Approaches to the development of broadly protective HIV vaccines: challenges posed by the genetic, biological and antigenic variability of HIV-1

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Introduction

The Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that since the start of the Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) epidemic close to 53 million people worldwide have become infected with HIV. An overwhelming majority of those infections have occurred in developing countries. Over the past years, the AIDS pandemic has claimed the lives of more than 18 million people, placing HIV/AIDS among three major fatal infectious diseases of mankind, together with malaria and tuberculosis.

At present, the HIV pandemic continues its accelerated spread at a rate of 15 000 new infections every day, generating a total of 5.4 million newly infected individuals only in 1999. Facing this situation, there is a growing consensus among scientific, public health and grass-root communities, that safe, effective and affordable HIV vaccine would offer the best hope and an important complementary tool for the future control of this pandemic.

The development of an HIV vaccine is confronting multiple scientific, economic, ethical and logistic challenges, which could adequately be addressed only through an extensive international collaborative effort. A key point in this strategy is the active involvement of developing countries, which would provide invaluable opportunities for steady and reliable progress towards a safe, effective and globally affordable HIV vaccine.

Since 1989, two UN agencies, first WHO and later UNAIDS have been actively involved in international aspects related to promotion and coordination in the area of HIV vaccine development and evaluation. A joint WHO-UNAIDS HIV Vaccine Initiative (HVI) was established from 1st January 2000, based on complementary expertise and advantages provided by both WHO and UNAIDS.

The WHO-UNAIDS HVI activities are guided by a Vaccine Advisory Committee (VAC). The VAC Members (listed in Annex I) represent leading national and international institutions from developed and developing countries involved in HIV vaccine development and evaluation. A meeting of the VAC took place from 21–23 February 2000 at the WHO Headquarters in Geneva with an objective of refining goals, objectives and priority activities to be pursued by the newly created WHO-UNAIDS HVI.

The meeting was chaired by Dr Barry Bloom and had two open scientific briefing session. The scientific session was attended by 54 international experts in the areas of virology, immunology, vaccine development,

*This report contains the collective views of an international group of experts, and does not necessarily represent the decisions or the stated policy of the World Health Organization.

**From the WHO-UNAIDS HIV Vaccine Advisory Committee (see Appendix I for members) and International Scientific Expert Group (listed in Appendix II).

Requests for reprints to: Dr Saladin Osmanov, WHO-UNAIDS HIV Vaccine Initiative, Health Technology and Pharmaceuticals, World Health Organization, 1211 Geneva 27, Switzerland. Email: osmanovs@who.ch

and clinical trials (list of scientific experts is given in Annex II), who addressed the following two major topics:

1. Approaches to the development of broadly protective HIV/AIDS Vaccines: challenges posed by genetic, biological and antigenic variability of HIV-1 (*This session was co-sponsored by the Office of AIDS Research (OAR) of the National Institutes of Health, NIH, U.S.A*), and
2. State of the art of therapeutic vaccines against HIV/AIDS.

This report provides summary of discussions, conclusions and recommendations that were made by the participants of the scientific briefing session.

Approaches to the development of broadly protective HIV/AIDS Vaccines: challenges posed by genetic, biological and antigenic variability of HIV-1

Genetic variability of HIV-1 (Presenters: F. McCutchan, M. Peeters, Y. Takebe , M. Kalish, J. Goudsmit, J. Mullins, B. Korber , D. Burke)

Molecular characterization and genetic classification of HIV-1

New and pertinent information was presented regarding the genetic diversity of HIV and its relationship to vaccine design. From an analysis of 170 virtually full-length (FL) genome sequences, several important observations were made. First, the international HIV database of published FL sequences has achieved a better balance with respect to the origins and geographical distribution of efforts to characterize HIV strains on a global basis, including more than 100 strains from Africa, 20 from Asia, and 50 from Europe and the Americas.

The newly generated data has permitted the identification of geographically restricted, significant sequence clusters within subtypes C, D, F and within a circulating recombinant form (CRF) AE, distinguishing strains from India versus African strains; Zaire versus East Africa; Cameroon as compared to other countries in Africa; and between HIV-1 strains circulating in Asia and Africa, respectively. A putative new subtype, possibly to be named subtype 'L', was described, and was found most commonly in Senegal.

The characteristics of inter-subtype recombinant HIV-1 strains prevalent in several heterosexual and injecting-drug user (IDU) epidemics were compared. Among HIV-1 isolates obtained from cases of heterosexual transmission in several African countries multiple recombinant HIV-1 forms were found, including ten AD recombinants, nine AC recombinants, and two BE recombinants, all of which represented unique recombinant forms obtained from a single individual. In all cases the inter-subtype recombination patterns reflected the local mixture of subtypes.

Viruses from multiple IDU epidemics have now been analyzed, mostly by C2V3 sequencing, but also by FL genome sequencing in cases of newly emerging epidemics in Russia (Kaliningrad) and China. Almost without exception, the inter-patient diversity of HIV-1 strains from these epidemics was extraordinarily low. For example, the analysis of FL sequences revealed the presence of two novel BC recombinant forms among IDUs in China, which varied, on average, by less than 1% for the full genome of isolates obtained from different patients. The reasons for such low levels of heterogeneity are not well understood yet, and could be due to sampling close to the time of introduction or emergence of new CRFs, although other factors of HIV transmission via IDU exposure may also contribute.

Sentinel surveillance studies continue to provide a rich source of new information of HIV genetic diversity. In a study of 450 seroconverters among military personnel in the U.S.A. studied over the past three years, approximately 5% of new infections were due to non-B subtypes; in all cases these findings were confirmed by FL genome sequencing. The study protocol was designed in a way to generate detailed epidemiological data regarding the perceived HIV exposures, their locations and estimated time of infection. The non-B strains identified in this study included one putative subtype L strain, one of the first BE recombinants found in the USA, the first case of AG (IbNG) recombinant, and the first case of AE CRF of African origin outside Africa, as opposed to the Asian AE subcluster. While the majority of infections were most likely linked to exposure overseas, the subtype C and AE infections were acquired in the USA, accounting for 20% of the non-B infections.

Molecular epidemiology of HIV-1

The genetic diversity of HIV-1 in Africa was the focus of another study sponsored by the Agence Nationale de Reserche sur le SIDA (ANRS, France), in which 1400 samples from 8 African countries were characterized by heteroduplex mobility assay (HMA) and sequence analysis. The predominance of subtype A, including the CRF AG IbNG was documented, which varied from more than 80% in West Africa to less than 30% in Central Africa, with a decreasing gradient from West to East. Overall, the most prevalent subtypes in Mali, Niger and Nigeria were subtypes A and G, while in Chad subtype A and a geographically distinct subtype D cluster were predominant. In Cameroon, subtypes A and a regional F2 sub-cluster were mainly found. The

highest diversity of subtypes was found in the Democratic Republic of Congo (DRC), the former Zaire. In most of East Africa, subtypes A and D were prevalent in varying proportions, while in Tanzania, subtypes A, C, and D were present in roughly equal proportions. The HIV epidemics in Ethiopia, Zambia and South Africa were overwhelmingly dominated by subtype C strains.

Inter-subtype recombination was frequently found in that study, which was ascertained by comparative partial *gag* and *env* sequencing, as well as by FL genome sequencing. The fraction of recombinants ranged from 30–35% in DRC and Nigeria to an intermediate level of 12–15% in Chad, Gabon and Senegal, and to a low level of 7% in Cameroon. The CRF IbNG appeared to be a major prevalent strain in Senegal, Nigeria, Gabon and Cameroon, but was found much less frequently in DRC and Chad. Four new FL AG(IbNG) sequences from Senegal and Cameroon were documented, confirming the maintenance of the IgNG structure through a massive epidemic spread in this region. Again a certain gradient was seen in the distribution of the IbNG strains with 84% of IbNG prevalence among the Senegalese samples, but only 15% in DRC. The prototype recombinant form A/G/K (I)/J (BFP90) was found at a noticeable level in Senegal, Mali and Nigeria, which qualified this recombinant stain as a CRF. In addition, a new A/J CRF with a different structure was described among samples from Senegal, Cameroon and the Central African Republic. This picture of an extraordinary diversity was further augmented by identification of significant sub-clusters within subtypes, and a high proportion of complex recombinant and unclassified forms. Based on sequencing of the protease and part of the reverse transcriptase (RT) genes, 17 samples in this study remained unclassified. Finally, a new recombinant between groups M and O of HIV-1 also was described.

Two regional studies of HIV diversity in Asia were presented. The initial study was conducted in Myanmar in 1997, which by that time had the second largest epidemic in South East Asia with a rapid spread of HIV among IDU. Since 1989, the HIV prevalence among commercial sex workers (CSW) and IDU had reached the levels of 30% and 62%, respectively. It was reported that subtype B' strains (a Thai-B cluster within subtype B) predominated among IDU, while CRF01_AE dominated the heterosexual epidemic. In a follow up study, 34 samples newly collected in Mandalay, central Myanmar in 1999, were characterized by partial *gag* and *env* sequencing, which revealed the presence of subtype C strains in 20% of cases, in addition to AE and B' strains. One-fourth of the strains exhibited discordance in *gag* and *env* sequencing, suggesting the presence of recombination. Based on the sequence of 1.6 kb derived from plasma RNA, three different recombinant forms were identified, including B'C (two of them with different recombination breakpoints), and B'CE recombinants. The 'parental' strains of these recombinants resemble subtypes C and B' circulating in Yunnan, China, suggesting close relationship of the epidemics in Myanmar and China.

A recent study of HIV diversity in the USA. was presented, in which 542 samples were studied, first by a V3-peptide-binding enzyme immunoassay (PEIA), after which samples with apparent non-B subtype reactivity were characterized by partial *env* sequencing. The samples were obtained from African Americans (52%), white (25%), Hispanic (19%) individuals, 71% were males. The distribution of major reported risks of HIV acquisition was as follows: 55% heterosexual intercourse, 31% homosexual, 8% heterosexual + IDU, and 6% homosexual + IDU. In the initial V3-serotyping test, 65% of the samples clearly reacted as serotype B, 15% showed single non-B sero-reactivity, 7% were reactive to multiple peptides, and 15% were untypable by this method. However, the great majority of samples with non-B sero-reactivity, appeared to be subtype B infections when characterized by genetic sequencing, and only 1.6% of cases were proven as non-B genetic subtypes, including 6 subtype A infections, 2 subtype F and one novel AG recombinant. Among 179 subtype B sequences, no sub-clustering by geography or route of transmission could be seen. The cases of discordant results between V3-serotyping and genetic sequencing were mostly associated with an increased diversity in the V3 loop. Most of the genetically confirmed non-B infections were identified in individuals born outside of the USA, and the strain subtypes were typically representative of the subtypes that had been reported in respective countries of origin. Two non-B infections, both due to subtype A strains, were from American-born persons who had never traveled outside the USA. In summary, the serotyping method is becoming more efficient for identification of non-B subtype infections; occurrence of non-B subtype infections remains uncommon in the USA. although, secondary transmissions begin to be documented.

Methodological aspects of HIV-1 genetic characterization

With this complexity in mind, subsequent speakers addressed issues related to: (a) need for universal, simple assays for HIV-1 subtyping, especially in the context of HIV vaccine trials in sites with high diversity of HIV strains; (b) problems of HIV nomenclature and classification; and (c) the impact of variation on selection of prototype HIV-1 strains to optimize chances of achieving broadly cross-reactive immune responses and vaccine-induced cross-protection.

The development of a one tube, real time HIV subtype identification test was reviewed. The test was designed

to overcome differences in detection sensitivity between different subtypes, extending to HIV-1 groups M, O, and N. The test is based on the use of conserved primer, capture and probe sequences in long terminal repeat sequences (LTR) with a distinct fluorescent end-point for as many as 8 subtypes/groups. In a pediatric study, there was 85% concordance between the test results at 6 weeks of age and the HIV serostatus of the same children at 18 months after birth. There are good prospects for the test encompassing subtypes A, D and AE in the near future.

The nomenclature of HIV is becoming increasingly challenging and complex. Criteria for identification of new HIV subtypes and CRF have been proposed, as follows: (1) the strain should be obtained from at least 3 individuals who are not linked by a common source of HIV transmission; (2) three FL genome sequences have to be obtained; (3) the inter-clade distances should be similar to those between subtypes B and D, which in retrospect should have been classified as one subtype; (4) for CRF, three FL genome sequences have to be obtained, demonstrating the same structure plus all the above. Presently, the subtypes A, B + D, C, F, G, H, J, K, and at least 4 CRF, including AE, AG (IbNG), AB and BC satisfy these criteria.

The current HIV sequence database includes more than 37 000 genetic sequence entries. The Los Alamos National Laboratory has organized these sequences on their web-site, so that sequences may be downloaded by country of origin, date, subtype, genome region, and other parameters.

