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Feature

Molecular Mechanisms of Viral Infection and Propagation: An Overview of the Second Advanced Summer School in Africa

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INTRODUCTION

Viral replication is dependent on a host; with their small genomes, viruses need to hijack host cellular machinery to complete their life cycle. The coevolution of intimate virus-host relationships has led to many viruses being able to successfully propagate without a significant detrimental effect to the host. However, in some instances, viral infection causes disease. The molecular mechanisms underlying virus-associated disease were the focus of an Advanced Summer School in Africa held in Hermanus, South Africa, from 6 to 14 March 2010.

Assembled in the coastal town of Hermanus were 45 participants, primarily PhD students and postdocs from less developed countries, and 18 speakers, leaders in their respective fields, from around the world (Fig. 1). The school, the second of its kind in Africa was sponsored by the International Union of Biochemistry and Molecular Biology (IUBMB), the Federation of European Biochemical Societies (FEBS), the Federation of African Societies of Biochemistry and Molecular Biology, the United Nations Educational Scientific and Cultural Organization, and the International Centre for Genetic Engineering and Biotechnology (ICGEB).

The course was honored by the presence of Brian Clark, Professor of Biostructural Chemistry at The University of Aarhus, Denmark. Prof Clark is a renowned scientist who participated in the discovery of the initiation codon for protein synthesis (*1–4*) and the first crystallization of a tRNA (*5*). Today, Prof Clark studies the molecular and cellular mechanisms of ageing using a systems biology approach. His presence was particularly significant at the Summer School since he has been involved in the organization of several similar events during his tenure as President of the IUBMB and FEBS, most notably the Spetses Summer School. Prof Clark stressed the importance of such meetings for the development, not just of young scientists, but of science itself.

The main themes of discussion at the summer school were: 1) why viral infection can lead to cancer; 2) how a greater understanding of the mechanisms underpinning human immunodeficiency virus (HIV) propagation can inform new antiviral strategies; 3) the abilities of viruses to evade the immune system and the obstacles to the development of effective vaccines; and, 4) the potential afforded by viruses as research tools. The importance of host factors became apparent in the discussion of all these topics, and how viral research has informed our general knowledge of cell biology. This report serves to summarise the findings presented at the summer school.

VIRAL TUMORIGENESIS: A RESULT OF VIRUS-HOST INTERACTIONS GONE AWRY?

Until Peyton Rous discovered the first tumor virus in 1911, viruses were viewed as peculiar infectious agents capable of inducing cancer in animals, but not in humans. However, it is now well-established that many different human viruses, including Human papilloma virus (HPV), Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpes virus (KSHV), Human T-cell leukemia virus-1 (HTLV-1), Hepatitis B virus (HBV), Hepatitis C virus (HCV), and Merkel cell polyoma virus (MCV) are the etiological agents of human cancers, encompassing at least 15–20% of all tumors worldwide (*6*). Hence, tumor virology was a focus of this summer school.

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Figure 1. Participants of the Second Advancement Summer School in Africa. 45 young researchers and 18 speakers were assembled in the beautiful coastal town of Hermanus, South Africa.

Human Papilloma Virus

Dr Lawrence Banks from the ICGEB, Trieste, provided an overview of HPV and the role of viral proteins in the transformation of cervical epithelial cells. Almost all (99.7%) cervical cancers contain HPV DNA, usually types 16 and 18 (*7*). Despite this statistic, cancer induction is not part of the 'normal' HPV life cycle. Only in rare cases where there has been a lack of immune clearance leading to persistent infection, along with additional changes in the cell, does cancer develop.

Banks described how HPV E6 and E7 are the major viral oncoproteins involved in the development of cervical cancer. The HPV life cycle is critically dependent on the differentiation of epithelial cells: there is usually no DNA replication, other than in cells of the basement layer, but HPV E6 and E7 induce a pseudo-S phase, creating a permissive environment for viral replication (*8*). In addition, E6 and E7 target cellular substrates for proteasome-mediated degradation, notably the tumor suppressors p53 and pRb, respectively (*9, 10*).

HPV DNA exists in an episomal form but may be found integrated in malignant cells. Integration often involves loss of viral DNA. Indeed, malignant cells often display no viral replication but maintain expression of E6 and E7 (*11*), further emphasizing the roles of these proteins in cancer progression.

