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THE ROLE OF THE IMMUNE RESPONSE DURING SIVAGM INFECTION OF THE AFRICAN GREEN MONKEY NATURAL HOST

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1. ABSTRACT

The African green monkey (AGM) is one of many African species endemically infected with simian immunodeficiency virus (SIV). Like the other natural hosts, AGMs do not succumb to AIDS and understanding the basis for this resistance to disease progression would be of enormous theoretical and practical importance. Early efforts by our group that concentrated on identifying immune mechanisms presumed to keep the virus under control failed to find any obvious candidates. The presumption of virus control was invalidated by the finding that SIVagm replicates in AGMs with the same vigor as HIV-1 does in humans. Focus therefore shifted to identifying possible immunopathologic features present in disease susceptible hosts but absent in the AGM natural host. The apparent immunologic tolerance of AGMs to the SIVagm core protein led to the development of a hypothesis implicating anti-Gag antibodies in the formation of immune complexes, virus trapping in the lymph nodes and immune dysfunction. The idea proved difficult to test *in vivo* and present work focuses on the possibility that Gag tolerance at the T-cell level plays an important role in preventing the catastrophic demise of the immune system characteristic of immunodeficiency virus infection of the heterologous primate host.

2. INTRODUCTION

Despite all the ingenuity and efforts of human science, the global catastrophe that is the HIV/AIDS epidemic continues unabated. We know more about this virus and it's interaction with the human host than about any other pathogen and yet we are not significantly closer to an effective vaccine than we were when the disease was first recognized twenty years ago. More than anything, the explosive nature of this 'modern plague' and our inability to combat it has revealed how vulnerable we remain to the myriad of pathogens that exist in nature. We not only still lack a vaccine but also still do not fully understand the cause of the profound immune dysfunction known as AIDS.

HIV did not appear from nowhere and it was quickly realized that different African primate species in the wild, animal facilities and zoos (and even people's homes) carry HIV-related lentiviruses – the potential source of a zoonoses has been with us all the time (Figure 1). Infections of humans have probably been happening sporadically for millennia and why the epidemic only exploded in recent decades remains a matter of debate and speculation. The most intriguing aspect of these natural host primate species is, however, that they remain healthy despite being infected for their entire adult lives with retroviruses that readily cause disease and death in other primates, including man. Understanding the reasons for this indifference to SIV infection could help us unravel the basis for AIDS in humans and could open up new avenues for the development of AIDS therapeutics.

In this review we will summarize the work others and we have done in an attempt to understand the reason for the lack of pathogenicity in one of these natural host systems: SIVagm infection of the African green monkey. Much of this work concentrates on aspects of the immune responses to SIVagm and on the degree and pattern of virus replication *in vivo*. We will also put forward some hypothetical models for disease resistance and discuss how this fits with the known data.

3. NATURAL HOSTS OF SIV

During the last decade, it has become increasingly clear that many African primate species act as natural hosts to their own HIV-related lentivirus. In a recent surveillance of 16 diverse West African primate species for example, Peeters *et al* (1) found evidence of

Figure 1. African green monkeys enjoying breakfast in the grounds of an African hotel (with kind permission of Dr. Brigitte Beer).

infection in thirteen. Furthermore, for those species testing negative, two have been independently shown to carry their own SIVs (2-4) and for the other, only one juvenile pet monkey was available for testing. There is now a total of at least thirty primate species known to harbor SIV (1) and the list is growing continuously. It is therefore likely that a great proportion of African lower primate species are endemically infected with one variant or another of SIV.

Genomic sequencing of these viruses has revealed both the long association of many viruses with their respective natural hosts and the ease with which the viruses can spread to other primate species. Cross-species transmission between primates has been recorded in the wild (5-7) and the mosaic nature of the genomes of many SIV isolates suggest multiple infections and transmissions have occurred in the past (3, 8-10).

The study of the history, distribution and nature of these viruses is more than just an exercise of academic interest. The isolation and characterization of the closely HIV-related SIVcpz from chimpanzees (11-13) suggests that our closest living genetic cousins are the natural host of the virus that has recently entered the human population with such devastating consequences (14, 15). Indeed, the pattern of genetic diversity of HIV-1 and HIV-2 in the human population strongly indicates that several independent transmissions of SIV to humans have recently occurred, an observation that highlights the ease with which such zoonoses can occur. By identifying which species carry viruses able to potentially infect humans it may be possible to take measures to prevent the threatened emergence of HIV-3.