A review of an extensive database of HIV cytotoxic T lymphocyte (CTL) epitopes was presented, with an emphasis on CTL variability among and within HIV-1 subtypes in conjunction with an extensive HLA complexity contributing to epitope recognition. With respect to defined CTL epitopes, a correlation was noted between epitope density and sequence conservation; possibly the use of subtype B strain for screening is skewing the database. The conservation of CTL epitopes varied by gene, with the highest in *gag*, and the lowest in *env*. In view of these complexities, there was no common agreement with regard to the effect of CTL epitope variation for the breadth of HIV vaccine protection

Evolutionary aspects of HIV variability

The origins of HIV-1 subtypes were briefly discussed in terms of exploration of retrospective diversification rates, attempting to pinpoint an estimated time of emergence for individual subtypes and CRFs. For this analysis, all known recombinants and unclassified strains were excluded, and a uniform divergence rate was assumed. According to this study, the common ancestor of the HIV-1 subtypes was present around the year 1930, although these estimates had large margins of error. Application of somewhat variable evolutionary rates gave essentially the same answer. In addition, the common ancestor of subtypes B and D was estimated to be present in 1950, and the emergence of subtype B, probably, occurred in 1970s.

The theme of evolutionary rates was continued with a presentation of an investigation that applied coalescent methods to determine the timing of the most recent common ancestor of HIV strains in several regional epidemics. The results of this study were concordant with other data for two subtype B epidemics in the USA and Thailand. However, application of the same methodology suggested that the start of the epidemic with AE virus in Thailand should have been 7 years earlier, than it was actually detected there. To explain this discrepancy, unequal evolutionary rates among HIV subtypes in the *env* gene were suggested. Problems concerning the origins of AE as a result of unequal evolution rates in the *env* gene versus recombination were discussed, although the origins of a number of other recombinants did not seem to pose this type of problem, and could be attributed to the phenomenon of inter-subtype recombination. The question as to the probability of differential evolution of different *env* genes was raised.

With regard to selection of a prototype strain that would represent the most common and cross-reactive immunogens, it was hypothesized that the use of the most recent common ancestor (MRCA) may help in 'taming' the extensive HIV diversity. Experimentally, in a model system where macaques were infected with an *in vivo* passaged SIV-BK28 strain, this approach allowed the generation of a sequence for the MRCA strain, which corresponded with 97–98% of the input sequences.

Search for correlation between HIV-1 genetic subtypes and immunotypes

The session concluded with a presentation on genotype/immunotype congruencies in HIV and other viruses. The possible relationships can range from no relationship to a universal relation (one strain covers all), to diffuse immunotypes, to the extreme of totally discrete immunotypes for each strain. The earlier data on cross-reactivity in neutralization serotypes and CTL was reviewed, noting that in one study correspondence of genetic subtype neutralization patterns between subtypes B and AE, while in other studies that relationship between genetic subtypes and neutralization serotypes was not observed. With regard to CTL cross-reactivity, at least one study was published showing a gradient of cross-reactivity in different genes, ranging from the best in *gag* to the worst in *env*. The general picture is one of a complex matrix of partial cross-reactivity of humoral and cellular immune responses

against HIV-1, but still not excluding the possible existence of discrete HIV-1 immunotypes.

Viewpoints on this relationships have shifted several times over the recent time. In 1984, a universal HIV vaccine was first envisaged. By 1988, with the recognition of the principal neutralization domain (PND) in the V3 loop, the pendulum swung to the other extreme, with some data suggesting that each strain would represent a unique immunotype. Following the initial recognition of HIV genetic subtypes in 1993, a model of discrete immunotypes, perhaps corresponding in part to genetic subtypes was anticipated. By 1997, the generated data on antibody and CTL cross-reactivity resurrected the idea of a universal, extensively cross-reactive HIV vaccine ensured by a single strain. For 2000, two ideas were entertained: (1) the idea of diffuse immunotypes with the quantitatively best reactivity on the same subtype and weaker reactivity as genetic distances increases and (2) the idea of discrete immunotypes with quantitatively best reactivity within the same immunotype, which could contain members of several genotypes.

When compared to other viral diseases, in general, a good congruence could be seen between genotypes and immunotypes where divergence greater than 10% typically predicted a separate immunotype. It remains to be seen whether HIV will conform to this paradigm.

Lessons learned from studies on natural history and HIV/AIDS pathogenesis (Presenters: H. Sheppard, B. Walker, J.-L. Virelizier, G. Scarlatti, A.M. Aubertin)

Viral load and disease progression

A set of data on the role of viral load as a predictive marker for disease progression, and as an end-point for HIV vaccine efficacy trials was reviewed. It was suggested that viral load measurement may lose its predictive value due to possible interference by early initiation of highly active anti-retroviral therapy (HAART). The studies presented included two natural history cohorts, the San Francisco Men's Health Study (SFMHS) and the HIVNET Vaccine Preparedness Study (VPS). In the first study, 1034 HIV-negative men-who-have-sex-with-men (MSM) were recruited in a cohort since 1984, in which 400 cases of HIV infections were observed. The infected individuals from the SFMHS presented with baseline viral load levels similar to those described in other MSM cohorts, which were highly predictive of clinical outcomes of HIV infection and disease progression. The VPS cohort was composed of 4892 seronegative individuals considered at a higher risk for HIV infection, and were followed since 1995. In the course of 18 months follow-up there were 104 HIV infections, who were subsequently enrolled in another study on natural history of primary HIV infection. Additional 52 seroconvertors from the SFMHS cohort were also included in this study of primary HIV infection. The preliminary results showed that at the time of diagnosis most individuals had already reached the viral set point with mean viral load levels at approximately 15 000 copies/ml. The distribution of mean viral load levels was similar to those observed in other natural cohort studies. The predictive value of the viral load level as a prognostic marker will be further evaluated.

Host factors for the control of virus replication and disease progression

Data was presented on correlation between the Δ32/CCR5 heterozygous mutation and disease progression in the SFMHS cohort. The results of the study confirmed earlier reports on the association of this mutation with a significantly delayed disease progression. Specifically, this study demonstrated a 2–5-fold reduction in mean viral load levels among individuals who had the Δ32/CCR5 heterozygous mutation ($p = 0.02$). These results provided additional indirect evidence that even a modest 0.5 *log* reduction in viral load levels may have a significant impact on clinical outcomes of HIV-1 infection, and, possibly, on the efficiency of virus transmission.

The results of another natural history study of patients undergoing an interruption of HAART were further reviewed. It showed that HAART treatment interruption was followed by a rapid return to pre-treatment viral load levels, proving that a reservoir of viable and infectious virus pool persists in long-living infected cells despite a very aggressive anti-retroviral treatment. It was also observed that after the re-start of the HAART, viral RNA levels quickly decreased to undetectable levels, while pro-viral DNA load was maintained at a new steady state level after an initial decrease. Furthermore, the viral and pro-viral load levels were found to be in linear correlation with disease progression both in patients with a steady progression and long-term non-progressors (LTNP), although absolute viral load levels were lower in the latter group. Interestingly, the pro-viral DNA set point levels in the HAART-receiving group were comparable to those in the LTNP group, suggesting that pro-viral DNA load may be a useful marker for disease progression, and thus could be used as an end point in HIV vaccine efficacy trials. In addition, it was shown that the viral load dynamics were associated with cellular activation markers, such as β2-microglobulin and CD38+CD8+ cells, which also displayed a set point behaviour.

Further associations of the viral set point were identified, which were related to CTL activity against HIV-infected cells. While discontinuation of HAART coincides with a rebound of the viral load to pre-treatment levels, the repeated treatment interruption in some cases can result in a control of the viral load levels,

even in the absence of therapy, which was attributed to increased CTL responses. Also, it was shown that not all human leucocyte antigen (HLA) class I alleles could equally be involved in CTL responses. In fact, distribution by geography and ethnic background could be seen. Using the ELISPOT assay to map the genetic restriction in recognition of p17 and p24 CTL epitopes, an inverse pattern of immune dominance was observed, with a predominant response to p17 in the Caucasian population, and to p24 in the non-Caucasian group. Also, it was suggested that different CTL epitopes could be targeted at different stages of infection. The CTLs recognizing A201-restricted p17 (SL9) epitope were rarely associated with early/acute stages of infection, but appeared to be more prominent at late stages of disease. The use of peptide major histompatability complex (MHC) tetramers did not provide evidence of correlation between anti-p17 (SL9) CTL response and the control of viremia. However, it was felt very unlikely that recognition of a single epitope could correlate with the LTNP status, since in earlier studies it was demonstrated that the LTNP status was frequently associated with broad CTL responses directed against several epitopes. The results of this study indicated that the MHC-tetramer analysis was less sensitive, compared to the intracellular cytokine staining method.

Based on the fact that the Δ32-deletion in the CCR5 gene is not associated with any known pathological effect, it was suggested that β-chemokines, the ligands to the CCR5 receptor, could be used as a therapy or in conjunction with an HIV vaccine, in order to prevent HIV attachment and entry into cells. A potential approach for HIV vaccines could also target an increased production of antiviral chemokines. Indeed, as shown *in vitro*, some antigen-specific recognition was accompanied by significant stimulation of β-chemokine production by T cells. It is therefore conceivable that triggering the chemokine-release by CD8+ or CD4+ cells could result in down-regulation of chemokine receptors on the cell surface, and consequently limit HIV infection. This type of approach, if confirmed in humans, would likely broaden the repertoire for protection, since a large majority of primary HIV isolates of different subtypes appear to be susceptible to the anti-retroviral activity of β-chemokines, unlike the situation observed with the antibody-mediated neutralization.

Biological variability of HIV-1 in vivo

The following presentation, focusing on HIV-1 biological variability, confirmed previously reported results demonstrating that the majority of HIV isolates obtained close to early stages of HIV infection both in adults and children were characterized by an R5-phenotype. Disease progression in HIV-infected individuals was shown to be associated with a switch in chemokine receptor usage of viral isolates from monotropic R5 to dual tropic R5X4, and further to monotropic X4 isolates in patients with a progressive course of infection. A study of the viral co-receptor usage in 5 slow-progressing and 4 fast-progressing HIV-infected children, demonstrated that multi-tropic HIV strains were predominant in fast progressors, while only monotropic X4 strains were seen in children with slowly progressing disease. In addition, it was found that HIV isolates at late stages of the disease were characterized by an increased resistance both to α- and β-chemokines, the natural ligands of the CXCR4 and CCR5 receptors, confirming a virtual relationship between viral phenotype/co-receptor usage, sensitivity to chemokines and disease progression, which merits further investigation.

The determinants of HIV-1 biological phenotypes and co-receptor usage were investigated *in vitro* by conducting multiple passages of primary dual tropic X4R5 isolates in cell lines expressing different chemokine receptors. These experiments showed that the viral population within a single individual could vary substantially over a short period of time, within a few months. The V3 sequence analysis of selected viral variants supported earlier published data on correlation between biological phenotype, V3 genotype and patterns of amino acid substitutions in the V3 loop for monotropic R5- and X4-using strains, in close analogy with the non-syncytium-inducing (NSI) and syncytium-inducing (SI) phenotypes/genotypes, respectively. Similarly, the overall amino acid net charge of the V3 and V2C2 regions was higher in X4 viruses as compared to R5-using strains. However, the V2-C2 amino acid sequences both in R5 and X4 viruses varied widely, and no specific amino acid variation could be identified as a determinant of co-receptor usage. Interestingly, the dual tropic X4R5 viruses were preferentially characterized by X4-related sequences in the V3 loop, but resembled more the R5 variants in the C2V2 region.

Additional studies were conducted to explore the role of viral heterogeneity in HIV infected children and disease progression, using HMA analysis of the V1C2 envelope region. Two of 4 children with fast disease progression displayed a widely heterogeneous viral population at the time close to birth, whereas in slowly progressing children this level of heterogeneity could be seen only 6 months after birth. These data indicate that transmission of multiple variants may be a more frequent phenomenon than previously believed, the mechanisms of which need further investigation.

The role of the HIV-1 biological variation in relation to HIV vaccine development was investigated in animal models, using infection of rhesus macaques with chimeric simian/human immunodeficiency viruses (SHIV)

viruses, which encode either X4 or R5 HIV-1 envelope proteins. In previously conducted studies, most of the chimeric SHIVs were based on the use of X4- or dual X4R5-tropic prototype strains, which is in contrast with the genuine situation, where the majority of transmitting HIV-1 strains are R5-tropic. In order to improve on this discrepancy, two chimera SHIVsbg strains containing R5-type envelopes from subtype B (SHIV Bx08) and subtype A (SHIV RW) were designed and characterized in full detail. It was demonstrated that both chimeric strains replicated well in human peripheral blood mononuclear cells (PBMC). However, their replication was less efficient in PBMC derived from macaques, while both of the SHIVs could effectively infect macaques *in vivo* via intravenous challenge. Sequential passages of the SHIV strains in macaques revealed an increasing replication capacity of both chimeric strains, as measured by the levels of the DNA and RNA viral load in infected macaques. The SHIV strain carrying X4 envelope, as compared to the R5- based SHIV strains, was characterized by higher replication capacity, higher number of infected cells, plasma RNA viral load and a more pronounced CD4 depletion. Furthermore, data were presented comparing the biological properties of the *nef* gene, derived from SIV and HIV. The results showed that substitution of the SIV *nef* gene by that one of HIV in SHIV chimeras did not have any significant effect on replication capacity and viral load levels in macaques, as compared to wild-type strains. These experiments demonstrated the usefulness of the SHIV/macaque model in determining the contribution of specific genes to the pathogenic properties of HIV and SIV.