But why do only a few HPV infections result in malignancy? This issue was tackled by Dr John Doorbar, National Institute for Medical Research, London. HPV-associated cancers are more predominant in some tissues than others: there are ~500,000 cases of cervical cancer reported each year. In contrast, HPV-associated penile cancers are rare (~40,000 cases per year), despite no evidence of a disparity in primary infection rates. This implies a difference in the propensity of HPV to form neoplastic lesions at different sites. Doorbar speculates that this is due to a graded ability of HPV to replicate in different tissues. As further evidence, HPV-1 causes warts but no lesions at mucosal sites. Doorbar believes that the transformation zone in the cervix may be particularly susceptible to neoplasia formation. The exact host factors responsible for the variation in HPV replication ability are unresolved.

Doorbar's group is also investigating the molecular basis of the different outcomes resulting from HPV infection. HPV gene expression occurs in an ordered manner as the infected cells move through the epithelial tissue. Doorbar described how the host protein mini chromosome maintenance protein 7 (MCM7) could be used as a marker for cell proliferation and, thus, as a surrogate for E6/7 activity. MCM7 was expressed in the lower layers of HPV lesions, as shown by laser capture microscopy, and the viral protein E4 was abundantly expressed in the upper layers (*12*).

Interestingly, Doorbar's group have shown that high-grade squamous intraepithelial lesions (HSILs) are associated with increased MCM7 expression in upper epithelial layers with almost no E4 expression (*12*), revealing that viral expression patterns reflect the severity of disease. In addition, proliferation of the basal cells does not stop when the basement layer is complete in high-risk lesions, in contrast to low-risk lesions. The increased basal layer proliferation is correlated with beta-catenin activity and E6 levels (*13, 14*). What drives increased E6 activity in some infections is unknown: wound healing,

episomal copy number, DNA methylation patterns, and host genetic background could all contribute and require further investigation. Nevertheless, these observations have the potential to tremendously improve existing cervical screening practice, which relies heavily on cytology.

Though *in vitro* studies are invaluable for identifying candidate molecules involved in pathogenesis, conformation of their role often requires an *in vivo* approach. Dr Paul F. Lambert, University of Wisconsin Medical School, described transgenic mouse models of HPV where the human keratin promoter, hK14, was used to drive expression of HPV genes in stratified squamous epithelia (*15*). Previous studies had shown that expression of HPV genes was insufficient to cause cervical cancer in mice (*15*). Lambert's group hypothesized that estrogen was a cofactor for cervical cancer. They generated hK14-HPV16-estrogen receptor transgenic mice, which did develop cervical cancer (*16–18*). This may account for observations that >5 years of contraceptive pill usage and pregnancy increases the risk of women developing cervical cancer.

HPV is not just associated with cancer of the cervix, however. Prof Iqbal Parker of the ICGEB, Cape Town, showed that papilloma viruses typically considered low-risk could be involved in fatal cancers of the oesophagus. Parker's group found that about 40% of oesophageal cancer (OC) patients had integrated HPV-11 and -39 DNA in their tumors (*19*). Brush biopsies of healthy people confirmed that HPV was enriched in OC patients. However, smoking, alcoholic home brew consumption and cooking on indoor fires in conjunction with certain Sulpho-transferase alleles were also risk factors associated with the development of OC in Africa (*20*). Whether HPV infection is an early or initiation event in OC development requires further investigation.

The molecular alterations that direct progression from productive infection to HSILs in HPV infection are not fully understood. HPV oncoproteins alone are not capable of transformation, as shown in human keratinocytes *in vitro* (*21*) and murine cervical epithelia *in vivo* (*15*). Rather, papilloma virus-mediated oncogenesis requires supplementary genetic changes that occur over time following the initial infection. Whilst it is clear that integration of viral DNA into the host genome is crucial to HPV-induced tumor development (*22, 23*), whether this is a cause or consequence of wider-spread chromosome instability is not clear.

Despite the activity of various oncogenic HPV proteins, viral DNA integration and cancer development does not often follow. The import of the host, including an ability to regulate viral gene expression in different tissues and to mount an effective immune response, is becoming increasingly apparent in determining the molecular basis of HPV-associated tumor progression.

Kaposi's Sarcoma-associated Herpes Virus

Dr Ethel Cesarman, Weill Cornell Medical College, described Human Herpes viruses and focused on KSHV, also known as Human Herpes Virus 8 (HHV8). KSHV causes a cancer often found in immunosuppressed individuals, such as those suffering from AIDS, including Karposi's sarcoma primary effusion lymphoma and multicentric Castleman's disease. There is >50% KSHV seroprevalence in sub-Saharan Africa but, as with HPV, the majority of individuals present with no disease.