The one striking feature of lentivirus infection of African nonhuman primates is the apparent lack of disease in the natural host species. Animals usually become infected when first sexually active (16) and remain productively infected for the rest of their lives without succumbing to AIDS. It is only when the viruses enter a new species that disease ensues. Many, if not most, pathogens are relatively harmless in their natural host species due presumably to the millennia of co-evolution and these states of harmonious co-existence must have a biological basis. By comparing the infections of natural host species with those of disease susceptible heterologous hosts (Asian macaques, humans) it might be possible to learn more about the underlying mechanisms of AIDS and perhaps to develop novel strategies for therapy.

Probably the most closely studied natural host system is that involving SIVsm infection of sooty mangabeys. SIVsm is of particular interest because it is (beyond reasonable doubt) the source of HIV-2 in humans and of SIVmac in macaques (17). It is therefore possible to directly study disease susceptible heterologous hosts and disease resistant natural hosts after infection with highly related or even identical viruses. These studies are covered in detail in other contributions to this volume.

The second natural host system which has been studied closely is the SIVagm infection of African green monkeys (18-21). This is primarily due to the presence of AGM colonies in many Western primate facilities, usually for historic reasons linked to the testing of poliovirus vaccines. Unlike SIVsm, SIVagm does not appear to infect humans, which is fortunate given the extensive use of AGM tissues to prepare live human vaccines and the close contact of AGMs with humans in many parts of Africa. However, as will be described later, SIVagm infection of AGMs is strikingly similar to the infection of other species with SIV or HIV and therefore provides an addition model with which to study the enigma of SIV infection of the natural host.

During the course of evolution African green monkeys have split into four distinct subspecies – grivets (*Chlorocebus aethiops aethiops*), vervets (*C. a. pygerythrus*), tantalus (*C. a. tantalus*) and sabaeus (*C. a. sabaeus*) - now living in largely non-overlapping regions ranging from the West African coast to the tip of South Africa. SIVagm has been isolated from all four subspecies and genetic analysis shows that the viruses cluster with their hosts and are quite distinct from one another (22). Indeed, even viruses from geographically separated populations of the same AGM species cluster separately (23). These data strongly suggest that infection of AGMs with SIVagm initially occurred before subspeciation occurred and that the virus and host have been co-evolving ever since.

4. POSSIBLE REASONS FOR APATHOGENICITY

4.1. Lack of Inherent Pathogenicity

The first and most obvious question to ask when investigating possible reasons for apathogenicity is "does the virus have the potential to induce disease?" It is conceivable that SIVagm has adapted itself so well to the natural host that it has lost "pathogenic genes or gene sequences". It is known that clones of SIVmac with deletions in non-essential genes (e.g. *nef*) have a vastly reduced pathogenicity in adult macaques although this is almost certainly due to lower levels of replication (24). If adaptation were indeed the cause of viral apathogenicity, comparing at the genetic level viruses such as SIVagm with HIV-1 might allow identification of an "AIDS gene" which would itself be an extremely important discovery. However, a comparison of the genetic sequences of the pathogenic and non-pathogenic primate lentiviruses does not reveal a clear candidate "pathogenicity" gene. Attempts to exchange parts of the genome between the viruses yields ambiguous results, usually because the hybrid viruses are initially poorly replicating *in vivo*. The search for 'pathogenic' genes is rendered somewhat academic by the observation that a virus may cause rapid AIDS in one species but not in another. The best documented example is the obvious

