of CC-chemokines (ligands or receptors)

Searching the literature revealed several SNP’s (and one gene cluster of three SNP’s) associated with reduced susceptibility to HIV-1 in EU cohorts: a) CCR2 V64I (82), b)
MCP-1 -2136T/MCP-3 767G/ Eotaxin -1385A (83), c) CCL4L (84), d) CCL2 -2578G (85), e) CCR5 T303 (86), f) CCR5 -2459A/G (82). DNA was extracted from PBMC by an automated Magnapure extractor (Roche Diagnostics Scandinavia) using the LC Total Nucleic Acid Isolation kit (Roche). Determination of alleles for rs1799864, rs2857657, rs1799987, rs4796195, rs3917885, rsCCR5T303 and rs479589 in DNA samples was performed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (SEQUENOM Inc., San Diego, California) of allele-specific primer extension products. All assays were designed using the SpectroDESIGNER software (Sequenom Inc., San Diego, California). Primers were obtained from Metabion GmbH (Planegg-Martinsried, Germany). The reaction volume of 5 µl contained 2.5 ng of genomic DNA, 2 pmol of the sequence specific primers, 0.5 mM of each dNTP, 2.5 mM MgCl2, reaction buffer, and 0.2 units of Thermo-Start DNA Polymerase. PCR conditions were an initial 10 minutes at 95°C, and then 45 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, and a final extension step for 10 min at 72°C. Allele-specific primer extensions were conducted using 5 pmol of a extension primer and the Mass EXTEND Reagents Kit and and analyzed using a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were processed and analyzed for peak identification using the SpectroTYPER RT 2.0 software (Sequenom).

For rs1799987 and rs1024611 the following procedure was used:

The primers (designed using Primer 3) optimal annealing temperature was tested by gradient PCR. Amplification reactions for the patient material were subsequently run in a total volume of 5 µl, with 10 ng of genomic DNA, 0.2µM of each primer (rs1799987 from ProOligo; rs1024611 from Metabion), 0.2 mM dNTP, 2,5mM MgCl2 and 0,25U/µl of HotStarTaq (Qiagen, Venlo, The Netherlands). Reactions were first heated to 95 °C for 15 min, and then subjected to 40 PCR cycles (30 s at 94°C, 15 s at 65°C, and 30 s at 72°C) followed by a 7 min extension at 72°C. PCR-products were cleaned by incubation with 2 U/µl Exonuclease I (NEB/InVitro) and 0.4 U/µl SAP for 30 min at 37°C followed by a 15 min, 80 °C. For the sequencing reaction, 5 µl PCR-product, 1 µl primer (5µM) and 4 µl Sequencing Premix (Amersham Biosciences, Uppsala, Sweden) in a total volume of 10 µl, was run for 25 PCR cycles using the following program; 95°C for 20 s, 60°C for 75 s. Reactions were performed in both forward and reverse direction. The sequencing reactions were
cleaned using a Multiscreen HV filter plate (Millipore, Bedford, Massachusetts, USA) containing a Sephadex-G50 Super fine (Amersham Biosciences) solution according to the suppliers instruction. The sequences were then obtained using the MegaBACE1000 Automated Capillary DNA Sequencing System (Molecular Dynamics, GE Healthcare, Uppsala, Sweden). The sequences were analyzed using the MegaBACE Sequencer Analyser software (3.0) (Amersham Biosciences) and the Staden Package computer Programs Pregap4 and Gap4 (MRC Laboratory of Molecular Biology, Cambridge, U.K.).

Enzyme-linked immunospot (ELISPOT) assay to detect HIV-specific T-cell responses

The HIV-Gag p55 consensus B peptide pool was obtained from JPT Peptide Technologies GmbH (Berlin, Germany). This pool consisted of purified peptides (>70%) and was used at a final concentration of 1 μg/ml. The HIV group M consensus Gag and Nef peptide sets were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. These HIV peptide sets were reconstituted and divided into three Gag peptide pools (p15, p17 and p24) and one Nef peptide pool and were used at a final concentration of 2 μg/ml. The peptide sets were all 15mer long overlapping with 11 amino acids. Staphylococcal Enterotoxin B (SEB) from Sigma-Aldrich Logistik GmbH (Schnelldorf, Germany) was used as a positive control.