KSHV contains up to 90 ORF with at least 15 accessory genes. Among the accessory genes identified to date, ORF74, named KSHV G protein-coupled receptor (KSHV-GPCR), has become a major focus of investigation. Though it is expressed in a very small number of KS lesion spindle cells (*24*), it is known to be involved in cell transformation through induction of angiogenic cytokine expression (*25*). For example, vascular endothelial growth factor is one cytokine secreted from cells expressing KSHV-GPCR, which can induce proliferation and angiogenesis in KS tissue (*26*).

Following the observation that proteins expressed by lymphomagenic viruses during latent infection activate NF-κB, Cesarman's group searched for a viral protein whose activity correlated with NF-κB. They identified vFLIP, an homologue of cFLIP proteins, and showed that anti-vFLIP siRNAs induced apoptosis in KSHV-positive cells only (*27*). Work is continuing to develop drugs that are vFLIP-specific, in the hope that these will prove effective anti-KSHV agents.

Epstein-Barr Virus

During his lectures, Dr Ingemar Ernberg, of the Karolinska Institute in Stockholm, recoined the acronym EBV as "Every Bodies Virus" because >95% of humans are infected from childhood, though infections are typically subclinical. A member of the HHV family, EBV was initially isolated from a Burkitt's lymphoma cell line (*28*), though it was later found associated with a number of different tumors, such as undifferentiated nasopharyngeal carcinoma, Hodgkin's disease, nasal T cell lymphoma and gastric carcinoma.

Ernberg described the four programs of latent viral gene expression (L0–L3) and how all 12 viral genes are expressed in L3, which is associated with cell proliferation (*29*). Therefore, Ernberg's group looked for the signal controlling the switch from L1 to L3. They showed that in L1, there was low expression of the viral gene EBNA1, but high levels of the cellular transcription factor Oct2, and vice versa in L3. Work is ongoing into deciphering the reason for a decrease in Oct2 levels that is concomitant with a switch to the L3 program of gene expression. Hopefully this will shed light on why EBV set points vary between individuals and, further, what differs between the B cells of those who appear immune to EBV infection and the majority of the population who are susceptible.

Human T-cell Leukemia Virus-1

Dr Aluisio Segurado, University of São Paulo, described HTLV-1, a retrovirus that can result in Adult T cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy (HAM/

TSP). It is estimated that 15 to 20 million individuals are infected with HTLV-1 worldwide, yet the vast majority remain clinically asymptomatic–only 2–6% develop ATLL (*30*).

The reason for the low frequency of HTLV-1-associated disease is unknown but Segurado hypothesises that host genetics and environmental factors play a role, rather than infection with different strains of HTLV. Tax (p40tax) is one of the HTLV-1 proteins that acts as a transcription activator in ATLL (*31*). However, the low incidence rate and the long latency period prior to development of ATLL suggests that, in addition to viral infection, accumulation of mutations in host genes is required for cellular transformation *in vivo*.

Hepatitis B and C Viruses

Among the hepatitis viruses, HBV and HCV cause chronic infection leading to the development of cirrhosis and hepatocellular carcinoma (HCC). HBV is a DNA virus that integrates into the host genome. Dr Shahid Jameel, ICGEB, New Delhi, described how this integration deregulates expression of the viral protein HBx, which is able to induce HCC, either alone or in synergy with different cellular proteins (*32*). Jameel also discussed tumorigenesis induced by HCV: chronic immune-mediated inflammation and associated oxidative chromosomal DNA damage probably play a role. Interactions of viral proteins with pRb and p53 may also predispose to carcinogenesis (*33*).

Summary

Although not all the viruses discussed induce the same pathway to cancer, some parallels can be drawn. A requirement for viral oncogenes indicates that the viruses play a crucial role in progression to malignancy. Both RNA and DNA tumor viruses promote growth of infected tissue by activation of cellular oncogenes and inactivation of tumor suppressor genes. Interestingly, many cellular oncogenes and tumor suppressor genes (e.g., p53, pRb) were identified through studies of RNA and DNA tumor viruses, respectively. After infection, these viruses can establish either latent (HPV, EBV, KSHV, HTLV-1) or chronic (HBV, HCV) infection. Activation from latent infection may result from changes in the cell environment, an accumulation of viral stress on the cell and/or a decreased ability of the host to maintain latency or clear infection. Such activation signals may be dependent on the host's genetic make-up (*34*) and epigenetic factors (*35*).