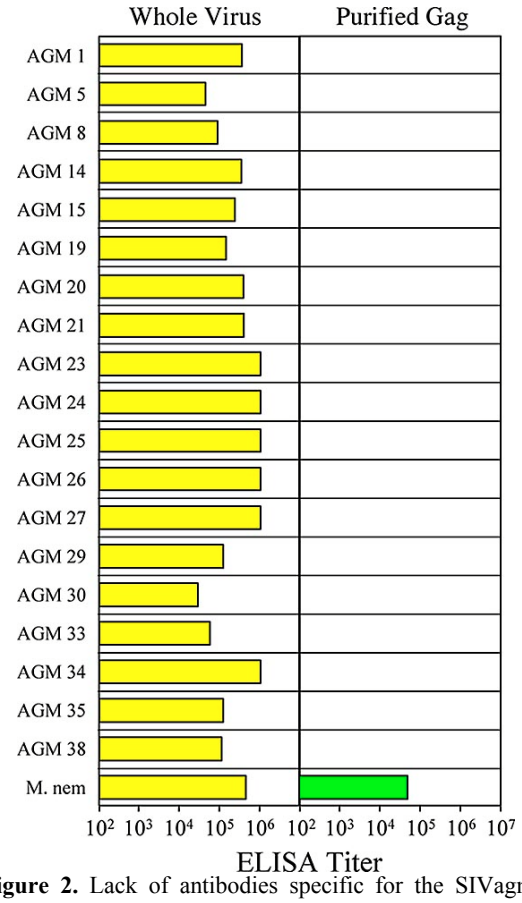

Figure 2. Lack of antibodies specific for the SIVagm Gag protein in naturally infected AGMs. Sera were titrated in ELISA plates coated with either whole SIVagm lysate (left) or with purified SIVagm Gag (right). M. nem = Serum from a pig-tailed macaque (*M. nemestrina*) artificially infected with SIVagm.

pathogenicity of SIVmac and HIV-2 in Asian macaques and humans respectively. Both, as mentioned above, are closely related at the genome level to SIVsm of sooty mangabeys (17). The argument that SIVsm may have evolved to a pathogenic virus in the heterologous host is negated by the observation that inoculation of a defined isolate or even molecular clone into Asian macaques will result in the rapid development of AIDS while infection of sooty mangabeys will be tolerated with impunity (25, 26). Similarly, infection of sooty mangabeys with pathogenic isolates of SIVmac does not result in disease (27). Furthermore, different isolates of SIVagm will induce AIDS in pig-tailed macaques but not in African green monkeys (28, 29). The susceptibility and response of the host to infection is therefore the major factor influencing progression to disease.

The high variability of HIV-1 is often cited as a reason for the failure of the immune system to control the virus. It is believed that constant changes occurring in the genome (and therefore the viral proteins) allow new variants to develop, which are not recognized by the antibodies and T-cells generated in response to the earlier viruses. By continuous generation of such escape mutants, the virus could constantly remain one step ahead of the comparatively sluggish immune response. It therefore seemed possible that viruses such as SIVagm and SIVsm were somehow constrained with regard to variability in their natural hosts. If this were the case, the viruses might never escape immune control and disease would not occur. The variability of the primate lentiviruses is due to the relative inaccuracy of the viral enzyme reverse transcriptase that has no proofreading capability. However, the fact that the SIVagm RT is intrinsically as error prone as that of HIV-1 was readily demonstrable *in vitro* (30). Furthermore, by inoculating animals with a molecular clone of SIVagm, it was possible to precisely measure the rate of mutation *in vivo* (31, 32). This, again, was found to be similar to that observed for HIV-1 in humans and incidentally indicated that the virus replicates at a similar rate in AGMs. The *in vivo* mutation rate of SIVsm was also shown to be high in sooty mangabeys (33). There is therefore no paucity of mutational capacity that could account for the lack of pathogenicity in the natural host species.

HIV preferentially infects cells of the immune system and this is almost certainly the major reason, directly or indirectly, for the eventual demise of the immune response characteristic of AIDS. If the T-cells of the natural host species had developed a mechanism preventing infection by their respective SIV's they would presumably not develop AIDS.