The production of IFN-γ by antigen specific T-cells was detected using the ELISPOT assay as previously described (87). In brief, PBMC samples obtained from the EU subjects and the low-risk controls were plated in duplicate with 2 x 10⁶ PBMC/well. The cells were stimulated over night with the HIV-Gag p55, p15, p17, p24 and HIV-Nef peptide pools to identify HIV-specific immune responses. SEB was added as positive control and medium containing 0.02% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Stockholm, Sweden), which corresponded to the DMSO concentration in the peptide pools, was added as a negative control. The net number of SFU/million PBMC was calculated by subtracting background, i.e. number of spots in the negative control. Response was considered positive if SFU/million PBMC were at least two
times the experimental background and more than 50 SFU/million PBMC. To ensure accurate results the response against SEB should be more than 1000 SFU per million PBMC for the cell sample to be considered viable.

**Intracellular cytokine staining (ICS) assay**

Identification of cytokine production and determination of HIV-specific CD4\(^+\) and CD8\(^+\) T-cells were made in an ICS assay using PBMC (88). Antibodies and reagents were manufactured by BD Becton Dickinson AB (Stockholm, Sweden). The cells were thawed and left to rest overnight at 37\(^\circ\) C. On day two, the PBMC were plated together with the peptide antigens, SEB or negative control as stated above. After one hour incubation 10\(\mu\)g/ml Brefeldin A (Sigma-Aldrich, Stockholm, Sweden) was added to each well. Plates were incubated for 6 hours at 37\(^\circ\) C, and stored in 4\(^\circ\) C overnight. On day 3, cells were stained with Vivid-Pacific Blue (Invitrogen, Stockholm, Sweden) for identification of live/dead cells together with extracellular antibodies against CD4-PerCp-Cy5.5 (clone SK3) and CD8-AmCyan (clone SK1). After staining the cells were fixed and permeabilized before intracellular staining was performed using a mixture of anti-MIP-1\(\beta\)-PE (clone D21-1351), anti-TNF-\(\alpha\)-PE-Cy7 (clone Mab11), anti-IFN-\(\gamma\)-FITC (clone B27), anti-IL-2-APC (clone 5344.111) together with anti-CD3-APC-H7 (clone SK7). The flow analysis was carried out using the FACS Canto\textsuperscript{TM} II instrument and data were analyzed using FlowJo (TreeStar, Ashland, USA). Using FlowJo we gated on single cells, and excluded dead cells (Vivid-Pacific Blue positive). We selected the lymphocytes followed by gating on CD3\(^+\) cells only. Analyses were made for CD4\(^+\)CD8\(^-\) cells and CD8\(^+\)CD4\(^-\) cells individually to evaluate cytokine production of MIP-1\(\beta\), TNF-\(\alpha\), IFN-\(\gamma\) and IL-2. A sample was considered positive (above the cutoff) when the response was two times the background, i.e. response induced in the negative control.
DNA extraction and amplification to exclude HIV-infection below detection levels

Extraction of DNA from PBMC of the EU was performed using the QIAamp DNA mini kit (QIAGEN, USA) according to the manufacturer’s protocol. Detection of any potential HIV-DNA was performed after nested PCR using the primer set JA79-JA82 (pol) as described previously (89, 90). More precisely, the sequences of primer set JA79-JA82 (pol) were as follows:

outer sense, 3’-ACAGGAGCAGATGATACAGTATTAG-5’ (HXB2 positions: 2328 - 2352); inner sense, 3’-GAAGATGGAAACCAAAAATGATAGG-5’ (HXB2 positions: 2371 - 2395); inner antisense, 3’-CAATTATGTTGACAGGTAGGTCC-5’ (HXB2 positions: 2508 - 2484); and outer antisense, 3’-CTGGCTTTAATTTTACTGGTACAG-5’ (HXB2 positions: 2593 - 2569).

Quantification of innate molecules in saliva

A sandwich immunoassay based protein array system (Biosource International, Camarillo, California, USA) was used to quantify the chemokines RANTES, MIP-1α, MIP-1β, MCP-1 and Eotaxin in whole saliva samples, according to the manufacturer’s instructions. Saliva samples were thawed, centrifuged and diluted 1:10 in PBS. The assay contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein. Antibody-coupled beads were incubated with the samples after which they were incubated with biotinylated detection antibody before finally being incubated with streptavidin phycoerythrin. A broad sensitivity range of standards (Biosource International) ranging from 1.95 to 40 000 pg/ml were used to help enable the quantifications of the chemokine concentrations and provide the greatest sensitivity. The immunoassay was then read by the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, California, USA) which uses Luminex fluorescent-bead-based technology (Luminex Corporation Austin, Texas, USA) and allows multiple measurements of the sample, resulting in effective quantification of the chemokines.

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used, according to the manufacturer’s protocols, to quantify the following molecules of interest
(additional saliva dilution in brackets): the alpha-defensins HNP-1-3 (1:1000), LL-37 (1:100) (both Hycult biotechnology, Uden, the Netherlands), SLPI (1:1000) (RD Systems Europe Ltd, Abingdon Oxon, UK).