Understanding why some individuals infected with tumor viruses do not develop cancer remains critical. It is anticipated that continuing research into what distinguishes 'normal' viral life cycles from the life cycles in malignant tissues, and the changes in host factors accompanying transformation, will address this problem. So far it has been demonstrated that changes in viral gene expression may accompany the development of high-risk lesions (e.g., HPV, EBV). But why are such changes occurring at a low frequency in infected populations? Are these changes in gene expression a result of a virus-host interaction disruption? Answering these questions requires a great deal more research, but certainly the speakers at the Summer School are on course to do just that.

ELUCIDATION OF THE MOLECULAR MECHANISMS OF HIV INFECTION AND PROPAGATION INFORMS ANTIVIRAL STRATEGIES

A detailed understanding of the molecular mechanisms governing virus infection and propagation is crucial to the development of antiviral strategies through identification of critical processes and drug targets. This was best exemplified at the Summer School by three speakers who are elucidating complimentary stages of the HIV life cycle: Ariberto Fassati, University College London, discussed nuclear import, Alessandro Marcello, ICGEB, Trieste, focused on integration and the spatial and temporal regulation of HIV-1 gene expression, and Hans-Georg Kräusslich, Universität Heidelberg, dealt with assembly and maturation. Although existing highly active antiretroviral therapy (HAART) can significantly reduce HIV-related illness, the emergence of drug resistant strains, toxic side effects and their ineffectiveness in latently infected cells has ensured that the development of novel HIV-1 therapies remains an important objective.

Nuclear Import

Lentiviruses, such as HIV-1, have the ability to infect terminally differentiated nondividing cells and, therefore, require nuclear import. However, the HIV-1 reverse transcription complex (RTC) is larger than the nuclear pore diffusion size limit. In addition, the RTC is enriched for nucleic acids and so must overcome the hydrophobic exclusion of nuclear pore complexes and a concentration gradient of DNA to enter the nucleus. The manner in which HIV-1 resolves this dilemma is worth investigating as nuclear import is critical to HIV-1 transmission and AIDS pathogenesis. Dr A. Fassati is doing just that and his group has identified some novel mechanisms by which HIV-1 enters the nucleus.

A number of viral elements have been shown to play a role in nuclear import e.g., the cPPT element, a nuclear localization signal within matrix and the viral proteins integrase (IN) and Vpr. Fassati's group was interested in identifying the host nuclear transport receptor responsible for HIV-1 import. They used purified RTC complexes in primary macrophages to show that importin 7 (imp7) is involved (*36*), an import receptor for RNA- and DNA-binding ribosomal and histone proteins, respectively. Further work revealed that the role of imp7 was HIV-specific, which correlated with its ability to bind HIV IN (*37*). RNAi-mediated knockdown of imp7 decreased import of DNA, but not RNA, indicating that reverse transcription is not a requirement for nuclear import (*37*). The DNA import-function of imp7 is likely hijacked by HIV-1 to facilitate import of the HIV-1 DNA genome, although it remains unclear whether the RTC remains intact throughout the nuclear import process.

Fassati's group continued to search for other nuclear import pathways. They used nucleic acid dye-labeled RTCs and cells treated with digitonin (to permeabilise the plasma, but not the nuclear, membrane) to monitor nuclear import in the presence of various cytosolic extracts. The almost homogenous fraction capable of inducing nuclear import of HIV-1 RTCs was found to contain tRNAs (*38*). Further, they showed that nuclear import of at least some species of tRNAs occurs in uninfected cells (*38*) –a previously undescribed cellular pathway of unknown function that HIV-1 appears to exploit. tRNAs are present in HIV-1 virions and it is speculated that the T-arm promotes nuclear import of the RTC via associations with host factors, which have yet to be identified.

Fassati's work not only describes novel import pathways for HIV-1, and thus, identifies potential drug targets, it has also enhanced our knowledge of cellular biology through identification of the tRNA nuclear import pathway.

Chromatin Organization and Latency

Latent viral reservoirs are established early during HIV infection, which prevents eradication of the virus, as they are not susceptible to antiretroviral treatment. In addition, latent proviral DNA can be reactivated to replenish viral loads on interruption of treatment. Therefore, understanding the molecular mechanisms governing latency and reactivation of viral expression is crucial to developing strategies aimed at complete eradication of the virus. To this end, Dr A. Marcello described his work into the impact of chromatin and chromosome territories on the control of HIV-1 transcription and latency.