Initially, *in vitro* and *in vivo* analysis of the phenotype of the cells used by SIVagm revealed no obvious differences from those used by HIV i.e. $CD4^+$ T-cells (19, 21) and macrophages (34). There did not seem to be any reason to believe that the target cells for SIVagm in AGMs were any different than those targeted by HIV in humans. With the identification of the elusive co-receptors used by HIV-1 (the chemokine receptors) it became almost dogma that the progression to AIDS is associated with a switch of virus phenotype from CCR5 using (macrophage tropic) to CXCR4 using (T-cell tropic). Although this is certainly not a general rule and indeed may be only true for a subset of clade B viruses, it was striking that SIVagm, like other SIVs, did not appear to usually use CXCR4 (35, 36). Experiments have therefore been performed in which the region of the SIVagm envelope glycoprotein responsible for co-receptor tropism was exchanged with that of a T-cell tropic HIV. Although the hybrid virus was indeed shown to switch its receptor usage to CXCR4 (and incidentally, to become susceptible to neutralizing antibodies) infection of even Asian macaques did not lead to AIDS (37). Receptor (and therefore target cell) usage therefore does not appear to account for differences in pathogenic potential. Indeed, more recently it has been shown that various SIV isolates, including SIVagm, can indeed utilize the CXCR4 receptor when necessary (38).

4.2. Immune responses

When looking for a host factor able to control the virus and prevent progression to disease, the obvious first candidate is of course the immune response. Functional specific immune responses include neutralizing antibodies, complement activating antibodies, antibodies able to stimulate ADCC, and of course, cytotoxic T-lymphocytes. Might one or more of these mechanisms be particularly active in AGMs compared with the human response to HIV?

4.2.1. Humoral Immune Responses

Humans infected with HIV-1 mount a strong serological response to the major structural viral proteins.

The response to the p24 Gag protein is particularly strong. It was therefore somewhat surprising to see that whereas AGMs developed very high titer anti-SIVagm antibodies as measured by ELISA using a whole virus lysate, none of this reactivity was directed to the SIV Gag protein when whole non-denatured Gag was used as antigen (34). Naturally infected AGMs were found to be totally devoid of Gag-specific antibodies (Figure 2), as were animals infected with a molecular clone of SIVagm. In contrast, macaques infected with the same molecular clone developed high titers of such antibodies. The AGMs therefore appeared to have some form of 'tolerance' to the SIVagm Gag protein and this observation was initially filed away as being 'interesting but of no obvious relevance to disease'. We shall, however, come back to this point later in the article.

In contrast to the humoral response to the Gag protein, the pattern of antibody reactivity to the SIVagm envelope glycoprotein in infected animals was found to be surprisingly similar to that of infected humans to HIV-1. By producing a panel of overlapping synthetic peptides spanning the entire SIVagm Env protein and using them in binding assays, it was possible to identify the immunogenic regions. Fine mapping of these regions then allowed the precise epitopes to be elucidated (39). An epitope corresponding to a region in the transmembrane glycoprotein known to be immunodominant in HIV-1 (and indeed often used as the basis of diagnostic tests) was found to be recognized by sera from virtually all infected AGMs (100% of captive animals and 98% of feral animals tested). Similarly, the region corresponding to the famous "V3" loop of HIV-1 was found to be immunodominant. For the envelope glycoprotein at least it would therefore appear that the interaction with the immune system resembles that in a pathogenic system even at the level of individual epitopes.

Most of our initial work focused on measuring the classical functional immune responses. It was known that many primary isolates of HIV are difficult to neutralize and indeed, most of the early work looking at neutralizing antibodies used virus isolates adapted to grow in continuous T-cell lines (CXCR4-tropic). If AGMs could be shown to develop high levels of neutralizing antibodies, it might indeed be significant. However, when four different isolates of SIVagm were tested for their susceptibility to neutralization by sera from infected AGMs, the titers were either extremely low or non-existent (34). Even homologous sera (i.e. sera from the animals from which the viruses were isolated) were unable to neutralize the virus. These experiments were performed using isolates adapted to growth in the human T-cells, similar to the commonly used lab-adapted strains of HIV. It was therefore surprising that, given the very high titers of antibodies specific for the Env glycoprotein, very little neutralization could be demonstrated. This was almost certainly due to the fact that, unlike HIV, SIVagm seldom uses CXCR4 as a co-receptor, even after long term-culture in T-cells. This is presumably because the virus already has the ability to use, in addition to CCR5, the GPR15 and STLR33 (Bob and Bonzo) already present on T-cells. Therefore, there is no selective pressure favoring the switch to an 'X4' virus. As mentioned above, replacing the portion of the Env glycoprotein responsible for co-receptor tropism with the corresponding region of an X4 HIV isolate, as well as conferring tropism for CXCR4 also rendered the variant SIVagm susceptible to neutralization (37). In addition to being neutralized by sera from macaques infected with same X4-SIVagm, sera from animals infected with the wild-type virus were also highly effective. The antibodies themselves are therefore produced in high amounts – it is the nature of the virus that is decisive. This was demonstrated more recently by others who found that specific isolates of SIVagm are indeed susceptible to neutralization depending on the cell line used in the assay (40). In this respect, it is interesting that, in contrast to HIV-1, SIVagm infectivity is enhanced by the addition of soluble CD4 (41) and that this enhanced infectivity can easily be abrogated by SIVagm-specific antibodies (42).