Role of cell-mediated immunity as a potential mechanism of HIV vaccine-induced protection (Presenters: G. Ferrari, D. Birx, A. McMichael, H. Cao)

Cell-mediated immune responses in HIV-infected individuals

Several reports in the scientific literature suggest that CTL responses can play a role in protection against HIV infection and disease progression. Data have been generated by studies in acute seroconverters showing a correlation between appearance of detectable CTL activities and decline of virus load. Moreover, in exposed uninfected individuals, detectable anti-HIV specific CTL responses seem to be the only sign of previous exposure to the virus. If the CTL response can truly impact the development of an overt disease, as suggested by studies in LTNP, a more recent set of data now indicates that a T-helper response may also be a critical component in delaying disease progression.

It is well documented that virus levels reach their peak approximately 3 weeks after infection, as shown in macaques infected with SIV, as well as in HIV infection in humans. The CTL response reaches its maximum activity a few days later following the establishment of the highest virus level. In studies of the acute stage of HIV infection, using MHC class I tetramers loaded with immunodominant epitopic peptides, it was shown that during peak CTL responses up to 1–8% of CD8+ T cells are directed against a single epitope. Before exposure, the frequency of such T cells is less than one in a million. Therefore, the T cells must be dividing in response to HIV infection at a rate of approximately 1 division per day. The intensity of this response may be comparable to, or slightly less pronounced, than in the case of an acute Epstein-Barr virus infection, which recruits up to 5–40% of CD8+ T cells. It is as yet uncertain how functional and effective these responses to HIV infection are, since tetramer staining allows only for ennumerating cells with a specific T cell receptor. After clearance of HIV infection, and in the absence of antigen stimulation, the acute CTL response starts fading very rapidly, since these cells have been shown to be highly prone to apoptosis *in vitro*, with a half-life of approximately 7 days. However, studies using limiting dilution and T cell cloning, indicate that soon after primary infection, there are T cells that are capable of multiple divisions *in vitro*, that are present at a frequency of between 1/1000 and 1/10 000, representing most probably long-term memory cells. In vaccination approaches based on the use of non-persisting antigen, any protective response would be originating from this population of memory cells. These memory cells usually are present in numbers at least 100 times higher than naïve T cells with an advantage of 6 times higher expansion rates. In addition, these cells can respond by secreting interferon-γ (IFN-γ), as well as other cytokines and chemokines within 6 hours after antigen stimulation. All of these data confirm that an individual primed for a CTL response can promptly react upon virus exposure. Studies in animal models indicate that a specific CTL priming by vaccination against a broad range of viral and parasite pathogens (e.g., influenza, herpes simplex, malaria and others) could be effective in preventing infection or controlling disease. The expectations for an effective HIV vaccine resides mainly on the objective of discovering vaccine approaches capable of induction of long-term HIV specific memory cells, that would ensure a rapid and broadly reactive CTL response against multiple HIV epitopes upon exposure to the virus. The induction of T helper responses to several viral domains should also greatly facilitate an accelerated and optimal CTL response, required for vaccine-induced protection.

Cell mediated immune responses in uninfected vaccine recipients

In this session, data was presented from the latest NIH-sponsored trials of prime-boost regimens with a subtype B based Canarypox-vector (ALVAC) expressing gp120 MN, the transmembrane portion of gp41 IIIB, the full

gag IIIB and protease as a priming regimen in combination with two doses of recombinant gp120 protein given as a boost. It was shown that this vaccination strategy could induce CTL responses that became detectable as early as 2 weeks after the second immunization and in a subset of responders lasted up to 2 years after the last immunization. The vaccinees demonstrated CTL responses with frequencies comparable to those previously reported in LTNP, and also effective in lysing autologous CD4+ lymphocytes infected with primary NSI strains of different HIV-1 subtypes, including subtypes A through F. The frequency of these broadly reactive responses was increased in cases when more than one HIV-1 antigen was present in the vaccine, i.e. more frequent in vaccinees who received vaccines including *env-gag* immunogens, as compared to those who received only *env*-based candidate vaccine.

The analysis of HLA restriction and epitope recognition in vaccine recipients produced somewhat surprising results. In fact, 4 out of 6 vaccinees recognized CTL epitopes not previously described in HIV-infected individuals. The possibility that uninfected recipients of HIV vaccines could mount CTL responses to immunodominant epitopes not described in infected individuals was further explored, focusing on the HLA A^*0201-restricted response against the p17 epitope SLYNTVATL. Surprisingly, in 10 HLA A^*0201^+ vaccinees with detectable anti-Gag CTL responses, none of them mounted an anti-p17 CTL response detectable by any of the described assays (51Cr release, tetramer, as well as IFN-γ ELISPOT assay). This lack of SLYNTVATL responsiveness occurred despite expression of this epitope in both of the recombinant constructs used as a vaccine and for the *in vitro* amplification of CTL. Taken together, these data suggest that currently available vaccines can elicit long-lasting, cross-reactive anti-HIV CTL responses in a subset of vaccinees, although these responses may involve recognition of different epitopes, as well as utilization of different HLA-restricting elements.

Another study was reported, in which CTL responses were evaluated in 20 individuals infected with either subtype A or subtype D strains of HIV-1. The possibility of dual infections was excluded by the V3 sequence analysis. *In vitro* PBMC stimulation of frozen samples with autologous subtype-specific antigens revealed the presence of cross-reactive CTL responses against cells expressing antigens derived from subtypes A, B and D. However, the highest response was directed against homologous subtype targets. The analysis of CTL frequencies showed that they were also characterized by preferential reactivity against the homologous subtype targets, which was further confirmed using the IFN-γ ELISPOT method. It was encouraging to see that subtype B-specific antigens were able to amplify cross-reactive CTL responses in subjects infected with subtype A and D strains of HIV-1. This study resulted in an important finding of at least 3 novel CTL epitopes, including A29/gp120/KMTFEPIPIH (subtype A), B7/gp41/IPRRIRQGF (subtype A) and B42,44/p24/LSGEGATPQDL (subtypes A and D).

Role of T-helper responses in the control of HIV infection

The role of the T helper response in delaying the onset of overt disease in infected subjects is still controversial, and was the topic of the following presentation. It appears that significantly different conclusions could be drawn depending on the study design. It was noted that using a cross-sectional approach, a positive correlation could be seen between T helper responses and delayed disease progression, while longitudinal studies failed to confirm these findings.

The results from studies of HIV-infected individuals may be of limited value, since in vaccination studies with seronegative volunteers significantly broader T-helper responses were observed. This is not always evident when T cell proliferative responses are measured using the whole protein antigens, but is more clear-cut when single 20-mer peptides are used. In general, the HIV-seronegative individuals are capable of recognizing a greater number of T-cell helper epitopes. Moreover, it becomes more evident in cases with different formulations of HIV-1 antigens in candidate vaccines (soluble proteins versus expression by replicating live vectors), that may result in different patterns of T-helper epitope recognition. These observations were made both in humans and in BALB/c mice studies. Comparative studies in HIV-negative and HIV-positive individuals will provide further opportunities in identifying novel T-cell helper epitopes, which may be more relevant for HIV vaccine-induced protection.

Also, it should be noted that the methodology to measure T-cell helper responses needs to be carefully standardized and quality controlled, which may have a significant impact on the quality and reproducibility of results. Optimally, single lots of antigens should be used throughout trials. In this regard, it was confirmed that frozen PBMC could effectively be used in T-cell helper assays with the advantage of a standardized preparation of larger stocks of frozen PBMC from a single source and at a single time point, that would significantly facilitate quality and interpretation of results.

Targeting CTL responses by vaccination

The design of HIV vaccines targeted at the induction of broad and sustainable CTL responses represents a major challenge. One of the approaches presented in

this session was based on a combined two-step vaccination approach. The first step involved primary presentation of selected CTL epitopes from several HIV genes recognized in the context of several HLA alleles and presented by means of DNA constructs. Animal experiments with these constructs confirmed them as an effective system correctly expressing the CTL epitopes with sufficiently good immunogenicity. Once the immune system had been primed and focused against these epitopes, the second step involves the use of an attenuated strain of the Modified Vaccinia Ankara (MVA) virus as a vector for expression of HIV antigens to further amplify the pool of CTL memory cells. The advantage of using the MVA vector is related to its high safety profile, in particular for use in areas with high HIV prevalence. In addition, the MVA vector may provide better chances for a prolonged period of antigen expression and maintenance of a long-lasting immune memory. This vaccination strategy was prompted by the observations from natural history studies in a cohort of female commercial sex workers (CSW) in Africa. In the course of this study over an extended period of time, 90% of CSW became HIV-infected. Of the remaining 10%, 5% were likely to become infected over the next year, and the remaining 5% appeared to be resistant to HIV, despite an estimated high frequency exposure to HIV as a result of unprotected sex with multiple clients. The results of these studies suggested that this type of resistance to HIV infection could be associated with either a continued exposure to HIV antigens or to allo-antigens, or both, leading to induction of CTL responses protective against HIV. However, it was also reported that in 6 cases, when women stopped practicing sex-work after a certain period of time they lost this type of 'immunity' and became infected with HIV. This seems to suggest the presence of a short-lived immunologic memory and a need for continuous antigen stimulation in order to sustain protective efficacy of an HIV vaccine. One of the key questions in the field is whether a possibility exists to select one HIV isolate of a certain subtype to develop a universal worldwide vaccine. Some of these aspects were addressed in this study, which showed that immunodominant subtype B-specific epitopes are not sufficiently conserved within subtype A isolates. Following these observations, a new DNA construct was designed to accommodate such variation in CTL epitope sequences matching HIV isolates that are prevalent in the target population.

In summary, the data presented in this session indicated that the quality of T-helper and CTL responses in HIV-negative vaccinees could be qualitatively different from what had been seen in HIV-infected individuals with regard to epitope recognition. The importance of the newly defined epitopes in the context of protective immunity is still under investigation. The HLA allelic prevalence in different populations influences greatly the selection of immunodominant CTL epitopes. In fact, new discrete CTL epitopes were identified in HIV isolates from individuals infected with non-B subtypes, i.e. subtypes A and D. It will be important to determine their immunodominance and relevance for vaccine protection against infection and/or disease.

Humoral anti-HIV immune responses as potential mechanism of protection against HIV/AIDS (Presenters: J. Weber, E.-M. Fenyo, S. Zolla-Pazner, G. van der Groen, J. Mascola)

Role of antibodies in prevention and/or control of HIV infection

A key question in HIV vaccine development relates to the role of humoral immune responses for HIV vaccine efficacy. The experience with other viral vaccines provides evidence that humoral responses can be an important factor of protection, by interacting with the virus and preventing its infection of target cells (neutralizing antibodies), by limiting its spread and dissemination in the host (binding antibodies), by facilitating recognition and presentation of the viral antigens (immune complexes), as well as by triggering antibody directed cellular cytoxicity (ADCC).

However, with regard to HIV infection the role of antibodies in protection still remains unknown and controversial. Most of the information on anti-HIV antibody responses is derived from studies of HIV-infected individuals, which suggest that the presence of neutralizing and binding antibodies of sufficiently broad specificity may be correlated with a more favorable prognosis. It was reported that an HIV-neutralizing antibody response is usually delayed until 6–8 months after infection, and in the course of infection the neutralizing antibody repertoire undergoes a process of broadening and maturation. However, these studies do not provide appropriate insight into mechanisms of protection mediated by a pre-existing humoral immunity in non-infected individuals, which is more relevant with regard to HIV vaccines.

The mechanisms of antibody-mediated neutralization of primary HIV-1 isolates are not yet well understood. Multiple stages of the virus-host interactions have been reported as important targets for HIV neutralization, including: (a) coating and blocking the virus circulating in blood or on the surface of infected cells by formation of immune complexes, (b) interference with the virus adhesion to primary and secondary receptors on the target cells, such as CD4, CCR5 or CXCR4 and other cell surface molecules; (c) inhibition of the virus fusion with target cells; and (d) suppression of virus replication by antibodies directed against the key regulatory proteins of HIV (e.g. TAT, Nef). Clearly, there is a whole array of antibodies with varying specificity and affinity to different HIV-1 epitopes,

which may play a role on their own or in different combinations in HIV neutralization and protection against infection.

A persistent question is raised by the genetic variability of HIV and its implication for the specificity and cross-reactivity of anti-HIV antibodies against different HIV-1 groups (M, N, O), group M subtypes (A through L) and inter-subtype recombinants. The available experimental data suggest that no straightforward correlation exists between genetic subtypes of HIV-1 and its neutralization patterns. In other words, the HIV-1 genetic subtypes do not predict HIV neutralization serotypes.

Methodological problems related to evaluation of antibody responses

The importance of standardized methodology for HIV neutralization studies was identified as an important prerequisite in this area of research. The experimental data presented indicated that HIV neutralization sensitivity could be a factor of conformational changes in the virus envelop structures, which in their turn are dependent on virus culture conditions. In this study, functional sequences derived from the *env* genes of neutralization sensitive (Ns) and neutralization resistant (Nr) HIV-1 isolates tested against a pool of polyclonal HIV-1 positive sera were analyzed. It was shown that sensitivity to neutralization was not related to a number of glycolysation sites or to the secondary receptor (R5 or X4) usage. At the same time, these results suggested that differences in neutralization sensitivity could be linked to sequence variation in conserved regions in gp120 and gp41, which may also be associated with conformational changes in the 'silent face' of the CD4 binding domain and gp140 oligomerization. Interestingly, it was found that gp140 oligomerization was influenced by co-variation in the gp41 domain. Neutralization studies with R5/X4 clones in PBMC and T-cell line adapted (TCLA) cultures showed that dual tropic clones do not change their tropism for secondary receptors, but may, in fact, shift in their neutralization sensitivity phenotype. It was therefore recommended that the database of Nr and Ns env sequences be extended and correlated with functional neutralization data, which could help define group specific neutralization antigens. In addition, a set of well-characterized reagents, such as Nr and Ns HIV-1 isolates fully characterized by full length *env* sequence, biological and neutralization phenotyping, should be established for future reference and comparative studies.