Marcello has shown that the histone methyltransferase Suv39H1, the chromodomain-containing protein HP1γ and histone H3K9 trimethylation is enriched at transcriptionally silent proviral DNA (*39*). RNAi targeting HP1γ can alleviate the chromatin repression on HIV-1 gene expression in PBMCs from HIV-1-infected donors (*39*). Whilst providing insight into the mechanisms underlying HIV-1 latency, the question remains as to how some cells come to be latently infected, with repressed chromatin architecture at the proviral LTR, given the propensity for HIV-1 to integrate into active transcriptional units.

The nucleus is a highly dynamic and organised entity and it is now widely accepted that active transcription is enriched at the nuclear periphery. Marcello's group examined the latently infected cell line, J-lat A1 cells developed by Jordan, et al (*40*), by FISH and showed that along with activated viral transcription occurring at the nuclear periphery, the latent provirus was located at the periphery too (*41*). Chromatin conformation capture (3C) analysis revealed an association of the latent provirus with a pericentromeric region of chromosome 12 in trans, which was lost on reactivation of transcription (*41*).

Although it cannot be ruled out that latency is a result of a rare integration event into an inactive gene at the nuclear periphery, Marcello's work supports a model in which integration into an active gene and clonal expansion of the activated T cell population is followed by a number of T cells becoming quiescent memory cells by chance, a transition that is accompanied by the repression of the provirus through epigenetic mechanisms. Disruption of this repressive chromatin in an HIV-specific manner is an ongoing challenge.

Virion Budding

The host cell is a crowded environment, so assembling virions need to form stable structures in the producing cell; but the virion needs to be rapidly destabilised upon entry into a new cell. Different viruses have evolved different solutions to this assembly–disassembly paradox. In HIV, the solution is provided by the Gag polyprotein. Initially, a stable, immature virion with a spherical, capsid shell composed of Gag is formed (*42*). During maturation, sequential cleavage of the Gag polyprotein by protease (PR) (*43*) results in a rearrangement of the virion to form a mature, infectious virus with a conical capsid, composed solely of the capsid (CA) domains of Gag, despite no change in the overall size of the virion (*44*).

Dr H. G. Kräusslich provided an overview of his work detailing the mechanisms of virus release and the immature to mature virion transition. Cryo-EM studies revealed that following proteolytic maturation of Gag, the mature capsid contains 1,000–1,500 CA proteins assembled into a hexameric lattice (*45*). A completely spherical immature capsid was predicted to contain ~5,000 Gag polyproteins, more than double the number present in the mature core. Subsequent cryoelectron tomography studies demonstrated that, in fact, Gag is arranged in an incomplete spherical hexagonal lattice which, in addition, contains holes (*46*). The "holes" may serve to alleviate the strain imparted by the lattice curvature, as there is no evidence of pentameric defects, as observed in the mature core.

The differing arrangement of Gag and its derivatives in the immature and mature stages may indicate a disassembly of the immature shell, prior to assembly of the mature core. The extent of disassembly is unclear, but such a process would be an attractive drug target.

If the immature virus contains an incomplete shell, how is the virus released from the cell? Previously, it was thought that Gag assembled at the plasma membrane into spherical shells that resulted in membrane budding, with subsequent cleavage of the resulting thin membrane tether by the cellular endosomal sorting complex required for transport (ESCRT) machinery (*47–50*). Kräusslich's group demonstrated that almost complete Gag spheres were only present at late-budding sites where functional ESCRT was absent (*51*). Along with their observations that the immature shell is composed of an incomplete Gag sphere, they proposed a novel model for the release of HIV-1 virions: budding is initiated by Gag assembly with ESCRT recruited early to drive release (*51*). The process can be, therefore, considered a kinetic competition: should assembly occur faster than ESCRT recruitment, release is incomplete. Using total internal reflection fluorescence (TIRF) microscopy, Kräusslich's group

was able to confirm that Vps4, an ESCRT-associated ATPase, appears in bursts at sites of HIV-1 particle production early in Gag assembly (*52*).

Kräusslich furthered understanding of the role of another host factor, CD137/tetherin, in HIV-1 release. Known to be an HIV-1 restriction factor, CD137 is downregulated by the HIV accessory protein Vpu (*53*). However, rodent and mouse CD137 potently inhibits HIV-1 release through its resistance to Vpu (*54*). This has consequences for the development of small animal models of HIV infection.