**Statistical analysis**

Preliminary tests for normality showed a skewed distribution of values for all parameters measured. Thus, all analyses of results were performed with non-parametric tests. Inter-group variations were analyzed in parallel by comparing a single parameter from one group of individuals against the corresponding parameter of each other group using the unpaired two-tailed Mann Whitney test. For qualitative analyses, Fisher’s Exact Test was used. Calculations were performed by using the GraphPad Prism 5 software and, for the questionnaires answered by the EU and control subjects, SPSS version 16.0. To test for single-point association, two-sided P values for different genetic models were calculated using a Fischer exact test with no correction for multiple testing (GraphPad Instat). Correlation between protein levels in saliva and genotype for variations within the corresponding gene was analyzed using the Kruskal-Wallis rank sum test in R.
5. RESULTS AND DISCUSSION

5.1 The nature of the present cohort’s exposure to HIV-1

By evaluating the self-reported questionnaires (documented as a reliable way of determining sexual behaviors (91)) from the EU individuals at each time point as well as the clinical records of the HIV-positive spouse, the route and magnitude of HIV-1 exposure was determined (paper I and II). All EU answered questions regarding sexual behavior for the last six months as well as the latest two weeks prior to each study visit, in ordinal and nominal data, respectively. The results were quite homogenous in the group; the vast majority practiced unprotected oral sex but either abstained or performed anal sex with adequate protection. “Oral risk” determined as having unprotected oral intercourse with HIV-positive partner or casual partner with unknown HIV-serostatus was attributed to 24 of 25 EU (one subject did not respond to the question). “Anal risk” determined as having unprotected anal sex was attributed to 6 of 25 EU, however only two of these responded “sometimes/often” and the other four reported sporadic occasions, and mainly active intercourse as opposed to receptive intercourse. In total, 9 of 25 EU engaged in sexual acts outside the partnership during the study period; however the level of protection were the same as with the regular partner (mainly protected anal sex and unprotected oral sex).

The questionnaires revealed that most of the EU subjects, while performing unprotected receptive oral sex, often did so without receiving in the oral cavity the ejaculate from the partner. Although this probably lowers the rate of viral exposure through semen, it is still not a safe mode of oral sex since it is known that also the pre-ejaculatory fluid contains HIV-1 virions (92).

At Venhälsan all patients, and when applicable their partners, are informed about the risks of unprotected sexual intercourse, by the anal but also the oral route. Oral sex has earlier been determined as a fairly common mode of transmission at this clinic; between 1990 and 1992 a pilot study detected 28 newly (<1 year) HIV-infected individuals, of which six (21%) reported only oral unprotected sex with the respective partner/partners prior to transmission (93). Thus, the present finding of an MSM mileu where exclusive oral sex is frequent confirms these earlier data.
During the study period of two years the questionnaires revealed few deviations from the initial reported sexual behaviors, thus the exposure over time continued to be attributed mainly to the oral route and only rarely via anal sex.

### 5.2 Samples from EU subjects capable of neutralizing HIV-1 in vitro

To investigate whether samples from the EU differed in anti-HIV-1 capacity, we performed PBMC-based neutralization assays using either salivary (paper I and IV) or plasma (paper II) samples. Neutralization assays can be performed in several ways. One of the most common, and principally quite a different model from PBMC-based neutralization, is the TZM-assay using pseudotyped viruses. This assay is using viruses where only the outer structure (envelope) of HIV-1 is expressed (it is thus not a replication-competent virus), and the TZM-bl target cell. The latter form is more scalable, less time-consuming and is adequate when looking at a specific envelope-structure (as for example when developing vaccine constructs). The PBMC-assay rely on blood-donor cells which can be variable in their quality, however it is an assay more alike to the natural mechanisms of HIV-1 transmission (please see (94) for a more extensive comparison). In a PBMC-based neutralization assay, other antibodies than those directed against HIV env-epitopes can exhibit their neutralizing potential.