An important methodological limitation for HIV neutralization studies has been the lack of a simple and reproducible techniques for the quantitative measurement of HIV-1 neutralizing antibodies. A novel method, an infectious centre (or plaque) reduction assay was presented, which was shown to be effective and sensitive for quantification of neutralizing, as well as enhancing anti-HIV antibodies in polyclonal HIV-1 positive sera, as well as for monoclonal antibodies. The technique is performed using HIV-1 infected sub-confluent mono-layers of the U87 CD4+ cell line, expressing either CCR5 and/or CXCR4 receptors. The principle is based on syncytia-inducing capacity of primary HIV-1 isolates, which could be seen after 5–7 days of incubation. The quantification of results is facilitated by direct counting of HIV-1 infected cells, which are stained by a mouse anti-p24 monoclonal antibody and anti-mouse IgG tagged with beta-galactosidase. After substrate addition the infected cells were stained in blue. Since blue cells were predominantly syncytial cells, the syncytia represented infectious foci, and could therefore be used to estimate 'plaque forming units' (PFU) in the seeding HIV-1 stocks. As such, neutralization could be estimated by a significant decrease in virus titre expressed in PFU/ml, or in the case of enhancement as a significant increase in virus titre, compared to the control sera. The positive control is measured against a multi-tropic HIV-1 strain, exhibiting 3 different co-receptor usage patterns (R5, X4 and R5X4). When compared with the conventional $TCID_{50}$ measured in the PBMC co-culture neutralization assay, the results obtained by the new method expressed in PFU/ml were generally higher, especially for R5-using viruses.

This assay was further used to test a set of plasma pools prepared from plasma of individuals infected with HIV-1 subtypes A, B, D and a mixture of all three. All plasma samples were collected as part of a WHO-UNAIDS-sponsored study. The plasma samples for these pools were selected based on their HIV neutralization activity, which gave more than 90% of neutralization at dilution of 1:64 against the HIV-1 MN strain. Also, a pool of subtype C-specific plasma was prepared based on screening data on neutralization of primary isolates. These experiments demonstrated that the new technique was simple and reliable for the quantification of anti-HIV neutralizing activity. In agreement with previously reported data, this study confirmed that there was no correlation between neutralization serotypes and HIV-1 genetic subtypes. Furthermore, no co-receptor-dependent differences in neutralization sensitivity to monoclonal antibodies or human sera could be seen. Both R5 and X4 HIV-1 strains were neutralized by subtype A, B and C pools, while only R5 viruses were neutralized by the subtype D pool. However, the preliminary data suggested that subtype D X4-using strains were not only resistant to neutralization, but rather had a tendency to enhancement by some human sera or plasma under the conditions of these experiments. It appeared that the observed enhancement was virus-dependent and not reagent-dependent, since the same plasma/sera could neutralize one strain, but enhance the infectivity of another.

These differences between the viruses may be related to genetic subtypes, co-receptor usage, or other viral properties, which is the subject of on-going experiments.

Antibody responses in HIV-infected individuals and vaccine recipients

Most of the presently available data on HIV neutralization is derived from studies using polyclonal sera/plasma from HIV-infected individuals or monoclonal (human or mouse) antibodies. However, this information needs careful assessment with regard to its relevance to vaccination studies in HIV-negative volunteers. The following presentation was dedicated to a comparative analysis of humoral immune responses in HIV-infected individuals and HIV vaccine recipients, with a focus on conservation of HIV epitopes and cross-reactive antibody responses. Two types of antibody responses were analyzed, including binding anti-V3 antibodies and HIV-1 neutralizing antibodies. The binding antibody response was measured by a V3-peptide-binding immune assay (PEIA) with 53 different peptides representative of various HIV-1 group M subtypes (subtypes A to H) and group O. The HIV-neutralization was analyzed by an original method, using of GHOST (human osteosarcoma cells expressing CD4, as well as R5 or X4 receptors and a green fluorescence protein as an indicator gene) neutralization assay with a measurement of results by flow cytometry. The neutralization activity was tested against primary HIV-1 isolates (subtypes B, C and F), which were grown only in PBMC with a minimum number of passages. The serum samples were obtained from HIV-seropositive individuals infected with subtype B, which were compared to antibody responses in sera from 20 volunteers participating in an HIV vaccine trial (AVEG Protocol 029) of an accelerated immunization schedule with Canarypox vector (vCP205), expressing HIV_{MN} envelope, and boosting with a recombinant $gp120_{SF2}$ protein.

In this study, it was shown that the breadth of the anti-V3 antibody reactivity induced by immunization with subtype B-specific immunogen was comparable to the reactivity observed in subtype B-infected individuals. In both cases, broadly reactive anti-V3 antibodies could have been detected. This cross-reactivity was most prominent against peptides derived from subtypes B, C and F. Less reactivity was seen with V3 peptides from subtypes A, D, G and H, and no significant cross-reactivity was detected with subtype E- and group O-specific peptides.

The breadth of neutralizing activity induced by immunization with a subtype B-specific vaccination regimen was more narrow as compared to sera from HIV-infected individuals. However, in a number of cases, sera from vaccinees displayed neutralizing activity against X4, R5 and dual tropic primary isolates of subtype B. In addition, some of the vaccines sera displayed lower but significant levels of neutralization activity against an R5 subtype C primary isolate.

Overall, the data presented constituted a proof of principle that candidate HIV vaccines can induce broad humoral antibody responses against strains belonging to heterologous biological phenotypes and heterologous HIV-1 subtypes. The discussion of these results also indicated that the use of multivalent vaccines containing a defined mixture of immunogens might be a feasible approach to achieve broadly-protective HIV vaccines.

Targeting the induction of antibody responses by vaccination

The following presentation described a strategy which is based on HIV neutralization data as a guide for the development of broadly protective HIV vaccines. This strategy was based on the analysis of broad spectrum cross-neutralizing (BSCN) activity against laboratory adapted and primary isolates, which was documented by various studies on natural history, animal experiments and HIV vaccine trials in humans. These studies showed that close to 10% of all HIV-infected individuals were capable of mounting BSCN antibody response, including cross-neutralization of 17 primary HIV-1 isolates from different group M subtypes (A through H) or their recombinants ($n = 14$), as well as group O strains ($n = 3$). It was demonstrated that the BSNC activity was antibody-mediated, since it could be absorbed and eluted by IgG fractionation. Also, the specificity of the BSNC to the envelope protein was confirmed by competition-neutralization experiments, in which a recombinant gp120 protein could absorb up to 50% of neutralizing activity. Interestingly, in the same study it was noted that BSNC sera was more frequently found in samples obtained from African women, as compared to African men or Europeans of both sexes.

Based on these and other data, it was concluded that BSCN phenomenon may be associated with certain conserved conformational epitopes, identification of which should be a priority for future HIV vaccine research. This strategy could be further pursued through the following phases:

(a) development of appropriate and standardized techniques for rapid screening of a large number of sera/plasma samples with BSCN activity;
(b) development of methodologies for in-depth analysis and interpretation of HIV neutralization data generated by experimental studies and HIV vaccine clinical trials;
(c) identification of conformational epitopes responsible for induction of the BSCN responses;

(d) generation of human monoclonal antibodies (HuMAb) with the BSCN activity.
(e) designing antigens, which specifically bind BSCN polyclonal sera, as well as BSCN HuMAb; and
(f) designing and inclusion of the identified BSNC immunogens into novel vaccine approaches.

Demonstration of protection by passive transfer of antibodies

Passive or active antibody-mediated immunity is known to be important for protection against several viral diseases in humans and animal retroviral infections. In the case of HIV infection, there is a limited amount of data in this regard. Several studies demonstrated that passive transfer of polyclonal anti-HIV IgG or an anti-V3 monoclonal antibody can protect chimpanzees against intravenous challenge. However, several methodological problems place under question the relevance of these results for the human situation, specifically the use of laboratory adapted HIV strains as a challenge, the intravenous route of challenge and lack of pathogenic effects of HIV-1 in chimpanzees. The advances in construction of chimeric SHIV strains based on the envelope genes from primary HIV-1 isolates with pathogenic capacities in infected macaques, along with standardized protocols for challenge experiments have provided new opportunities to address these questions.

A summary of experimental data was presented, which was generated using a challenge with a SHIV strain based on a primary isolate HIV-89.6, which was also shown to be relatively resistant to anti-HIV-1 neutralizing antibodies *in vitro*, and capable of producing significant pathogenic effects in infected macaques, as estimated by high plasma viremia and accelerated decline of CD4+ cells. The SHIV-89.6 strain was further standardized and titrated as a mucosal/vaginal challenge in macaques treated with progesterone (30 days prior to challenge), in order to optimize chances of consistent infection rates in challenged animals. The vaginal challenge used in this experiment was 600 $TCID_{50}$or approximately 40 animal infectious doses (AID) per challenge.

In passive transfer experiments, 24 hours prior to challenge, four groups of macaques were infused with anti-HIV antibody preparations: 2G12 monoclonal antibody which binds to a conformation sensitive epitope in C3-V4 gp120 region, 15 mg/kg (n = 4); 2G12 + 2F5 monoclonal antibody against a conserved epitope (ELDKWA) in gp41, 15 mg/kg (n = 5); 400 mg/kg of the HIV immune globulin (HIVIG) in combination with 2F5 and 2G12 monoclonal antibodies (n = 5). The control monkeys received 400 mg/kg of the intravenous immune globulin (IVIG) 400 mg/kg (n = 5).

After challenge, all 5 control monkeys became infected with SHIV with high plasma viremia and rapid decline of CD4+ cells. In contrast, no SHIV plasma RNA was detected in 8 out of 14 monkeys, passively transferred with anti-HIV antibodies. In protected monkeys, virus was not detectable either by PBMC co-culture or by DNA polymerase chain reaction (PCR) from inguinal lymph node cells. Therefore, it was considered that in 8 monkeys a complete protection was achieved. By this criteria, 4/5 were protected in the HIVIG/2F5/2G12 transfused group, 2/5 in the 2F5/2G12 group, and 2/4 in the 2G12 group. In the six protected monkeys, none of them developed a rapid CD4 cell decline, which was observed in all control monkeys. Additional analyses showed that after infusion of antibody preparations in the doses used in this experiment, the specific antibodies could be detected 24 hours after infusion both in plasma and in mucosal washes. The plasma of the anti-HIV antibody transfused monkeys also was shown to contain significant levels of neutralizing antibodies.

Overall, the results of this study lead to conclusion that protection conferred by anti-HIV antibodies could be more readily achieved in cases of mucosal exposure to SHIV, as compared to the intravenous challenge, although relatively high levels of antibodies were required for protection. These studies also suggested that anti-HIV-1 antibodies, especially neutralizing antibodies, may be an important factor (or correlate) of protection against infection or against disease. As a future direction, it was planned to investigate the breadth of the passive protection against SHIV isolates other than 89.6, representative of other subtype B and non-B strains. Also, it is of interest to evaluate, in the same model, the activity of anti-HIV antibodies from gp120-immunized human volunteers.

HIV envelope structure and function (Presenters: R. Wyatt, J. Nunberg, R. Desrosiers, H. Holmes)

Structural analysis of the envelope of HIV-1

The native HIV-1 envelope glycoprotein contains major functional domains responsible for the virus attachment to target cells (via CD4, CCR5, CXCR4 and other cell surface molecules) and fusion – the two most important stages of the virus life cycle, which makes it a natural candidate as an immunogen for HIV vaccines. Important progress has been made in studies on structure, composition and function of the envelope glycoprotein of HIV. The exterior viral protein gp120 (SU) and the transmembrane glycoprotein gp41 (TM) are generated from a gp160 precursor. These proteins, following cleavage, are assembled in oligomeric spikes and remain non-covalently associated on the cell or viral surface. The gp120 glycoprotein contains constant (C1–C5) and variable (V1–V5) regions, where the latter may contribute to evasion of the virus from host immune responses. In addition, the gp120 envelope proteins are characterized by a high degree of N-linked

glycosylation and high carbohydrate content, up to 50% of the molecular weight. This extensive glycosylation also was postulated as a significant factor for virus ability to resist antibody-mediated neutralization.

The heterotrimeric spikes mediate the virus entry into susceptible cells by sequential gp120 binding to the primary viral receptor, CD4, and subsequently to specific proteins of the chemokine receptor family, CCR5 or CXCR4, which serve as co-receptors. Primary HIV-1 isolates mostly use CCR5 as a co-receptor; but later in the course of HIV-1 infection, isolates dually tropic to CCR5 and CXCR4 are more frequently found.

A model of the virus entry mediated by the HIV-1 envelope glycoproteins was deduced as a result of various *in vitro* and *in vivo* investigations. Initially, gp120 after binding to CD4 receptor undergoes a conformational change that allows for high affinity interaction with the chemokine co-receptors. It is likely that conformational changes involve displacement of a large, disulfide-linked V1/V2 loop and formation of a mini-domain bridging sheet. The chemokine receptor binding site minimally involves elements in the V3 loop and residues within the C4 region. Interaction of gp120 with co-receptor triggers a second conformational change, which leads to insertion of a hydrophobic amino-terminal fusion peptide of gp41 into the target cell membrane. Once a critical number of gp41 molecules have achieved fusion peptide insertion into target cell, the membranes are then cross-linked via gp41 fusion elements. The following pre-fusogenic and fusion stages permit entry of an HIV-1 pre-integration complex into the cytoplasm, initiating the infection process.