Given the high dependence on virus strain and cell type for the *in vitro* measurement of neutralizing antibodies in the SIVagm/AGM system, it is difficult to draw any conclusions concerning the *in vivo* relevance of the data. We therefore decided to directly assess the anti-viral role of antibodies in African green monkeys. Uninfected AGMs were immunized with whole ßPL-inactivated SIVagm plus adjuvant and subsequently developed high levels of virus-specific antibodies. Furthermore, other AGMs received, by passive transfer, a mixture of immunoglobulins purified from the plasma of AGMs infected with the SIVagm3 molecular clone and from naturally infected AGMs (43). All animals were then challenged with 20 MID_{50} of the same SIVagm3 molecular clone. Despite animals having levels of antibodies at the time of challenge as high as those seen in infected animals, no protection from infection was achieved. If such physiological levels of antibodies are unable to prevent infection with just 20 infectious doses of the homologous molecular clone, it is difficult to see how they could be expected to keep an active, established infection under control.

Neutralization is not the only antibody-mediated mechanism through which virus (and virus infected cells) can be eliminated. Activation of complement after antibody binding to viral proteins present on the surface of the virion or of the infected cell can lead, in the case of many viruses, to lysis of the virus or cell. HIV avoids this by using host complement inhibiting proteins and it seemed possible that SIVagm might not have such a protective mechanism. However, no evidence for complement mediated lysis of SIVagm or SIVagm infected cells could be found (34). In contrast, SIVagm infected cells were readily lysed by effector PBMC in the presence of virus specific antibodies (34). Although the in vivo role of ADCC remains in doubt the levels of ADCC-activating antibodies are equally high in HIV-infected humans (44) and this mechanism could therefore not account for the differences in pathogenicity seen in the two systems.

The side-by-side comparisons of the antibody responses in the pathogenic (HIV/Human) and non-pathogenic (SIVagm/AGM) systems failed to pinpoint any mechanism capable of controlling virus replication in the natural host system. The only striking differences were negative: lack of anti-Gag antibodies and very low (or absent) neutralizing antibodies. Indeed, the failure of very high amounts of passively transferred specific immunoglobulin to prevent infection suggested that the humoral immune response in AGMs is largely ineffective.

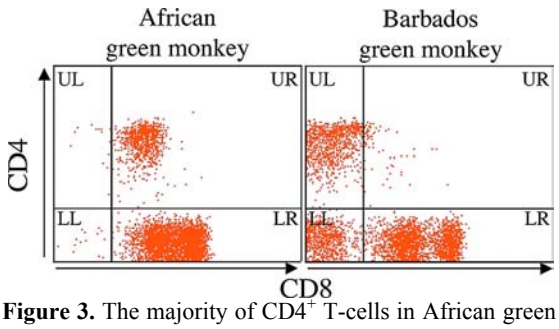

Figure 3. The majority of $CD4^+$ T-cells in African green monkeys co-express (UR) the CD8 molecule whereas AGMs from Barbados do not. Blood samples were taken from uninfected animals and FACS dot-blots gated on $CD3^+$ cells. Note also the high percentage of CD4/CD8 double-negative T-cells in Barbados monkeys (LL). Adapted from Holznagel *et al* (52). Both animals were of the C. a. *sabaeus* subspecies.