As whole saliva naturally contains known antiviral molecules, the neutralization capacity of whole saliva was fairly equal in EU and controls (15 of 25 EU and 9 of 22 controls-sic! Errata in paper I where it says 6 of 22 controls). When evaluating purified salivary IgA1 however, the differences between EU and controls were striking: 13 of 25 EU able to neutralize HIV-1 vs. 0 of 22 controls (p=0.001, Fischer’s exact test). An additional primary HIV-1 isolate (92/US/727) less sensitive to neutralization was used to confirm this activity, and 11 of 13 samples neutralized also this virus. Testing also sequential samples, the neutralizing capacity of the EU salivary IgA1 was largely unchanged over time (12 of 13 able to neutralize at last time point of the study). The neutralizing capacity was not due to salivary non-HIV-specific IgG, since we purified this antibody fraction in EU as well as controls, and did not detect any neutralizing capacity. Moreover, the possible effect of seminal remnants from the HIV-positive partners was excluded, since no prostate specific antigen (PSA) was detected in the salivary samples.
Since the HIV-1 exposure was attributed to the oral route we initially tested the saliva, but then went further to see whether a low-risk exposure orally could elicit also systemic neutralizing responses, in plasma. In whole plasma, 7 of 25 EU individuals at the first time point of the study could neutralize HIV-1 vs. 0 of 22 controls (p=0.01, Fischer’s exact test). We once again used BZ-167 as an initial screening virus, but further tested the seven neutralizing samples against two more primary isolates (92/US/727 and QZ-4589). Two of seven EU neutralized all three HIV-1 isolates, and the remaining five neutralized two isolates. Consecutive samples every six months were investigated for neutralizing capacity, and the results are summarized in Table 1.

Table 1. Longitudinal follow-up of the EU who neutralized HIV-1 in plasma samples at study start

<table>
<thead>
<tr>
<th>Study subject</th>
<th>0 mo.</th>
<th>6 mo.</th>
<th>12 mo.</th>
<th>18 mo.</th>
<th>24 mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3B</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6B</td>
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<td>1</td>
<td>2</td>
<td>2</td>
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<tr>
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<td>1</td>
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<td>*</td>
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</tr>
<tr>
<td>19B</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

0, 1, 2, 3 = number of HIV-1 isolates neutralized by each sample. * = sample not available

A total of 7/25 EU could neutralize HIV-1 at the study’s start, and those 7 were tested further for this functional activity over time. In contrast, none of 22 low-risk controls neutralized HIV-1 (p=0.01, Fisher’s exact test). The first and the last available plasma sample from each individual were tested against three HIV-1 isolates (BZ-167, 92/US/727 and QZ-4589), remaining samples were tested against two isolates (BZ-167, 92/US/727).

The mechanism of the neutralization was investigated by purifying IgA1 and testing this fraction as well as the IgA1-depleted fraction. Plasma IgA1 could alone neutralize in 6 of 7 EU subjects, whereas the IgA1-depleted fraction did not show any
neutralizing ability. Thus IgA1 seem to be the major mediator of this neutralization. The single EU having neutralizing capacity in whole plasma but in neither of the two isolated fractions is believed to neutralize with a synergistic effect of IgA1 and innate molecules. The specificity of the HIV-1 neutralizing IgA1 could however not be determined; no HIV-1 env specificity was detected either by ELISA or Western Blot. This argues that IgA1-mediated neutralization acts by one or several of the following ways: 1) by binding to HIV-1 targets such as the receptors CD4 or CCR5(95), 2) by binding to HLA-molecules (96), 3) by binding to epitopes displayed during the conformational changes when the virus fuses with its receptor(97), 4) by binding to internal HIV-1 proteins inside epithelial cells(98), 5) indeed by binding to HIV-1 env-epitopes, however too complex in their structure to be discovered by the methods used here(99).

In summary, all but one individual maintained HIV-1 neutralizing capacity at the last sampling; however the magnitude (as measured in numbers of isolates neutralized) were decreased in 4 of 6 EU subjects. This suggests that a low continuous (oral) exposure is able to maintain the anti-HIV-1 capacity but might not be sufficient to keep the initial breadth of the immune response. The only EU individual who completely lost his neutralizing capacity during sequential samples (19B), were infected with HIV-1 after the study had ended (this was later the case with one more of the EU subjects, however this individual was one of the two EU with a documented high-risk sexual behavior). The patient was however infected not by his regular partner but with a different HIV-1 virus clade (CRF-01AE). We can only speculate whether the initial neutralizing capacity was limited to the partner’s clade, if the response decreased also in vivo, and whether it was protective at all.

The obvious difference in neutralizing capacity between the oral mucosal samples (13 of 25 EU) and the systemic plasma samples (7 of 25 EU), can possibly be explained that the oral exposure more readily results in limited and local T-cell-independent anti-HIV IgA1 production at the site of exposure (48, 100), rather than a systemic involvement. Recent immunological research have contributed with clues why the oral mucosa might be a good inducer of T-cell independent IgA-production: in the gut-associated lymphoid tissue (GALT) there is co-localization of B-cells, A Proliferation-Inducing-Ligand (APRIL), activation-induced cytidine deaminase (AID) and other factors needed for class switch recombination (CSR) towards IgA.
independent of T-cells (87). Also there have been shown in human tonsils that viral double-stranded RNA can stimulate B-cells to Ig class switching through toll-like receptor 3 (TLR3)(101). This could be mediated through the exposure of virus debris or defective virus particles to the immunogenic sites of the tonsillar tissue. The EU having also a systemic neutralizing response, may have been exposed in a different and more profound fashion, as for example a single round of replication in the mucosa/submucosa that was self-limited or effectively hindered by local antibodies, T-cells or NK-cells (102). Supposedly this would have happened before this study started and several of these individuals had no awareness of their partner’s HIV-positive status, and subsequently a different sexual behavior together with a higher viral load in the partner might have existed.