Studies on the HIV-1 envelope crystal structure have revealed several interesting structural features of virus evasion from host antibody responses. The CD4 binding domain was described as a recessed region of gp120, which is likely to be protected from neutralizing antibodies by physical barriers set by the heavy glycosylation of the envelope protein and the major variable loops (V1, V2 and V3). Since all HIV-1 isolates utilize CD4 as the primary receptor, this region represents an attractive target for HIV vaccine design.

The results of biophysical studies of the gp120-CD4 interaction also indicated that *in vivo* free monomeric gp120 molecules are extremely labile and may be subject to multiple conformation changes. It is possible that this conformational flexibility may be diverting the immune system to production of antibodies which are not capable of recognizing the more structurally stable forms of the molecule located in the functional trimer on the surface of the virus. It was further discussed that this information might be exploited to improve the design of HIV vaccines targeted at induction of neutralizing antibodies. One possibility to stabilize gp120 conformational flexibility might be to covalently link gp120 to its natural ligand, the gp41 molecule. An approach was described to produce stable and soluble trimers, which better represent the conformation of native envelope structures of HIV. As a result of these experiments, cleavage-defective gp140 molecules were stabilized by covalent linkage of the GCN4 coiled coils. Sucrose gradient analysis and size exclusion chromatography studies confirmed the trimeric oligomerization of thus obtained gp140 (-)GCN4 molecules. It was shown that these gp140(-)GCN4 trimeric structures were well recognized by neutralizing antibodies, but were less antigenic with regard to non-neutralizing antibodies, when compared to monomeric gp140(-) preparations. Immunogenicity studies with the soluble and stabilized trimers are in progress.

Fusion-active envelope structures as targets for HIV neutralization

Approaches to induction of broadly reactive neutralizing antibody responses were further presented. This approach is based on a premise that all HIV isolates – regardless of geographic location or phylogenetic clade (subtype) – must successfully complete the identical processes of CD4 and co-receptor binding and membrane fusion, which constitute vital stages of the virus life cycle. Consequently, a hypothesis was put forward that: (a) key primary virus neutralizing determinants could be temporarily associated with the process of binding and fusion; and (b) immunogens representing functioning envelope protein associated with transient /complex structures, may be capable of eliciting broadly reactive neutralizing antibodies. The earlier experimental data from this project showed that fusion-active components of the virus are likely to be broadly conserved and could be an attractive vaccine target. In this approach, the fusion process was triggered by co-culturing two cell lines, one, the U87 cell line expressing CCR5 and CD4 receptors and another expressing functional gp160 derived form a primary HIV-1 isolate. The binding and fusion process was stopped by formaldehyde fixation prior to overt syncytium formation (2–5 hours after start of co-culture). These co-cultured fusion complexes were used to immunize transgenic mice, which express (and therefore are immunologically tolerant to) human CD4 and CCR5 receptors. Thus prepared fusion-competent sera from mice were shown to have potent neutralization against a primary isolate (168P) used to design the gp160-expressing cell line as an immunogen, as well as against the T-cell line adapted (TCLA) derivative of the same isolate. But most importantly, it was found that fusion-competent vaccine sera was capable of neutralizing a panel of primary isolates belonging to diverse genetic subtypes from A to E. Although some of the primary HIV-1 isolates (92RW008, subtype A) appeared to be

resistant to the fusion-competent vaccine sera. In summary, these results demonstrated that HIV-1 sequence diversity may not be a major obstacle for the development of broadly protective HIV vaccines, if highly conserved structures within functioning envelope protein are defined and used as immunogens. Clearly, to ensure a universal level of broad reactivity, there will be a need to design multivalent HIV vaccines, since several distinct immunotypes of HIV-1 may exist (e.g., 92RW008).

The current directions of this project acknowledge that for vaccine design the formaldehyde-fixed whole cell preparations may not be appropriate for clinical testing. The on-going efforts are therefore targeted at translation of the crude vaccine concept into a more acceptable formulation, including: (a) recombinant vaccine vectors, and (b) affinity-purified envelope-CD4-CCR5 protein complexes. In parallel, the existing crude fusion-competent vaccine preparations are planned to be tested for efficacy, using the SHIV/macaque model.

Approaches to improve immunogenicity of the HIV envelope glycoproteins

As mentioned earlier, the heavy glycosylation of the envelope of HIV and SIV, may represent a barrier for exposure of important neutralization epitopes in candidate vaccines based on recombinant envelope proteins. An experimental approach was reported, which attempted to improve the exposure of neutralizing determinants, using SIV strains with mutated envelope genes lacking different N-linked glycosylation sites. Initial results of the first study showed that removal of two carbohydrate sites in V1 and V2 regions was well tolerated in terms of the virus replication capacity. Thus mutated SIV strains (M5) proliferated well in macaques and induced neutralzing antibodies against the homologous SIV strain, but were also able to cross-neutralize the wild-type parental virus. The two macaques that were infected with the mutant M5 strain were resistant to super-infection with a challenge $SIV_{mac}251$ strain.

In further studies, an SIVmac239 molecular clone was manipulated by making deletions of 100 residues in the V1V2 region. The mutant strain was adapted for growth *in vitro* and was shown to have acquired 9 amino acid changes, which occurred both in gp120 and gp41. This ΔV1V2 virus was capable of infecting macaques, however, *in vivo* replication could be detected only for several weeks, which subsequently gradually decreased to undetectable levels.

Another set of mutants was generated using the same SIVmac239 molecular clone, in which mutations were created at glycosylation sites in the gp41 region. These mutations were designed either individually or in different combinations. Thus modified mutant strains are being tested for infectivity in macaques, and their ability to induce homologous and cross-protective neutralizing antibodies.

An additional approach was presented, which is aimed at induction of long-term persistent immune responses, using herpes simplex virus type 1 (HSV-1) as a vector for expression of *env* and *nef* genes. Preliminary results in macaques immunized with the HSV-1/*env* vector and challenged with $SIV_{mac}239$ six months after inoculation, showed that 2/7 animals were fully protected.

The last presentation of this session emphasized the importance of developing well standardized reagents for HIV vaccine research, including purified, biologically active recombinant HIV-1 envelope glycoproteins. For this purpose, stable CHO cell lines expressing functional envelope glycoproteins from subtypes A, B, C, D, F and group O were generated, using stocks of primary HIV strains (R5- or R5X4-users) collected mostly as part of the WHO-UNAIDS Virus Network. Soluble gp140 molecules produced by these cell lines possessed wild-type cleavage sites, but remained uncleaved through the production and purification process. The glycoproteins were purified either by C-terminally directed antibody or lectin affinity chromatography. The protein production levels ranged from 0.1 to 1 mg/L. The oligomerization state of envelope glycoproteins obtained in this way, was heterogeneous and consisted of aggregates, trimers, dimers and monomers. High salt treatment (2M NaCL) was able to reduce the oligomers derived from most of the subtypes to monomers with a corresponding increase in CD4 affinity. Several envelope glycoprotein preparations were recognizable by the conformation-sensitive and broadly neutralizing antibody, 2G12. Antisera to each gp140 preparation were raised in rabbits, all antisera (except for subtype F) recognized all seven gp120/140 proteins. The subtype F serum did not recognize subtype B antigens. It was shown that the recombinant HIV proteins were capable of inducing specific proliferative T-cell responses in mice. The results of neutralization studies are anticipated in the near future. As part of the European HIV vaccine programmes the same group is participating in the development of a subtype C specific vaccine, which will prepare batches of gp120/140 subtype C-preparations following the GMP standards for further evaluation in the macaque/SHIV model and in phase I clinical trials.

HIV vaccine design, immunogenicity and protection studies (Presenters: J. Heeney, L. Stamatatos, B. Ensoli, P. Berman)

Approaches to standardize animal models

Various aspects of pre-clinical HIV vaccine development and HIV vaccine trials were discussed in this session. The first topic was related to pre-clinical stages

of an HIV vaccine development, which emphasized the following issues:

(a) need for standardized approaches to pre-clinical evaluation of candidate HIV vaccines with regard to their immunogenicity and potential efficacy, using standardized immunological reagents, challenge stocks and laboratory assays;
(b) importance of research into potential correlates of protection, based on a well standardized SHIV/macaque model, which should generate data on the role of CTL and neutralizing antibodies for protection independently or in a complementary fashion.
(c) need for unified and standardized definition of vaccine protection (end-points) attainable in the SHIV/macaque model, which would facilitate interpretation of experimental data.

Current HIV-1 vaccine strategies employ different HIV-1 genetic subtypes for vaccine production. However, the importance of biological properties of the virus, such as virulence, have not been fully appreciated. To investigate the possible impact of viral virulence, as compared to its genetic diversity on vaccine efficacy, experimental data was analyzed from a series of studies using heterologous SHIV challenge of macaques vaccinated with a recombinant sub-unit HIV-1 vaccine. Twelve animals were immunized with a functional CCR5 binding envelope protein derived from an HIV-$1_{w6.1D}$ strain. In the first study, heterologous challenge included two non-pathogenic SHIV chimeras encoding envelopes of markedly divergent subtype B strains: HIV-1_{han1} and HIV-1_{sf13}. Subsequently, in the second study, all immunized and protected animals were re-challenged with a $SHIV_{89.6p}$ strain, which is closely related to the vaccine strain but is highly virulent. Protection from either of the divergent non-pathogenic strains was demonstrated in the majority of the vaccinated animals. In contrast, when challenged with a more related, but highly virulent $SHIV_{89.6p}$ strain, protection was achieved in only one of the previously protected immunized animals. These findings suggest that the use of genetic differences as a guide in HIV vaccine design may be misleading. Indeed, consideration of the viral biological properties and virulence may be more relevant for the selection of prototype vaccine strains. In addition to the envelope, it would make sense to consider the inclusion of immunogens targeting potential virulence and virus replication factors. Combination of different immune mechanisms directed to a number of conserved viral targets, including virus structural and regulatory targets, may result in a broad vaccine-induced protection against a diverse range of highly virulent HIV-1 strains.

Use of HIV regulatory proteins as targets for vaccination

As discussed earlier, an ideal outcome of an HIV vaccine would be the induction of sterilizing immunity. However, other alternative outcomes may be of value, including the control HIV-1 replication, resulting in lower virus load levels and reduction in the rate of disease progression. Different immune correlates have been suggested for these outcomes, including CTLs and neutralizing antibodies targeted at various functional epitopes in virus structural proteins. It was also suggested that targeting the immune responses to the HIV-1 regulatory proteins (e.g., Nef and Tat) would be a logical direction to pursue. One approach in this regard was presented, which was based on the use of HIV-1/Tat as a target for vaccination. Several reasons were discussed to justify this approach. The Tat protein is produced early after infection and is essential for viral replication and infectivity. Extra-cellular Tat induces expression of chemokine receptors (CCR5 and CXCR4), thus facilitating infection of both macrophage- and T cell-tropic HIV-1 strains. In addition, it was reported that Tat could play an important role in AIDS pathogenesis and the development of AIDS-associated malignancies, such as Kaposi sarcoma. *In vivo*, the presence of anti-Tat antibodies and anti-Tat CTL responses correlated with better control of disease progression. Finally, it was noted that Tat is well conserved among different HIV-1 subtypes.

Based on this rationale, researchers vaccinated cynomolgus monkeys (*Macaca fascicularis*) with biologically active HIV-1 Tat protein or Tat-expressing DNA construct. The Tat protein was expressed in *E. coli* and was adminstered as an active product combined with an adjuvant, Alum or Ribi. The DNA vaccine was delivered as a plasmid enriched in unmethylated CpG sequences. An intravenous challenge was performed with a highly pathogenic SHIV89.6P stock (10 MID_{50}). The results of these experiments showed that immunization with biologically active Tat and Tat-expressing DNA constructs were safe and without any undue side-effects. All control animals became infected, whereas Tat-vaccinees were either fully protected or were able to effectively control the infection. Viral load in Tat-vaccinated animals was suppressed or below the detection level, the CD4+ T-cell levels also remained stable within a normal range. The efficacy of the DNA-based Tat vaccine was comparable, if not better, to that of the sub-unit Tat protein. A number of Tat-specific immune responses were documented in animals vaccinated both with Tat- protein and Tat/DNA vaccine, including anti-Tat antibodies and Tat-specific CD8+ T-cells. The results of this study suggested that there was no straightforward correlation between anti-Tat antibodies and protection. In contrast, a strong correlation was found with the presence of anti-Tat specific CTLs and production of tumour necrosis factor

α (TNFα) by CD8+ T cells upon Tat stimulation. In addition, the analysis of CD8+ T cell responses indicated that protection by this arm was not limited to Tat-specific CTL, but also was linked to CD8-mediated antiviral activity (not restricted by MHC) and production of β-chemokines. This was confirmed in the protected monkeys with depleted CD8+ cells, who became susceptible again to super-infection with the same SHIV89.6P challenge. At the same time, the production of β-chemokines alone (RANTES, MIP1α, MIP1β) did not correlate with protection.