4.2.2. Cellular Immune Responses

The cytotoxic T-cell response to HIV-1 in humans is extraordinarily strong. Indeed, HIV-infection of humans is one of the few systems where specific CTL can be measured directly *ex vivo* using traditional chromium release assays. In the pathogenic SIVmac/macaque system, we were able to demonstrate that up to 1 in 20 total PBMC were CTLp specific for a single Gag epitope (measured by limiting dilution ^{51}Cr cytotoxity assay). These results were confirmed using specific peptide/MHC-I tetramers showing 25% of all circulating $CD8^+$ cells to be specific for this one epitope (45). Similar levels of activity have been demonstrated by others, both in the SIV/macaque and HIV/humans systems (46-50). It therefore seemed unlikely that AGMs would have a quantitatively stronger CTL response that could account for protection from disease. However, it seemed possible that qualitative differences (e.g. reactivity against particular gene products) might exist. Unfortunately, attempts to detect SIVagm-specific CTL using a range of different approaches to the cytotoxicity assay consistently failed. The work was hampered by the inability to transform AGM B-cells for use as stimulators and targets, necessitating the use of autologous fibroblasts or PHA blasts either infected with SIVagm vaccinia recombinants or pulsed with overlapping synthetic peptide pools. None of these approaches succeeded in unequivocally demonstrating virus specific CTL, despite the fact that identical protocols in the SIVmac/macaque system were successful. It could never be ruled out, however, that technical difficulties accounted for these failures. It is striking that in a system where direct comparison is possible, i.e. SIVsm infection of Asian macaques and the sooty mangabey natural host system, similar data have been obtained. Macaques infected with SIV develop high levels of virus-specific CTL while such cells are absent or rare in the natural host species (27, 51). Surprisingly, sooty mangabeys infected with the closely related SIVmac do develop 'normal' CTL responses (51).

Although all efforts to measure SIVagm specific CTL using traditional assays were unsuccessful (possibly because, as in the SIVsm/mangabey system, such cells are rare or absent) there is evidence of a massive expansion of CD8 cells in infected AGMs. The frequency of lymphocytes with a 'CTL-like' phenotype is significantly higher in infected AGMs compared to uninfected animals (52). Although such FACS analyses do not provide information about the functional specificity of the cells, it is a strong indication that the immune system of the AGM, although remaining healthy and functional, is drastically affected by the persistent SIVagm infection. Whether this represents an active stimulation of the specific immune response or a non-specific perturbation of the immune system in general remains to be seen. We are presently attempting to address this question using 'modern' techniques for measuring cell-mediated immune responses such as intracellular cytokine staining and ELISPOT.

One of the intriguing differences between AGMs and macaques/humans is the fact that most AGM T-cells carry both the CD4 and CD8 markers (52-55). Such double positive T-cells are rare in man, but in AGMs, they constitute the majority of T-cells (Figure 3). This is not a direct result of SIVagm infection, as both uninfected and infected animals show this unusual pattern of lymphocyte markers. This high percentage of cells carrying the CD8 molecule led to the idea that AGMs resist disease through an inherently high capacity for producing soluble, CD8-cell derived antiviral factors. AGM PBMCs were indeed shown to produce such factors *in vitro*, but not in quantities that might account for protection from disease progression (53). One antiviral factor, interleukin-16, was cloned from AGMs and was shown to inhibit both SIVagm and HIV-1 replication in cell culture (56-58).

Even more puzzling than the high percentage of double-positive T-cell in AGMs is the fact that Barbados green monkeys (BGMs) have a normal frequency of such cells (52). BGMs are descended from AGMs taken from West Africa by slave ships about 350 years ago. BGMs are SIV-free, presumably because the founder animals were not infected by SIVagm, either by chance or because only young animals were taken. Even if possession of a high frequency of double positive cells gave some form of selective advantage, 75 generations in the absence of SIVagm is hardly enough to evolve back to a 'normal' phenotype. Ongoing experimental infections of BGMs with SIVagm may help determine the role of the DP cells (if any) in protection from disease.