5.3 HIV-1 specific T-cell responses in these EU are rare

Having detected functional anti-HIV-1 capacity systemically (in plasma), we set out to investigate in paper III whether also T-cell mediated immune responses existed in these EU with fairly low exposure. In the literature, most studies on T-cell responses among EU have investigated subjects with estimated higher exposure, such as commercial sex workers (59) or MSM performing unprotected anal sex (61).

Of all EU assayed, PBMC from three individuals responded against HIV-peptide pools in the ELISPOT assay (none of the 13 tested controls had any activity). One of these three was indeed an individual who reported having unprotected anal sex, while the other two belonged to the majority of subjects who only reported unprotected oral sex. Subject 3B responded to the HIV-1 Gag peptides (p55 and p 24) and the other two (14B and 15B) responded against Nef peptides.

To exclude an eventual silent HIV-infection (below detection level in routine diagnostics) eliciting these T-cell responses, we performed a highly sensitive polymerase chain reaction (PCR) in 12 EU individuals (including the three T-cell responders), but none HIV-DNA could be detected (<40 copies/µg DNA).

Of the three responders, one (3B) had a unique exposure profile since the partner of this individual had a treatment interruption during the course of the study. The partner subsequently experienced a sharp increase in HIV-RNA viral load, after which
treatment was re-initiated and the viral load once again quickly normalized. This made us specifically interested in the response profile of 3B, and thus intracellular staining was performed on two consecutive PBMC-samples to determine the nature of his HIV-specific cellular response. During the time when the partner had a high viral load, 3B exhibited strong HIV-specific responses both in the CD4+ and CD8+ subsets of T-cells. The CD4+ population secreted IFN-γ, MIP-1β and TNF-α, however not IL-2. The CD8+ population secreted IFN-γ, MIP-1β and TNF-α as well as IL-2. One year after the first sampling, when viral load in partner was once again undetectable, the CD4+ population still had HIV-specific responses secreting IFN-γ, MIP-1β and TNF-α, however at a lower rate. CD8+ responses were now undetectable.

The most probable way of eliciting HIV-specific T-cell responses in the CD4+ population is the exposure of virus debris leading to phagocytosis and presentation by MHC class II molecules(103). Since CD8+ T-cells classically recognize endogenous molecules, we do not dismiss that during the time of high viral load translation of viral proteins might have occurred. This can of course be due to a local infection that was successfully cleared; more plausible is perhaps the already known event of translation of un-integrated HIV-DNA, which is enough protein expression to promote cell activation (104), but not sufficient to establish an infection. Lastly, there is the possibility of cross-priming by professional antigen presenting cells (APCs), priming the CD8+ T-cells with exogenous antigens from for example virus infected cells (105).

Despite this very interesting individual and two more cases of HIV-specific T-cell responses, it is however obvious that this type of cellular immunity are rare in an EU cohort with low continuous HIV-1 exposure.
5.4 Innate soluble molecules increased in EU individuals

The natural anti-viral property of human saliva is well known, and several innate immune factors have been suggested to display an anti-HIV-1 activity, possibly contributing to the low rate of oral sexual transmission of HIV-1.

In paper IV we thus set out to determine the levels of CC-chemokines (RANTES, MIP-1 alfa, MIP-1 beta, MCP-1 and Eotaxin), alpha-defensins, LL-37, SLPI and lastly IgA2; being an antibody subtype engaged in innate as well as adaptive immunity(106). First we concluded that EU individuals had increased levels compared to controls of IgA2 (p=0.001) and all CC.chemokines measured (however the difference in MIP-1 alpha did not reach statistical significance). We could however not detect any differences in alpha-defensins, LL-37 or SLPI.

Moving on to the contribution of these CC-chemokines in the neutralization mediated by IgA1-depleted saliva (of both EU and controls), we showed that all CC-chemokines except MCP-1 indeed associated with the neutralizing capacity. Even though this have been discussed in theory, the only prior investigation of salivary innate factors comes from our own group(79), however this study was on highly HIV-1 exposed individuals with less documentation about the specific route of exposure. Moreover, we could see an association between having high levels of CC-chemokines and reporting having more oral unprotected sex and/or with several partners, further discussed in the next paragraph.