Approaches to optimize antibody immune responses against HIV

Based on previously discussed information on HIV-1 envelope conformational structure and glycosylation and its impact on immunogenicity, a study was presented that targeted the development of envelope-based immunogens with deletions in V1 and V2 regions, thus anticipating a better exposure of neutralizing epitopes normally hidden in the virus envelope. In this study, V1 and V2 deletions were introduced into the envelope of SF162 (subtype B) strain with an NSI and R5 biological phenotype. Two constructs were prepared, including a 20 amino acid deletion in the V1 loop (SF162ΔV1), and a second construct with a 30 amino acid deletion in the V2 loop (SF162ΔV2). Both constructs were shown to preserve their biological phenotype in co-receptor usage, and capability of attachment, entry and replication in stimulated human PBMC. The SF162ΔV2 virus was more susceptible to neutralization than parental SF162 strain by sera from patients infected with subtype B and non-B subtypes HIV-1 strains. These results suggested that deletions in the V2 loop could result in a better exposure of broadly reactive neutralizing epitopes contained in the viral envelop structures, which are well conserved among various primary HIV-1 strains of different subtypes.

In further experiments to evaluate immunogenicity of the envelope protein derived from the SF162ΔV2 strain, two rhesus macaques were immunized with a DNA/ SF162ΔV2 construct, using a bimodal immunization regimen, i.e. 3 DNA immunizations at monthly intervals followed by a 'resting' period for five months, after which animals received additional immunization with the DNA construct and with purified CHO-produced SF162ΔV2 gp140 glycoprotein. Two months later, animals received a final boost with the gp140 glycoprotein. The analysis of immune responses showed that both immunized animals developed T-lymphoproliferative and humoral responses. The neutralizing antibody titres against SF162ΔV2 as well as against the parental SF162 strain were induced and maintained for extended period of time. Most importantly, immunization with the SF162ΔV2 construct resulted in induction of neutralizing antibodies to heterologous primary isolates, although at a lower and less frequent level, as compared to the parental SF162 strain. It is noteworthy that sera from macaques immunized with monomeric SF2gp120 did not neutralize the SF162 strain. Anti-HIV envelope-binding antibodies, both against gp120 and gp41, were induced during the DNA phase of immunization, and the gp140 binding antibodies were induced throughout the immunization. Currently, studies are under way to determine whether both anti-gp120 and anti-gp41 antibodies are responsible for the observed neutralization. Additional experiments also are planned to assess the protective role of the observed immune responses in the SHIV/macaque model.

The last presentation of this session focused on selection of representative envelope immunogens based on immunological and molecular epidemiology data. The rational for this strategy aimed at induction of broadly reactive binding and neutralizing antibody responses. It was estimated that gp120 envelope protein contained up to 5 different potential target sites for HIV-1 neutralizing antibodies. These sites are relatively conserved, but not very immunodominant and display a certain level of polymorphism. For example, one of these sites could be located at the bottom of the C4 region, which was mapped by 3 different Mabs, including 13H8, 1024 and 5C2, capable of blocking CD4 binding. A common polymorphism that was found in this site among subtype B strains was characterized by substitution of lysine for glutamic acid, which affected binding of neutralizing antibodies, and rendered strains relatively resistant to neutralization. These observations indicate that although there is a common polymorphism and sequence variation at most HIV-1 neutralization sites, this polymorphism is finite, and a limited number of immunogens could be identified covering the antigenic spectrum of such variation, which justifies an in-depth research into the development of multivalent candidate vaccines.

As part of this strategy, the presenting team produced 2 candidate vaccines, which are presently being evaluated in the first-large scale efficacy trials. The first candidate vaccine is a bivalent BB rgp120 protein produced in CHO and derived from 2 subtype B strains, including MN (T-tropic, SI and X4 strain) and a second strain designated as GNE8 (M-tropic, NSI and R5 strain), which was identified as a result of the analysis of breakthrough infections in trials with the rgp120-MN vaccine. Immunization of rabbits with this bivalent BB candidate vaccine showed that the combination of two immunogens resulted in broadening of neutralizing antibody responses, which cross-reacted against the HIV-1_{MN} strain, as well as against several breakthrough HIV-1 isolates obtained from previous phase II trials. This candidate vaccine is being evaluated in a phase III trial in the USA, which started in 1998.

The second candidate vaccine being tested in a phase III trial in Thailand was produced by the same group as a bivalent BE rgp120 vaccine, based on subtype B HIV-1_{MN} and a subtype E (NSI, R5 strain), designated as A244. This vaccine is being evaluated in a cohort of injecting drug users in Bangkok, which started in 1999.

The production of another variant of this vaccine would be based on triple combination of globally prevalent HIV-1 strains, including rgp120 immunogens derived from subtypes A, C and D, for evaluation in African countries.

An additional problem that needs to be addressed with this approach is related to the relatively low immunogenicity of the envelope glycoproteins, which could be improved by using adjuvant preparations. Experimental data was presented indicating that the adjuvant QS21 can significantly enhance immunogenicity of rgp120 proteins. It was demonstrated that the use of QS21 with rgp120 immunogens achieved the same levels of immunogenicity with significantly lower doses of the immunogen, e.g. the dose of 0.5 μg of rgp120 formulated with QS21 was equally immunogenic as 300 μg of rgp120 in Alum, resulting in an overall 600-fold increase in potency, the fact that should largely contribute to reduced costs of a future vaccine.

State of the art of therapeutic vaccines against HIV/AIDS (Presenters: E. Tramont, P. Salk, R. Moss, X. Jin, M. Liu, R. Redfield)

General considerations

Proponents of therapeutic vaccination hypothesize that post-infection therapeutic vaccination might induce HIV-specific immune mechanisms, which would result in down regulation of HIV *in vivo*. The clinical consequences of this intervention might be decreased transmissibility, lower chances for antiviral treatment failures, re-establishment or maintenance of viral control with intermittent or no anti-retroviral drug therapy, delayed progression and/or stabilization of disease progression, and prolonged survival of HIV-infected individuals.

There are examples of successful use of therapeutic vaccines for the treatment of several infectious diseases, e.g. rabies, herpes simplex virus, hepatitis B and more recently combination chemotherapy of leprosy. However, the feasibility and clinical utility of this approach for most of the chronic viral infections, including HIV/AIDS, remains to be demonstrated.

Early studies of therapeutic vaccines demonstrated the proof of principle. Post- exposure immunization with candidate HIV vaccines was shown to be capable of safely augmenting host-directed immunity against HIV, including both innate and adaptive immune effectors. Of note is the ability of therapeutic vaccination to induce novel immune responses, which are poorly elicited, if at all, as a result of a natural infection with HIV, including specific T helper and CTL responses to various HIV antigens. However, efficacy studies to date have failed to demonstrate that good immunogenicity and safety results obtained in phase I and II trials with candidate therapeutic vaccines could be translated into clinical benefits for HIV-infected individuals.

The early clinical studies of therapeutic vaccines were conducted at a time when effective anti-retroviral therapy was not available. In the setting of uncontrolled viral replication, therapeutic immunization failed to result in measurable improvements in clinical outcomes. In addition, the efficacy of the first candidate vaccines was grossly defined in terms of clinical end-points and the ability to achieve modification of disease progression and extending survival rates.

Introduction of HAART resulted in a significant impact on an underlying clinical progression, which in its turn has largely complicated feasibility of therapeutic vaccination studies based only on clinical end-points. Fortunately, the development of accurate assays for viral load measurement and demonstration of their prognostic value have provided for alternative approaches to the evaluation of therapeutic vaccine efficacy. In addition, recent advances in anti-retroviral therapy, resulting in an efficient control of viral replication in the setting of natural infection, also allow for new alternatives to explore clinical end-points in the setting of chemotherapy combined with therapeutic vaccination, including sustainability of the viral control by HAART, kinetics of emerging viral resistance, viral load set points following structured treatment interruption or intermittent chemotherapy.

Several recent factors have lead to re-examination of the potential role of therapeutic vaccines in the global efforts related to HIV prevention and treatment, including: (i) recognition of limitations of combination chemotherapy regimens (e.g., toxicity, viral resistance, lapses of patients' compliance), as well as the broadening gap for a global access to the anti-retroviral treatment due to prohibitive costs and complex logistics for delivery; (ii) generation of scientific data confirming the importance of HIV-specific immunity for the control of virus replication and the disease outcome; (iii) documented facts indicating that despite sustained viral suppression as a result of anti-retroviral treatment, its discontinuation is associated with a rapid viral rebound; (iv) observation that HIV-specific immunity can be suppressed as a consequence of effective chemotherapy; (v) generation of promising data in animal studies, demonstrating complementary beneficial effects of combined chemotherapy with therapeutic vaccination; (vi) broader acceptance of non-sterilizing immunity as a realistic goal for preventive HIV vaccines, and

inclusion of post-infection viral set-point as a surrogate marker for vaccine efficacy; (vii) recognition that research into therapeutic vaccines may be an integral step towards the development of a successful preventive HIV vaccine. It was therefore concluded that world-wide research efforts should be refocused to include therapeutic vaccination strategy as a potential to improve the existing treatment strategies, as well as a complementary tool for a successful development of preventive HIV vaccines.

State of the art: What is known and what do we think we know?

The central hypothesis driving the development of therapeutic anti-HIV vaccines is an expectation that post-infection vaccination would induce HIV-specific immune regulatory mechanisms, resulting in an effective control of HIV replication *in vivo*. The consequences of such improved viral control may allow for improvement of several clinical parameters: decreased transmissibility, improved clinical outcomes of anti-retroviral treatment, re-establishment or maintenance of the viral control during intermittent or in the absence of anti-retroviral therapy, delayed disease progression and/or disease stabilization and prolonged survival.

The host directed immunity can be an extremely potent antiviral 'agent', when immune mechanisms capable of interrupting viral life cycle are induced as a consequence of natural infection. However, in the context of chronic viral infections, the host fails to develop an immune response capable of clearing the pathogen from the host, due to a variety of mechanisms developed by viral pathogens. At the same time, this is not to say that the host is completely deprived of the ability to mount an effective immune response to viral antigens administered to the infected host as a therapeutic vaccine. Different vaccine delivery strategies could have a different impact on vaccine potency. An example was presented demonstrating that a formulated DNA construct had a 100–1000-fold immunogenic potency compared to a naked DNA construct. Furthermore, vaccine potency could have been increased by priming with DNA and boosting with a recombinant protein. Another example was mentioned where an alpha virus was used as a vector for DNA vaccine delivery (SIN-GFP), which resulted in significantly increased vaccine potency.

Several cohort studies have implicated that the status of the host immune system can have an impact on the course of HIV infection and the disease outcome. This was demonstrated in studies of LTNP, cases where broad and enhanced anti-HIV humoral or cell-mediated immune responses were associated with delayed disease progression.

One of the concepts being exploited in the development of therapeutic vaccines against HIV/AIDS is based on an assumption that the long incubation period between infection and the onset of disease may be due to an effective immune control of initial infection, which persists with health and wanes with the development of the disease. An additional rationale for this approach was derived from clinical observations made during studies on HAART, which indicated that an effective anti-retroviral therapy also resulted in a progressive loss of HIV-specific immunity. This observation lead to the concept of structured treatment interruption, where anti-retroviral treatment is temporarily discontinued to allow for an increased antigen exposure as a result of the viral rebound, which in its turn would serve as a boost for anti-HIV immune responses ('auto immunization'). In support of this approach data was presented from clinical studies with anti-retroviral treatment (DDI + hydroxyurea), which did not completely suppress viral replication, but was able to maintain it at a very low level. When compared with patients receiving HAART, the patients treated only with DDI and hydroxyurea were characterized by more pronounced anti-HIV immune responses, as assessed by an increased lymphocyte proliferation to p24 antigen. In addition, a separate study also demonstrated that in a setting of structured treatment interruption, patients who had been on HAART developed significantly higher set points of the viral plasma RNA at week 9 after treatment interruption, as compared to patients receiving DDI and hydroxyurea alone. Altogether these data suggested that patients receiving less aggressive anti-retroviral treatment, allowing for a low level of viral replication, establish more effective immune mechanisms for the control HIV replication.

To date, several candidate therapeutic vaccines have been tested in clinical trials. The results of trials with one candidate therapeutic vaccine (Remune) were presented at the meeting. This candidate vaccine is based on the use of inactivated (gp120 depleted) whole virus prepared from an HIV-1 strain originating from Africa, which appeared to be an inter-subtype A/G recombinant. The overall results demonstrated the ability of post infection immunization to induce lymphocyte proliferative (T helper) responses to homologous (AG) antigens, which also cross-reacted to other antigens derived from subtypes B, C and CRF01_AE. In a selected number of volunteers it was observed that this candidate vaccine also stimulated production of beta-chemokines (MIP1-beta, MIP1-alpha and RANTES) associated with down regulation of CCR5-receptor expression on T cells. This is of particular interest in light of growing body of evidence that dynamics between production of beta chemokines and expression of their receptors may represent a novel and potentially important target for therapeutic interventions. Additional clinical trials have been implemented

or are under way that were designed to assess clinical efficacy of this candidate vaccine.