Presumably because transmission first occurs during sex or fighting, juvenile AGMs have never been found to harbor SIVagm. Like other primates, including man, an analysis of lymphocyte subsets in AGMs revealed that very young animals had a much higher percentage of $CD4^+$ cells and a lower percentage of $CD8^+$ cells (55). This observation suggested that the higher availability of target cells and a lower number of putatively antiviral $CD8^+$ cells might render newborn AGMs more susceptible to disease. This is known to be true for neonatal macaques, for example, which succumb to AIDS when infected with even highly attenuated variants of SIVmac (59). We therefore infected matched pairs of adult and newborn AGMs with a variety of SIVagm isolates via different routes and measured virus loads and clinical parameters (55). Surprisingly, the newborn AGMs were, if anything, more resistant to SIVagm infection. Virus loads were lower and no

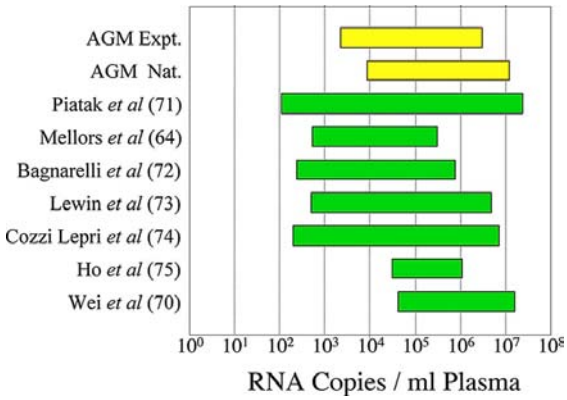

Figure 4. Plasma virus loads in SIVagm infected African green monkeys are similar to those in HIV-1 infected humans. Chronic virus loads in experimentally (Expt.) and naturally (Nat.) infected AGMs were determined by real-time RT-PCR (69) and the range of RNA copies / ml plasma plotted. Published ranges for HIV-1 infected humans determined using similar methodologies are given as comparison.

clinical symptoms were observed. In contrast, a newborn cynomolgus macaque infected with SIVagm developed high virus loads (compared to an adult cynomolgus macaque) and symptoms associated with AIDS (though no classic immunodeficiency). Any putative protective mechanism therefore appeared to be fully operational in the newborn AGM.

4.3. Viral Loads

The work described above was aimed at one major goal: identification of a unique mechanism in AGMs able to control SIVagm replication and therefore prevent onset of disease. The association between virus loads and disease progression is well documented in HIV-infected humans and, in the days before measurement of viral RNA in the plasma became routine, the best comparative marker for viral load was the number of infected cells in circulation. Analysis of cell-associated viral loads in AGMs (by limiting dilution co-culture) and quantitative PCR gave figures equivalent to those seen in asymptomatic HIV-1 infected humans, but not as high as those reported for patients progressing to AIDS (60). It was therefore assumed that AGMs indeed possessed a mechanism for maintaining virus loads at 'asymptomatic' levels for the lifetime of the animal. It was also interesting that the cell-associated virus load in the AGM lymph nodes were similar to those in the PBMC (61), unlike the situation in HIV-infected humans where loads in the lymphoid tissues are usually much higher (62, 63). Correspondingly, there was no obvious damage to the lymph node architecture in AGMs, even in those infected for many years, which is in contrast to the progressive loss of structure and function occurring in humans.

Knowing that progression to disease in HIV infected humans and SIVmac infected macaques is tightly associated with the plasma virus load (64, 65) and that immunization with a recall antigen such as tetanus toxoid can lead to an increase in virus load (66-68) - presumably by providing additional activated T-cell target cells - we decided to analyze the effect of immune stimulation in infected AGMs. Animals were given a standard tetanus immunization and later received booster immunizations with the same antigen. Surprisingly, no significant change in the plasma virus load was detected following this booster immunization. Furthermore, it was not possible to demonstrate tetanus-specific memory T-cells in the immunized animals. One possible explanation could be that as the AGMs were already SIVagm-positive at the time of initial immunization, reacting T-cells were infected and eliminated before expansion into memory cells. There would therefore be no pool of tetanus specific cells to react to the booster immunization. Although this precluded the possibility of assessing the effects of increased virus load in the AGMs, it raises the question of how well the immune system in infected animals reacts to pathogens in general. It is possible that although SIV infection of the natural host does not lead to the complete collapse of the immune system seen in heterologous species, infection may indeed compromise the animal's ability to combat infection. The precise study of survival rates and disease incidence in populations of infected and uninfected AGMs that might reveal such an effect has unfortunately not yet been carried out.