5.5 Associations with HIV-1 neutralizing capacity

Already in paper I we could see a trend regarding the EU able to neutralize HIV-1 and the viral load of the respective HIV-positive partner. This was further demonstrated in paper II, where the EU able to neutralize had had a partner with 1) higher viral load during the relation compared to non-neutralizing EU (p=0.01, Mann-Whitney test), and 2) the highest viral load occurring at a time point significantly closer to study start than compared with partners of non-neutralizing EU (p=0.02, Mann-Whitney test). (Figure 9, 10)
Peak viral load (VL) is defined as the highest level of HIV RNA copies measured in the respective HIV-positive partner during the time of relationship with the EU partner.

“Neutralizing” = the 7 EU with HIV-1 neutralizing capacity in plasma (median 96%, range 81-100%). “Non neutralizing” = the 18 EU lacking HIV-1 neutralizing capacity in plasma (median 0%, range 0-39%). Time was measured in months; from the date of the highest measured viral load in partner to the date of study start = first plasma sampling of the EU. (Hasselrot et al, AIDS Sep 2009)
This is the first time an association is seen between functional anti-HIV-1 responses in EU and the level of exposure, and suggests that these neutralizing responses indeed are acquired and not predestined. The finding of the time elapsed as a significant factor also suggests that earlier events (at least in the case of systemic neutralizing capacity, maybe not in the case of salivary neutralizing capacity) may have been important to elicit these responses, and the continuous (lower) exposure have been able to maintain it. Of interest, the 7 EU with neutralizing capacity was also over-represented among the couples who were informed of the HIV-positive diagnose during the relationship, thus previously having a sexual relation unaware of the infected status.

When determining the effect of salivary innate molecules on HIV-1 neutralization (paper IV), we could see an association between oral sex practices and the levels of CC-chemokines (RANTES, MIP-1 alfa, MIP-1 beta and Eotaxin) in IgA1-depleted saliva (in this case both in EU and controls). This is also suggesting environmental factors as eliciting (innate) immune responses as opposed to the studied genetic factors: the distribution of the investigated genetic polymorphisms were equal in EU subjects and controls, and when we compared the level of expression compared with the corresponding genotype in each individual we did neither see a correlation. In paper IV we discuss the possibility of allo-immunization causing these increased levels of pro-inflammatory molecules (107, 108), however further studies using for example the respective partners mononuclear cells (not available in our study) would be required to answer this question.
6 CONCLUSIONS

6.1 The oral route as an immunogenic environment

The oral mucosa has gained very diverse forms of attention during the course of HIV research (reviewed in (109)). While in the early 80’s a fear existed of contracting HIV via sharing a glass or a spoon with a HIV-positive individual, later on the time changed as to describing oral sex as almost “safe”.

No doubt there is a lot of HIV target cells in the oral cavity (foremost the tonsils and the adenoid lymphatic tissue) (110, 111) and animal models have shown rapid transmission via the oral route (112), there is however a greater antiviral defense in the hypotonic saliva compared to for example the genital tracts mucosal secretions, which can explain the lower infectivity by HIV-1 attributed to the oral route. The turnover in the oral cavity is also enormous; while approx 1 l of saliva is produced every day (113) (and swallowed down to the gastric acidic environment), seminal fluid can be present (as detected by prostate-specific antigen, PSA) in the vagina at least 48 hours after sexual intercourse (114). Still, we have here shown that the oral exposure to HIV, even though it might have been induced by earlier high-risk behaviors (before the present study started), is at the least able to maintain an anti-HIV capacity in salivary and systemic IgA1 (paper I and II), and to a lesser extent in systemic cellular immune responses (paper III). This could hypothetically be translated into a natural low-level boosting via the oral route of a presumably earlier initiated immune response. In paper IV, we detected increased innate immune responses in EU subjects compared to controls, however if this is due to HIV-1 exposure and/or an increased rate of unprotected oral sex acts is difficult to say.

Either way, if the stimulus of oral sex (with or without contagious HIV-1 material) is sufficient to induce functional innate immune responses, the immunogenic properties of the oral cavity may not be underestimated.
6.2 Acquired and not predestined immune responses

Since the beginning of all studies on EU individuals, there have been efforts to explore both possible genetic factors as well as acquired immune responses. Indeed, the only mechanism which deserves to be called “protective” in its proper meaning is a genetic mutation; the homozygous CCR5 Δ32 genotype (32). Different HLA alleles have also been found to correlate with reduced (and also increased) susceptibility to HIV-1 (70, 115).