The work of Kräusslich, Marcello, and Fassati reveals that continued efforts into discerning the molecular mechanisms of HIV-1 replication facilitates development of effective antivirals, an approach applicable to all pathogenic viruses.

VIRAL EVASION OF THE IMMUNE SYSTEM AND PERSPECTIVES ON VACCINE DEVELOPMENT

Human Papilloma Virus

The immune system is important in curtailing the detrimental effects of HPV infection: more lesions are observed in Severe Combined Immunodeficiency (SCID) patients (*55*). Further, detection of HPV DNA in women is age-dependent (*56*), which may be due to changes in the immune system.

Dr J. Doorbar described how the immune system struggles to clear HPV: Langerhans cells are not very effective as viral proteins are expressed at low levels in the lower epithelial layers. In addition, HPV proteins E6 and E7 downregulate cytokines and interferons, respectively (*57*). E5 is also thought to play an immunosuppressive role through inhibition of major histocompatability (MHC) maturation (*58*).

Hence, without intervention, HPV-associated warts and verrucas can take months to disappear. It is also this delay in immune clearance of the virus that may predispose individuals to cancer. Fortunately, an HPV vaccine is available. Whilst effective, the vaccine protects only against the two most predominant high-risk types, so that it is 75–80% protective (*59*). Nevertheless, widespread administration of this vaccine would greatly reduce the cervical cancer health burden.

Influenza

Fear of influenza pandemic has enveloped the world over recent years. Prof J. L. Virelizier, Pasteur Institute, Paris, addressed the immune response to this virus. Typically, humans successfully eradicate influenza, following the production of antibodies, although the virus is transferred to another individual prior to antibody production. The fear of a pandemic is born out of concern for a gene reassortment giving rise to a virus that is able to both infect humans and cause fatality.

Antibodies to the envelope protein haemagglutinin (HA) are protective against influenza (*60*). However, the virus is able to avoid the immune system through antigenic drift and periodic antigenic shift, the latter having the potential to cause pandemics (*61*). Passive transfer of polyclonal antibodies specific to influenza PR8 HA protected against homologous PR8 virus infection in mice, but transfer of cross-reactive anti-FM1 HA antibodies did not (*62, 63*). This highlights the problem that influenza protection requires strain-specific, not cross-reactive, antibodies, which needs to be taken under consideration in vaccine development.

Virelizier also discussed the relevance of lymphocyte memory to the antiviral immune response. He described the original antigenic sin phenomenon, in which immunisation with one variant and boosting with another recalls not just cross-reactive antibodies but also antibodies to variant-specific determinants (*64, 65*). For example, immunisation with HA strain 1 (H1) will not protect against another strain e.g., H3, but will improve the primary response to another H1 variant years later. This relates to the dual function of B cells as both antibody producers and antigen presenting cells: immunisation primes T cells, via B cell antigen presentation, not just B cell antibody production. Thus, following primary exposure to another variant, T cell help facilitates a more rapid antibody response, including to newly encountered epitopes (*66*). This is important in vaccine administration practices as it emphasises the importance of vaccinating not just the older members of society but also the young, so as to afford them lymphocyte memory.

Coronavirus

Severe Acute Respiratory Syndrome (SARS) was first reported in Asia in 2003 and within a few months the virus had spread to more than 24 countries in Asia, North America, South America, and Europe (*67*). Prof Luis Enjuanes, Consejo Superior de Investigaciones Cientificas (CSIC), Madrid, gave a detailed lecture on his development of a SARS-CoV vaccine. His group has demonstrated that recombinant virus rSARS-CoV-Δ*E* protected BALB/c mice from fatal respiratory disease when challenged with SARS-CoV, and partly protected mice expressing the viral receptor, hACE2 (*68*). Hamsters immunised with rSARS-CoV-Δ*E* showed decreased lung inflammation and clinical illness on SARS-CoV challenge (*69*). The recombinant strain also induced antivirus T cell and antibody responses (*68*). Work is ongoing to elucidate the role of *E*, but differential gene expression studies implicate regulation of the unfolded-protein stress response (*70*).

Whilst highly promising, the vaccine is not yet considered safe for humans, particularly given the prevalence of coronaviruses and the risk of recombination. Existing vaccine production involves chemical attenuation, but this also carries safety concerns, as inactivation can be incomplete. The use of rSARS-CoV-Δ*E*, rather than wild type SARS-CoV, in chemical attenuation could improve the safety of vaccine production, without compromising immunogenicity.