Preliminary results were presented from a study on safety and immunogenicity of another candidate vaccine (ALVAC vCP1452) combined with rgp160 boost in HIV-infected volunteers, who subsequently underwent a structured treatment interruption. This group comprised ten patients who had been treated with HAART within 90 days of acute infection and demonstrated evidence of complete virus suppression for at least 24 months after the start of treatment. The vaccine candidate was based on multiple immunogens, including MN/gp120, LAI/gp41, LAI/gag, LAI/protease, CTL epitopes (LAI/pol, BRU/nef) expressed by ALVAC and rgp160 (MN/gp120 and LAI/gp41), which was administered at days 0, 30, 90 and 180 by IM injection. The immunogenicity assessed in 6 volunteers showed the induction of binding antibodies to the HIV envelope in all 6 volunteers, binding antibodies to p24 in 2/6 volunteers, and an increased T helper response in 3/6 volunteers; CTL responses were observed in 4/6 volunteers, in 2 volunteers the CTL responses were of polyclonal nature. The structured treatment interruption data was presented for 4 volunteers, all of whom demonstrated virological rebound after discontinuation of HAART. However, in 2/4 volunteers a delayed viral rebound was observed with an initial doubling time varying between 3.2–4.5 days as compared to 2.2 days observed in the (historical) control group. Interestingly, the volunteers with delayed viral rebound had a polyclonal CTL response to multiple immunogens present in the ALVAC vCP1452/rgp160 constructs.

The results above described demonstrate the importance of detailed characterization of effective immune responses that are potentially capable of controlling virus replication and improving clinical outcomes of infection, which should represent an important objective for future investigations. In parallel, clinical trials with selected candidate therapeutic vaccines should continue, since they could also contribute towards determination of immune mechanisms of suppressing viral replication and preventing disease progression.

The key scientific questions and future directions

One of the challenges in relation to therapeutic vaccination is related to the ability of HIV infection itself to alter the capacity of the host to mount effective immune responses to a candidate immunogen. A number of immune suppressive mechanisms have been suggested, including clonal depletion of CD4+ T lymphocytes; gp120-CD4 interaction; suppression of T cell proliferation by structural regions of the virus envelope protein; certain anti-HIV antibodies, e.g. antibodies directed against CD4 binding site; biological activity of the viral regulatory proteins, such as Tat; post-translation modifications (glycosylation) of the viral envelope protein.

Several questions still remain concerning: (a) potential impact of an original antigenic exposure or pre-priming on the capacity to re-direct specificity of the host immune responses by means of therapeutic vaccination; (b) impact of HIV infection on maturation of an antibody response: a primary response to infection and a secondary response to candidate immunogens; (c) impact of anti-retroviral treatment on host capacity to orchestrate an HIV-directed immune response; (d) whether innate and/or adaptive immune responses should be modified and in which direction: augmented, suppressed or newly induced. The complexity of potential options for immune manipulations is obvious. With little data available to date, addressing these questions will require a strategic approach, which would include a stepwise implementation of clinical trials to bring the development of an effective therapeutic vaccine against HIV/AIDS to fruition.

An intriguing relationship was suggested between *in vivo* viral diversity and disease progression. It was noted that patients who had the greatest degree of viral diversity were also characterized by broad and potent immune responses, both of which were associated with delayed disease progression. On the other hand, patients with limited viral diversity and restricted anti-HIV immune responses were more frequently associated with an accelerated course of the disease. The researchers hypothesized that this phenomenon could reflect successful suppression of major viral forms by the host immune system, which as a result would drive diversification of minor viral forms with a net outcome in this setting being a more successful control of infection and delayed disease progression. In cases of restricted and less effective immune responses, there would be less pressure on the virus to diversify which remains uncontrolled in its capacity to cause the end organ damage and rapid disease progression.

Another part of the scientific unknowns is related to immunological targets for therapeutic vaccination. These may include HIV structural antigens, viral enzymes, regulatory proteins, human cell receptors, cytokines, etc. Most of the therapeutic vaccine approaches to date have focused on structural HIV proteins as primary immunogens. This is largely based on historical precedent and previous experience with other viral pathogens. However, non-structural proteins (e.g., Tat) might also be reasonable targets as a method to block their biological activity and interrupt the virus life cycle. For example, *in vitro* studies demonstrated that Tat enhances HIV replication by increasing HIV transcription, as well as by up-regulation of HIV-1 co-receptor expression (CXCR4 and CCR5). Also, *in vitro* studies have demonstrated that

Tat can inhibit host directed immunity, causing premature apoptosis of by-stander T cells, as well as by blocking antigen specific T cell recognition, resulting in T cell anergy.

The approaches to rationalize therapeutic vaccine design should also consider factors that could have an impact on quality and quantity (or both) of vaccine-induced responses, including: immunogen primary structure (e.g., genetic subtypes, R5/X4 phenotypes); immunogen secondary and tertiary conformation; glycosylation and expression systems used for production; formulation with adjuvants and immune modulators; route of administration, and the cytokine milieu in the host at the time of vaccination.

Although the recent advances in anti-retroviral drug development have been significant, it has become clear that this does not represent a sustainable therapeutic strategy for a majority of HIV-infected individuals on a worldwide basis. The development of effective therapeutic vaccines, if possible, could provide an affordable alternative complementary tool for improvement and feasibility of strategies for treatment of HIV/AIDS. Basic logistic characteristics make therapeutic vaccines an attractive target from public health point of view, since they are expected to be relatively inexpensive, easy to deliver, adherence friendly, with a minimal need for toxicity monitoring, not associated with expensive-to-treat complications, not requiring sophisticated medical training or facilities for application, and minimal concomitant need for clinical/therapeutic monitoring.

The long-term goal of therapeutic vaccination is to develop a sustainable and feasible treatment of HIV/AIDS on a worldwide scale. However, a number of immediate goals should also be pursued. First, to enhance *in vivo* antiviral activity by achieving an improved control of viral replication; prolonging efficacy of primary anti-retroviral drug therapy; permitting for drug-free viral control; decreasing the rate of emergence of viral resistance; modifying transition of co-receptor usage phenotypes of the virus; promoting and facilitating viral clearance. The second goal is targeted at enhancing the host immune reconstitution, which includes the induction and maintenance of HIV-specific immunity in the setting of prolonged anti-retroviral treatment; stimulation of innate anti-HIV immune effectors (e.g., beta-chemokines); correction of cytokine disregulation; correction of immune hyper-activation state; and promote primary immune reconstitution. Thirdly, therapeutic vaccines could be useful for enhancement of HIV prevention by facilitating identification of immune correlates of protection against HIV/AIDS and thus facilitating the development of effective HIV vaccines; allowing for prioritization of preventive vaccine candidates for efficacy trials; and potentially, when used on a large scale population basis, they could result in reduction of infectivity of the human pool and thereby modifying the dynamics of the epidemic.

Summary & conclusions for the scientific briefing session

In the summary cross-cutting issues were discussed relative to the pressing global need for an HIV vaccine, which could be summarized as follows: (i) need to demonstrate some vaccine efficacy; (ii) develop ways to improve it; and (iii) understand how it works. In addressing these issues the following questions were addressed by the participants:

1. ''*What is the relevance of HIV genetic diversity for vaccine efficacy?*'', and ''*Is there a need to match candidate vaccines to HIV-1 genetic subtypes prevalent in a specific country where the vaccine is going to be tested and eventually used?*''

Most participants agreed that the state-of-the-art in HIV vaccine development is sufficiently unsettled and there are many more pressing issues in developing a vaccine strategy/formulation that would have a high likelihood of success. A number of concerns and uncertainties remain despite substantial progress in technology and information on HIV virology, molecular genetics and immunology, including the following:

a) No correlate of protective immunity against HIV-1 has been defined so far, and this is unlikely to be defined without large scale trials, which should be adequately designed to address this question.
b) The immunogens, which are currently being tested in human trails have not yet demonstrated consistently high frequency of anti-HIV CTL responses, although some encouraging results have been recently reported.
c) None of the candidate HIV vaccines that have been tested in human volunteers have been capable of inducing broad neutralization of viral strains, in particular neutralization of primary HIV-1 isolates, even those that are closely related genetically to the prototype strain used for vaccine preparation.
d) There are virtually no data that could elucidate the links between genetic sequence and HIV-specific immune responses, allowing for informed development of HIV vaccine products.

The data presented at this meeting and elsewhere argue that for an HIV vaccine targeted at induction of T-cell responses, particularly against Gag and Pol proteins, which appear to be conserved in HIV, there is little evidence that matching at the genetic subtype level

would be of any benefit for the current immunogenicity trials. This is true for several reasons.

First, the HIV variation and recombination occurring within and across international borders is expanding in complexity more rapidly than previously anticipated. Thus, the selection of a predominant subtype or circulating recombinant form today will almost certainly be inappropriate or less than perfect in the course of time when a specific vaccine concept or candidate vaccine is optimized and ready for testing or ultimate use, should the preliminary trials demonstrate any level of efficacy.

Second, as discussed above, several studies have now demonstrated CTL cross-reactivity between different HIV-1 subtypes to varying degrees of conservation in structural and regulatory genes: *pol* > *gag* > *nef* > *env*.

Third, although understanding of functional antibody responses to HIV-1 is still a major scientific challenge, it was documented that sera from HIV-1 infected individuals and some vaccinees, as well as monoclonal antibodies, are capable of cross-recognition independently of genetic subtypes. Unfortunately, the possibility of inducing broadly reactive HIV-1 neutralizing antibodies in human immunization studies is still awaiting confirmation, although there are several promising new approaches that might improve this aspect of vaccine design.

The state of uncertainty surrounding the criteria for selecting virus strains for HIV vaccine production should not inhibit or paralyze the progress in HIV vaccine development and evaluation. Vaccine research teams that are initiating new HIV vaccine studies, particularly those in international partnerships, will need to justify the selection of HIV-1 variants that would appear close to the consensus sequence of globally dominant genetic forms of HIV-1.

Important information with regard to the possibility of inducing cross-protection against different HIV-1 subtypes, could be generated by specially designed phase I/II trials to be conducted in industrialized and developing countries. These trials could evaluate immune responses to subtype B-specific immunogens in selected populations from developing countries, while in parallel the non-subtype B immunogens could be evaluated in a limited number of volunteers from industrialized countries.

2. *Would the criteria for selection of prototype HIV vaccine strains be different for different types of vaccines and different vaccine approaches?*

There was a general consensus that matching of HIV vaccine candidates to the prevalent HIV-1 strains might be substantially less important for vaccines targeted at induction of T-cell responses to conserved proteins (e.g., Gag and Pol).

With regard to HIV vaccines aiming at induction of antibody responses, there may be a need to consider matching to some type of still unknown immunotypes of HIV-1, due to the fact that the anti-HIV antibody responses are often related to specific conformational determinants, which is particularly pronounced when candidate vaccines are based on HIV-1 envelope glycoproteins.

Based on existing data from studies of HIV-infected individuals, monoclonal antibodies, and human vaccine trials, it seems highly unlikely that this matching would be dependent on the known genetic subtypes. However, due to the lack of knowledge in this regard, it is advisable that HIV vaccine efficacy trials of different strategies be designed and implemented to provide more definitive answers to these questions. From this point of view, the issues of appropriate trial design and possibilities of generating relevant data from any HIV vaccine trial becomes even more important than the vaccine candidate itself which is being tested.

3. *What will be the significance of HIV-1 molecular epidemiology studies?*

It was felt important to continue supporting international HIV-1 genetic subtype surveillance studies linked to monitoring the epidemic in potential HIV vaccine evaluation sites. Data from different populations within a specific country might provide valuable information for vaccine trials. These data could be used to track genetic shifts of HIV-1 in a population and might be particularly useful at the stage of interpreting vaccine failures. In addition, genetic sequence data may serve as an important maker to monitor social and behavioural networks that are contributing to the spread of the epidemic in a specific country.

Conclusions and Recommendations from the WHO-UNAIDS HIV Vaccine Advisory Committee (VAC)

The WHO-UNAIDS VAC members analyzed the data presented at the scientific/briefing session and made the following conclusions and recommendations.

The increase in the AIDS pandemic emphasizes the urgency of developing new and more effective preventive tools against HIV. Vaccines represent an important component of future global strategies to combat the epidemic, and, perhaps, the best long-term

hope for the control of HIV/AIDS, especially in developing countries. However, the development of HIV/AIDS vaccines is still facing a number of difficult scientific challenges.

Great scientific progress has been made over the past year, providing unprecedented opportunities for developing and evaluating new candidate vaccines:

(1) genetic characterization studies of viral strains and variants sponsored by several agencies, including WHO-UNAIDS, have provided abundant information regarding global distribution and dynamics of HIV-1 subtypes and their recombinants;
(2) a significant number of candidate vaccines have been developed, and are entering clinical trials for safety and immunogenicity, and two large-scale trials have been initiated to test protective efficacy;
(3) new methodologies have been developed to assess immunological outcomes in vaccinees in a more precise way than previously available;
(4) introduction in some countries of highly active antiretroviral therapy, that can significantly reduce viral load, has opened new opportunities for the development of therapeutic vaccines; and
(5) guidance document on ethical considerations relevant to HIV preventive vaccine research, based on extensive discussions with interested parties from many countries, was developed by UNAIDS, providing an ethical framework for the design and conduct of clinical trials.

WHO/UNAIDS recognizes that vaccine development is an iterative process, which may require several trials and gradual improvement of candidate vaccines, before a highly effective vaccine is developed. Many agencies and organizations, in developed and developing countries, from the public and private sectors, are essential partners in this collaborative effort.