In the present endeavor however, our findings suggest that these EU individuals immune responses against HIV-1 is rather a cause of environmental factors, such as viral load in the respective partner or the time since high viral load occurred (paper II). This is in agreement with some other related investigations in EU individuals (66), although our study was first to associate a functional response (in vitro) with the exposure measured as the partner’s viral burden. While we are careful not to call these responses protective, they are at the least believed as a footprint of HIV-1 exposure in an individual who clearly has not been infected. Also, we have found an association between sexual behavior and the amount of CC-chemokines in saliva (paper IV), something that leads us to speculate in the alloreactive potential of the immune response. Allo-immunization as a mechanism to elicit anti-HIV responses is very interesting and has maybe not gained the attention it deserves within the field of HIV research.

It needs to be underscored however, that the results presented here are laboratory and should not be implied in real life – having unprotected sex with multiple partners might induce more chemokines or other immune responses, but is certainly a very bad strategy in order to not acquire HIV.

6.3 Implications for vaccine design

Since the obstacles in investigating human mucosal samples are significant as already mentioned, the evaluations of mucosal immunology in HIV-vaccinated individuals (in clinical trials) are alarmingly absent. To facilitate future investigations, a standardization of sampling procedures would be helpful, both in the case of liquid samples (cervicovaginal and rectal lavages and saliva) and tissue samples (biopsies,
cytobrushes etc) (116). Mucosal samples are scarcer in volume than peripheral blood, thus there is a need of optimizing investigative techniques as to be able to use less material. Technology such as for example multiplex bead immunoassays available today to determine peptide or protein levels are working excellent when using plasma or cell supernatant samples, but need thorough evaluation when assessing mucosal samples due to higher viscosity. The present studies (paper I and II) have shown that assessing systemic and mucosal samples in parallel is feasible and reproducible, and in the aspect of IgA the results differ depending on the respective compartment: to evaluate the efficacy of a vaccine to induce IgA-responses, investigating plasma would be a very insensitive methodology. Although mucosal sampling and evaluation of for example IgA-responses is more difficult than systemic investigations as here mentioned, it is by all means not impossible and it is time to change the attitude and practices in order to better encounter the task of an effective HIV-vaccine.

How is it possible to optimally reinforce mucosal immunity by vaccination, either by using different routes, modes of delivery or by new adjuvants? In order to investigate this, HIV researchers have nothing to lose by collaborating even more closely with scientists in the field of basic immunology. At the international AIDS vaccines conference 2009 a few weeks ago, the proposal to use several different mucosal pathways in vaccinology against HIV-1 was discussed, especially in order to create an effective mucosal response (117). The studies of this thesis imply that the oral route may not be forgotten, if not to induce but to maintain, humoral and possibly innate mucosal immune responses.

6.4 Implications for clinical counseling

This thesis has discussed HIV-1 exposure, foremost via the oral route, in a group of MSM. In the MSM setting, both partners are usually exposed to the potentially infectious seminal fluid. Due to the fact that some couples do not practice anal sex at all or to a lesser extent (as in the present cohort), oral sex is probably a proportionally more important and more often practiced sexual behavior compared to the heterosexual setting. Lastly, it is suggested that unprotected oral sex have increased on the behalf of anal sex due to the HIV-epidemic as it is attributed a lower risk of HIV-transmission compared to the latter behavior (118). In summary, it is reasonable
to suggest that the oral sex practices and the subsequently risk-taking are different in this group compared to heterosexual couples. Combined with the fact that the MSM group the last decade experiences increased numbers of HIV-transmissions (in Sweden (10) as well as in other countries), it is the authors belief that clinical counseling regarding oral sex practices and its following risk-taking should be emphasized in this group of individuals. Oral sex presumably suffers more in the aspect of sexual satisfaction from using protection compared to anal sex; however the individual per se should be informed as belonging to a special risk group in this matter, in order to be able to take an informed decision. Even though no comparative studies between MSM and heterosexuals on HIV-transmission due to exclusive unprotected oral sex have been performed, I have here argued that the former group likely through this particular sexual behavior takes a greater risk of being HIV-infected.

6.5 Final words

Still after 20 years of research on EU individuals(119), the absence of the CCR5 receptor are still the only finding of a single protective mechanism. We might never find another “factor X”, but are forced to investigate the complex system of synergisms and cooperation in the delicate immune system. Furthermore, the field of HIV research has experienced many set-backs in vaccine trials where results from animal models (NHP) have differed from the results of (human) clinical trials – there is a discussion ongoing whether NHP studies really are worth the time, money and ethical issues it raises(120). Although the year 2009 hopefully will be remembered as the turning-point for HIV vaccine research after the first positive results ever in a clinical trial (121), there is unfortunately much work to be done before high-efficacy prevention is a reality. This underscores the importance of investigating the human immunophysiology, where samples from EU individuals are invaluable. They represent an experiment in nature, hopefully with effective mechanisms yet to be found against a very difficult virus.