Human Immunodeficiency Virus

HIV has infected more than 60 million people worldwide, mostly in the developing world, and nearly half of these indi-

viduals have died (*71*). The epidemic is driven, in part, by the exquisite ability of HIV to evade the immune system.

Dr S. Jameel discussed various mechanisms used by HIV to escape the host immune system. The multifunctional HIV accessory protein Nef optimises the cellular environment for viral replication via associations with a number of host factors. It has been shown that Nef is able to downregulate expression of major histocompatability molecule (MHC)-I and -II and the MHCII-binding protein CD4 (*72*). Jameel's group has also identified a role of Nef in the downregulation of costimulatory molecules CD80 and CD86 (*73*). Their ongoing work is investigating the potential role of another HIV accessory protein, Vpu, in decreasing antigen presentation through interactions with the MHCII invariant chain, CD74 (*74*).

Given the ability of HIV to evade the immune system so successfully, considerable efforts are being devoted to the development of a vaccine. Despite a decade of research, an effective vaccine remains elusive. Three characteristics of HIV infection point to the difficulties associated with developing an effective vaccine: 1) persistent replication, 2) cellular and humoral immunity escape, and 3) immunosuppression.

Neutralising antibodies have been shown to provide protection in primate models (*75*). Dr Jeffrey Dorfman, ICGEB, Cape Town, described how he believed a successful HIV-1 vaccine would elicit broadly neutralising antibodies (bNAbs). Dorfman is hoping to identify novel bNAbs by screening sera with a pseudovirus neutralisation assay. Such bNAbs will be useful for the identification of epitopes that will inform vaccine design. Identification of bNAbs is hampered, however, by technical difficulties associated with their isolation and production, particularly as they are rare. Dorfman has developed a novel method for their production, which involves fusion of a PBMC with a myeloma (*76*), sidestepping the requirement for EBV-mediated transformation.

Prof Carolyn Williamson, University of Cape Town, discussed the genetic bottleneck of HIV transmission, i.e., only a single, or very few, viruses establish infection (*77*). Interestingly, these strains appear more neutralisation-sensitive, which bodes well for bNAb-inducing vaccines. Whether there is an infectious advantage in mucosal sites for a less stable viral envelope that is more likely to expose conserved epitopes is unclear.

Although NAbs are the basis of protection for most antiviral vaccines, there are concerns over their efficacy in preventing HIV-1 infection. The bNAb response would have to be sufficiently strong and rapid at mucosal sites to prevent any infection, even low-level. This is because bNAbs are unable to neutralise a virus transmitted through a virological synapse, due to steric hindrance (*78*). In addition, any virus that does not contain the epitope will be rapidly selected for. Therefore, no matter how broad the NAbs are it is likely that a vaccine would need to elicit a number of bNAbs to increase its protectiveness. Nevertheless, bNAbs are the only way of conferring sterilising immunity and, as such, are still the Holy Grail of HIV vaccine design.

Other scientists believe an effective HIV-1 vaccine will elicit cytotoxic T lymphocyte (CTL) responses. However, Prof J. L. Virelizier described how there is no precedent that CTLs, in any animal against any virus, kill virus-infected cells by a cytotoxic mechanism. Conversely, Williamson reported on studies revealing a correlation between specific HLA alleles and the breadth of HIV Gag-specific T lymphocyte responses with the control of viral replication (*79*), although there have been conflicting results (*80*). And, of course, correlation is not causation. That is not to say that T cell immunity is not protective: CTLs injected into a mouse liver expressing an HBV transgene resulted in decreased viral replication (*81*). Histology revealed that the protection was not conferred through a cytotoxic mechanism, rather through production of TNFα, as replication was restored in the presence of an anti-TNFα antibody (*81*).

T cell activation can also confer protection against HIV through production of chemokines. RANTES and MIP1α and SDF1 are the host ligands for HIV coreceptors CCR5 and CXCR4, respectively (*82*). These chemokines can block HIV entry because they compete with HIV for the receptors. Virelizier described the ability of truncated $RANTES_{9\text{-}68}$ to inhibit HIV infection, whilst no longer acting as an agonist, as truncation prevents signaling through G proteins (*83*). There are reports, however, that HIV can use other coreceptors, including CCR3, STRL33/Bonzo/TYMSTR, and BOB (*84*), complicating the use of chemokines or their mimics as a therapy. In addition, chemokines will suffer the same limitations of bNAbs at the virological synapse.