The first two trials to test vaccine efficacy are underway in the United States and Thailand, and it will take some time before results are available. Several new candidate vaccines remain to be tested for safety and immunogenicity, before being considered for large-scale efficacy trials. It is therefore essential that every effort be made to support and increase current HIV prevention efforts, because it will take at least several years before we have an effective HIV vaccine (especially one with high protective efficacy). Consequently, funding for AIDS vaccine research should be seen as additive to that already being used for other HIV/AIDS-related interventions, including care.

The WHO-UNAIDS VAC Members made the following recommendations:

1. While candidate vaccines entering Phase III evaluation are expected to show some level of protective efficacy, many questions remain regarding what antigens, and what immunological mechanisms, are necessary and sufficient for protection. In addition to continuing studies in animal models, it will be *essential to learn from human clinical trials, including efficacy trials*, what are the essential requirements and immunological correlates of protection.
2. Informative clinical vaccine trials will require *genuine and equal partnerships between scientists in developing and industrialized countries*. These partnerships should be developed at the earliest possible time, and should include planning for capacity building and infrastructure development. It is also essential to strengthen *partnerships between WHO-UNAIDS and other sponsors of vaccine research*, including national AIDS research programs in industrialized countries, the International AIDS Vaccine Initiative, the World Bank, the European Community, the pharmaceutical industry, and others. Likewise, building on previous work spearheaded by WHO-UNAIDS in 1992–1993, National AIDS Vaccine Programmes and/or Strategies in developing countries should be stimulated and supported. Because of the nature of HIV vaccine research, these collaborations and partnership should be conceived as *long-term and sustainable efforts*.
   Vaccine research for different diseases (especially the three major killers: HIV/AIDS, malaria, TB) could benefit from a coordinated approach, tackling common problems with common strategic approaches. This could be especially important in preparation of sites for the conduct of trials in developing countries. WHO-UNAIDS, through its Intercluster Vaccine Research (IVR), should establish necessary links with the Global Alliance for Vaccines and Immunization (GAVI), the Children Vaccine Programme (CVP), the International Vaccine Institute (IVI) and other relevant agencies.
3. Despite the lack of scientific evidence on the immunological relevance of HIV-1 genetic diversity, it *remains reasonable to initially design candidate vaccines based on the strains prevalent in the country in which trials are to be conducted*. However, because of the uncertainty of how important virus variation is for vaccine-induced protection, and because of the complexities of developing multiple vaccine candidates, it would be also important initially, wherever possible, to test vaccine efficacy against more than one strain in multiple arms of the trials. *Ideally, every Phase III trial should aim*

at testing more than one hypothesis or scientific question, so that maximum information could be obtained from each trial.

4. *In every Phase I, II or III trial, one goal should be to gain the most information relevant to the identification of immunological correlates of protection*, that could ultimately predict the effectiveness of future vaccine candidates, and reduce the time for development of second and third generation vaccines. This should include at least activities to develop protocols and reagents to assess:

   a. Antibodies, particularly neutralizing and mucosal antibodies;
   b. Cell mediated immunity, including cytotoxic T lymphocytes;
   c. T helper cells and immune memory;
   d. Antigenic characteristics of intercurrent viruses that might indicate some immune escape as evidence for selective pressure following immunization.

5. To facilitate the comparability of data from trials of different vaccines in different countries, WHO-UNAIDS should consider mechanisms to *assess needs for providing reference reagents and possibly standardized protocols* for immunological and epidemiological measurement methods, quality assurance and control.
6. With the introduction of highly active antiretroviral therapy for HIV/AIDS in some countries, which is capable of reducing viral loads to low levels in HIV-infected individuals, there may be an *opportunity to learn whether after reduction of viral burden, it is possible to immunize with vaccines* to engender a virus-free state, reduce viral loads, or reduce the chances of emergence of drug resistant strains. Such studies could also be helpful for the design of preventive vaccines, by more rapidly identifying immune correlates of protection, as well as effective candidate vaccines. However, at the present time it is *not recommended that such trials be undertaken in countries not using combination antiretroviral therapy* in their national programs.
7. WHO/UNAIDS should contribute to *supporting trials in developing countries by assisting in the planning process, and in strengthening their capacity, including opportunities for training*. Among the areas in which there is a need for expertise are: ethics, epidemiology, biostatistics, trial design and analysis, Good Clinical Practices, data management, culturally appropriate counseling to avoid HIV risk-related behaviour and provide prevention information, laboratory tests for virus and immune parameters assessment, economic analysis, intellectual property rights, and biological regulatory procedures. WHO-UNAIDS should also provide advice for the development of proposals for vaccine trials in developing countries, including plans and budgets for improvement of scientific facilities and ethical review capacity. An area in need of additional support is in relation to the natural history of HIV/AIDS in different populations, which may be required to interpret data from future efficacy and effectiveness trials.

   WHO-UNAIDS should *also facilitate the establishment of South-North and South-South collaborative networks* for exchange of information and experiences. Information on training opportunities should be made widely available.

   It is recognized that the human and financial resources needed to strengthen infrastructures for vaccine trials in developing countries are substantial. *WHO-UNAIDS can only provide seed funding to some of these activities, encouraging other partners to contribute to the effort*. In addition, *WHO-UNAIDS is encouraged to seek additional funds for their HIV vaccine activities*.
8. *The WHO-UNAIDS Network for HIV Isolation and Characterization*, aimed at isolating and characterizing viral strains and variants from different parts of the world and providing strains to all interested researchers and vaccine manufacturers, *should be maintained and strengthened*.
9. WHO-UNAIDS should *establish an information base, focusing on clinical trials (especially those conducted and planned in developing countries)* that would minimize unnecessary duplication of efforts, conserve human resources and maximize the new information to be gained from each trial. It would be helpful to learn which countries are planning to carry out trials, what are the participating institutions, industrial partners and scientists implementing the various aspects of the research, which vaccine candidates are to be tested, and the design and end points of the trials.
10. WHO-UNAIDS *should provide reliable and readily understandable scientific information to the public, media and advocacy organizations that can serve to mobilize support in countries for vaccine trials*. Of particular importance are epidemiological surveillance information on the burden of disease, incidence and prevalence rates, mortality rates due to AIDS-related conditions, and effectiveness of other non-vaccine interventions, which will ultimately be required to assess the impact of any vaccine on transmission and reduction of disease burden. Since many more HIV vaccine trials will be conducted in the future, it is important to develop information strategies targeting the media and advocacy groups, to educate the public on the rationale for conducting multiple HIV vaccine trials.

Annex I:

Members of the WHO-UNAIDS Vaccine Advisory Committee (VAC)

Dr. Francoise Barre-Sinoussi, *Institut Pasteur, Paris, France*
Dr. Seth Berkley, *International AIDS Vaccine Initiative, New York, USA*
Dr. Natth Bhamarapravati, *Mahidol University, Bangkok, Thailand*
Dr. Deborah Birx, *Walter Reed Army Institute of Research, Rockville, USA*
Dr. Barry Bloom, *Harvard School of Public Health, Boston, USA* **(Chair)**
Dr. Jose Carvalheiro, *Institute de Saude, Sao Paulo, Brazil*
Dr. Nirmal Ganguly, *Indian Council of Medical Research, New Delhi, India*
Dr. Margaret Johnston, *Division of AIDS, NIH, Bethesda, USA*
Dr. Michael Kazatchkine, *Agence Nationale de Recherche sur le SIDA, Paris, France*
Dr. William Malegapuru Makgoba, *Medical Research Council, Tygerberg, South Africa*
Mrs. Sophia Mukasa Monico, *The AIDS Support Organization, Kampala, Uganda*
Dr. Neal Nathanson, *Office of AIDS Research, NIH, Bethesda, USA*
Dr. Geoffrey Schild, *National Instititute for Biological Standards & Control, London, UK*
Dr. Hans Wigzell, *Karolinska Institute, Stockholm, Sweden*
Dr. Shudo Yamasaki, *National Institute of Infectious Diseases, Tokyo, Japan*

Annex II

Participants of the scientific expert group

Dr. A-M. Aubertin, *Institut de Virologie, Strasburg, France*
Dr. Michel Bartos, *La Trobe University, Melbourne, Australia*
Dr. Phil Bermann, *VaxGen, Brisbane, USA*
Dr. James Bradac, *Division of AIDS, NIH, Bethesda, USA*
Dr. Donald Burke, *Johns Hopkins School of Public Health, Baltimore, USA*
Dr. Huyen Cao, *Massachusetts General Hospital, Charlestown, USA*
Dr. Ronald Desrosiers, *Harvard Medical School, Southborough, USA*
Dr. Barbara Ensoli, *Instituto Superior di Sanita, Rome, Italy*
Dr. Eva Maria Fenyö, *Karolinska Institute, Stockholm, Sweden*
Dr. Guido Ferrari, *Duke University Medical Center, Durham, USA*
Dr. Bernardo Galvao-Castro, *Fundacao Oswaldo Cruz, Salvador, Brazil*
Dr. Tore Godal, *GAVI, Geneva, Switzerland*
Dr. Jaap Goudsmit, *University of Amsterdam, Netherlands*
Dr. Marie-Joelle Frachette, *Aventis Pasteur, Marcy L'Toile, France*
Dr. Richard Ginsberg, *Wyeth Lederle Vaccines, Pearl River, USA*
Dr. Jonathan Heeney, *Biomedical Primate Research Center, Rijswijk, Netherlands*
Dr. Harvey Holmes, *National Institute for Biological Standards & Control, UK*
Dr. Xia Jin, *Aaron Diamond AIDS Research Center, New York, USA*
Dr. Marcia Kalish, *Centers for Disease Control and Prevention, Atlanta, USA*
Dr. Michel Klein, *Aventis Pasteur, Marcy l'Etoile, France*
Dr. Bette Korber, *Los Alamos Laboratory, Los Alamos, USA*
Dr. Margaret Liu, *Chiron Corportaion, Emeryville, USA*
Dr. Andrew McMichael, *John Radcliffe Hospital, Oxford, UK*
Dr. Francine McCutchan, *Henry Jackson Foundation, Rockville, USA*
Dr. Fred Mhalu, *Muhimbili Medical Center, Dar es Salaam, Tanzania*
Dr. John Moscola, *Walter Reed Army Institute of Research, Rockville, USA*
Dr. Ronald Moss, *The Immune Response Corporation, Carlsbad, USA*
Dr. Bonnie Mathieson, *Office of AIDS Research, NIH, Bethesda, USA*
Dr. Edward Mbidde, *Uganda Cancer Institute, Kampala, Uganda*
Dr. Souleymane M'Boup, *Universite Cheikh Anta Diop, Dakar, Senegal*
Dr. James Mullins, *University of Washington, Seattle, USA*
Dr. Rosemary Musonda, *Tropical Diseases Research Centre, Ndola, Zambia*
Dr. Jack Numberg, *Montana Biotechnology Centre, Missoula, USA*
Dr. Malaki Owili, *Society of AIDS in Africa, Nairobi, Kenya*
Dr. Martine Peeters, *Centre ORSTOM, Montepellier, France*
Dr. Michel Pletschette, *European Commission, Brussels, Belgium*
Dr. Rober Redfield, *Institute of Human Virology, Baltimore, USA*
Dr. Mary-Paule Roudil, *UNESCO, Paris, France*
Dr. Peter Salk, *The Jonas Salk Foundation, La Jolla, USA*

Dr. Grabiella Scarlatti, *San Raffaele Scientific Institute, Milan, Italy*
Dr. Alan Schultz, *International AIDS Vaccine Initiative, New York, USA*
Dr. Haynes Sheppard, *California Department of Health Services, USA*
Dr. Leonidas Stamatos, *Aaron Diamond AIDS Research Center, New York, USA*
Dr. Ruengoung Sutthent, *Mahidol University, Bangkok, Thailand*
Dr. Yutaka Takebe, *National Institute of Infectious Diseases, Tokyo, Japan*
Dr. Edmund Tramont, *University of Maryland, Baltimore, USA*
Dr. Guido van der Groen, *Institute of Tropical Medicine, Antwerp, Belgium*
Dr. Jean-Louis Virelizier, *Institut Pasteur, Paris, France*
Dr. Gerald Voss, *Smith Kline Beecham Biologicals, R'Xensart, Belgium*
Dr. Bruce Walker, *Massachusetts General Hospital, Charlestown, USA*
Dr. Jonathan Weber, *Imperial College School of Medicine, London, UK*
Dr. Richard Wyatt, *Dana Farber Cancer Institute, Boston, USA*
Dr. Peter Young, *Alphavax, Durham, USA*
Dr. Susan Zolla-Pazner, *New York University Medical Centre, NY, USA*

WHO-UNAIDS Secretariat

Dr. Awa Coll Seck, *Director, Policy, Strategy and Research, UNAIDS*
Dr. Valentina Dias-Ferrao, *WHO-UNAIDS HIV Vaccine Initiative (temporary adviser)*
Dr. Jose Esparza, *Coordinator, WHO-UNAIDS HIV Vaccine Initiative*
Dr. Bjorn Melgaard, *Director, Vaccines and Biologicals, WHO*
Dr. Saladin Osmanov, *WHO-UNAIDS HIV Vaccine Initiative*
Ms. Claire Pattou, *WHO-UNAIDS HIV Vaccine Initiative*
Dr. Peter Piot, *Executive Director, UNAIDS*
Dr. Roger Salla Ntounga, *African Desk, UNAIDS*
Ms. Coumba Toure, *WHO-UNAIDS HIV Vaccine Initiative*