Runtom i världen studeras grupper av s.k. HIV-exponerade icke infekterade individer (Exposed Uninfected = EU). Det är personer som varit utsatta för HIV men undgått att bli infekterade, t. ex. prostituerade kvinnor i Afrika, bebisar till HIV-positiva mödrar, intravenösa missbrukare eller hetero- alt. homosexuella par där en partner är HIV-positiv och den andra HIV-negativ (s.k. diskordanta par). Alla dessa individer studeras för att man tänker sig att de har ett naturligt starkare immunförsvar mot HIV än andra människor. Man undersöker både ärfliga faktorer och icke ärfliga. Om forskare kan förstå mer exakt hur deras immunförsvar bemöter HIV, hoppas man kunna föra den kunskapen vidare och på detta sätt hjälpa till i framställningen av ett framtida vaccin mot HIV.

I den här avhandlingen har 25 diskordanta par bestående av homosexuella svenska män studerats. De har samlats in på Södersjukhuset i Stockholm under åren 2001-2004, och utgör den enda studerade EU-gruppen i Skandinavien. EU-partnern (den HIV-negative) har läkarundersökt, provtagits samt lämnat in enkätser om sexuella beteenden var sjätte månad under två år.
Resultaten vi har sett efter att ha undersökt denna grupp kan sammanfattas i de fyra artiklarna som avhandlingen bygger på:

I. Expositionen för HIV i denna grupp skedde i majoriteten av fallen via munslemhinnorna, då de allra flesta hade skyddat analsex (eller inget analsex alls) men konsekvent oskyddat oralsex. Detta gjorde gruppen speciell, då andra studier av diskordanta par beskriver exposition via genitalia. Vi undersökte därför deras saliv för att se om deras antikroppar av IgA-typ (den vanligaste antikroppen i slemhinnor) kunde hindra HIV-infektion i labmiljö (*in vitro*). Den förmågan hade 13 av 25 EU-individer, jämfört med 0 av 22 kontrollindivider. Vidare såg vi att denna HIV-neutraliserande förmåga kvarstod hos 12 av 13 individer även vid sista provtagning, dvs höll i sig över tid.

II. Nu undersökte vi blodplasma, och även här kunde vissa EU neutralisera HIV *in vitro* (7 av 25 mot 0 av 22 kontroller). Neutralisationen berodde främst på IgA i blodet. Vi testade blodet mot flera HIV-varianter, och såg att – trots att svaret även här kvarstod över tid hos de flesta – bredden av svaret verklade minskas (dvs att de kunde neutralisera färre HIV-varianter vid sista provtagning). Här kunde vi också se något intressant: de EU som kunde neutralisera HIV hade haft en partner med högre HIV-virusmängd i blodet under förhållandet, och detta under en tidpunkt närmare studiestart, jämfört med EU som inte kunde neutralisera HIV.

III. Eftersom anti-HIV-svar fanns även i blodet och inte bara i slemhinnan, gick vi vidare och undersökte de immunceller (lymfocyter) som cirkulerar i blodet. Detta kan man säga är ett andra linjens immunförsvar efter antikroppar. Här minskade andelen ”svarare”, så att endast 3 av 25 EU hade celler som aktivt ”kände igen” HIV. En av de tre hade haft oskyddat analsex, men övriga två rapporterade endast oral exposition, vilket får anses anmärkningsvärt när man har ett cellulärt HIV-svar.

IV. Tillbaka i saliven undersökte vi nu om det allra första, mest direkta immunsvaret var bättre på att bemöta HIV hos våra EU-individer. Det direkta (även kallat ”innate”, som kommer före en antikroppspröduktion) immunsvaret består av lösliga faktorer (peptider eller proteiner), och är ett ospecifikt försvar mot många mikrober. Vi kunde se att EU-individerna hade högre halter av sådana faktorer jämfört med kontrollerna. Det
konstaterades även en association mellan sådana faktorer och förmågan att neutralisera HIV *in vitro* med saliv (där man tagit bort IgA). Här undersökte vi även kända genmutationer som kan reglera produktionen av dessa faktorer, men sådana fanns inte hos dessa EU. Däremot kunde vi se en association mellan högre frekvens av oralsex och/eller flera partners och nivån av anti-HIV faktorer.

Sammantaget har de ovan beskrivna resultaten bidragit till kunskapen om EU och deras immunsvar mot HIV på följande sätt:

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