Although T cell activation can be antiviral (through the production of interferons, chemokines, and TNFα), it may be a double-edged sword: Virelizier proposed the hypothesis that T cell activation contributes to persistent replication, through NF-κB signaling initiating HIV transcription. Nevertheless, given the help that T cells can provide to B cells and antibody production, it is likely that an ideal HIV vaccine would elicit both humoral and cellular immunity.

There is a school of thought that a vaccine is not required to prevent HIV-related deaths at all. HIV-infected patients die as a result of their immunosuppression. Conversely, primates are able to survive with host-specific SIV infection indefinitely because their immune systems are not destroyed. Virelizier speculates, therefore, that research should be focused on combating HIV-mediated immunosuppression.

In summary, understanding the immune response to viral pathogens is critical, to counteract viral evasion of immune responses and to discern what responses are protective to inform vaccine design. This will enhance our ability to induce effective antiviral immune responses, reducing the probability of disease.

VIRUSES AS RESEARCH TOOLS

Beyond the enormous health concern that arose with the SARS epidemics and the major economic losses caused by this family of viruses, coronaviruses (CoVs) have the potential to be

promising delivery vectors for vaccine development and gene therapy (*85*). A highlight of the course was a presentation by Dr L. Enjuanes, on the mechanism of transcription used by the Coronaviridae family. He emphasised the importance of RNA chaperones, which are transcribed by the viral genome and are essential for efficient CoV replication (*86*).

Enjuanes' group identified a CoV transcriptional enhancer, based on a long distance RNA–RNA interaction in the transmissible gastroenteritis coronavirus (TGEV) (*87*). The additional discovery of transcription regulatory sequences permitted genetic manipulation to improve gene expression. As well as enhancing basic knowledge of CoV biology, these findings facilitated development of a safe and successful CoV-based delivery vector.

Advantages CoVs possess over other viruses as expression vectors include: 1) the possibility of spike protein manipulation, to engineer virus tropism (*88, 89*); 2) the replication of the RNA genome in the cytoplasm, side-stepping potential problems associated with integration (*90*); 3) the existence of nonpathogenic strains that infect a wide range of species of health and economic importance; 4) the ability to carry large genomes (~27–30 kb), which could favor the introduction of extensive foreign genes (*91*); and 5) the availability of cDNA clones derived from infectious strains (*92, 93*).

Two expression systems have been developed based on CoVs: one is a helper-dependent expression system, where the production of large amounts of heterologous antigen (2–8 μg/10^6 cells) has been achieved and synthesis has been retained for around 10 viral passages (*85*). In addition, there are the single genome CoV vectors, which can either be constructed by target recombination or by using an infectious CoV cDNA clone derived from the TGEV genome. This system has been shown to express a foreign gene for at least 20 passages (*94, 95*), demonstrating its high stability. Ongoing research is exploring the possibility of manipulating the species and tissue tropism of these CoV expression systems. Enjuanes' work reveals that these CoV vectors are flexible, robust tools with the potential to aid vaccine production and gene therapy.

CONCLUDING REMARKS

Virus-associated disease may arise for a number of reasons: a virus replicating in a suboptimal host or tissue, the emergence of a new, more pathogenic variant, an accumulation of detrimental effects when an infection clearance fails, or an enhanced susceptibility of the host. Research into the molecular basis of virus infection and propagation will enhance our understanding of virus-associated disease and inform new antiviral strategies and vaccine design. In addition, ways in which viruses can be harnessed for our own means will emerge.

Participants at the Summer School learnt a great deal about how to approach their work from the top-quality research presented. The School embodied not just good science, but also a great atmosphere (Fig. 2), epitomised by the inclusion of everyone in the birthday celebrations of speakers L. Enjuanes and J. L. Virelizier. Informal interaction throughout, and plenty of time allocated for discussion, ensured participants gained invaluable input from lecturers. Allowing all participants to present their work at the School further stimulated discussion and has led to new collaborations; an opportunity that would not have been afforded to the developing country scientists without the Summer School.

Figure 2. Food for thought. Speakers Dr. Alessandro Marcello (left) and Prof. Jean-Louis Virelizier (right) enjoy a discussion with speaker and course organizer Prof. Iqbal Parker (center) over dinner.

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