The search for a 'virus-suppressing' mechanism was finally rendered irrelevant by the development of techniques to measure virus loads in AGM plasma. Due to initial difficulties in identifying suitable regions for primers and probes, and because we wished to directly compare loads of different immunodeficiency viruses in different species using one common assay, plasma loads were initially measured using a polymerase-enhanced reverse transcriptase (PERT) assay. The results of these studies were surprising: AGMs appeared to carry as many virus particles in plasma as did SIVmac infected macaques and HIV-infected humans. The PERT assay, however, was not generally accepted as an established technique for assessing viral loads. The RT-PCR method had become the gold standard for measuring plasma virus because, being based on virus-specific PCR, it can unequivocally determine the number of genomic copies in circulation. In order to confirm the rather worrying result of the PERT assays, effort was therefore put into developing an RT-PCR assay for SIVagm.

The results of the RT-PCR analyses confirmed those of the PERT assay: AGMs experience virus loads comparable to those seen in pathogenic heterologous host systems (69). During acute infection, SIVagm plasma loads in AGMs peaked at $2.9x10^5$ to $4.2x10^7$ RNA copies/ml and were maintained at set-points of $2.1x10^3$ to $2.3x10^6$ copies/ml for at least a year. Naturally infected AGMs were also shown to have virus loads in the range of $8.3x10^3$ to $1.1x10^7$copies/ml plasma. These values fall within the range described for HIV-infected humans (64, 70-75, Figure 4) and cover those considered diagnostic for eventual disease progression in SIV infected macaques (65). Others have described similar levels of SIVagm in the plasma of infected AGMs. Broussard *et al* (76), for example, found virus loads greater that 6×10^6 per ml of plasma in two subspecies of AGMs and, like HIV-1 in humans, distinct populations of virus in neurological tissue. Goldstein *et al* (77) measured levels of plasma viremia similar to those found in HIV-infected humans progressing to disease and found virus extensively in the gut-associated lymphoid tissue and alveolar macrophages – again with no sign of pathogenicity. Finally, Diop *et al* (78) found peak levels

of plasma viremia as high as $2x10^8$ RNA copies/ml dropping rapidly to set-points similar to those seen in pathogenic systems but found no evidence for lymph node pathology. There is therefore no doubt that SIVagm is able to replicate with the same impunity in it's natural host as do HIV-1 and SIVmac in their heterologous hosts.

SIVagm infection of AGMs is not the only natural host system in which virus loads are equivalent to those seen in pathogenic systems. Using techniques similar to those described above, it quickly became clear that sooty mangabeys too support the replication of SIVsm at high levels (33) with plasma loads ranging from 10^5 to 10^7 RNA copies/ml. In a more recent study, virus loads in a third natural host system – SIVmnd infection of mandrills – were measured during both the acute and chronic phases of infection. The time course and magnitude of the virus loads were again found to parallel those measured in pathogenic systems but in the absence of concurrent pathogenic effects (79).

4.4. Immunopathology

The situation in the natural hosts of SIV is therefore that animals support the replication at "pathogenic" levels of viruses able to cause immunodeficiency in heterologous hosts. The virus is not controlled by a particularly vigorous adaptive or innate immune response, the target cells for replication are the same and the rates of variability are similar. Virtually every major aspect of the infection in natural hosts reflects the situation during infection of heterologous host species with one obvious difference: the natural hosts do not develop AIDS. Logically, there are two possible scenarios to explain this puzzle. The first is that the natural host species have independently evolved mechanisms to prevent or repair the damage to the immune system resulting from prolonged support of active virus replication. One could imagine, for example, the evolution of highly efficient mechanisms to replace those immune cells affected by the virus. Alternatively, the production of factors able to block or overcome the effects of a putative harmful viral protein might prevent the demise of the immune system. So far, evidence for such mechanisms or factors has not been found (although their existence cannot be ruled out) but it seems unlikely that such systems would evolve independently in the many natural host systems. The second possible scenario that could explain the lack of pathogenesis in the natural hosts is, ironically, the *failure* to mount a particular response to the virus. If, for example, a major contributor to the immune dysfunction that is AIDS were an inappropriate immune response, then a failure to mount the response would allow continued health despite active virus replication. In evolutionary terms it is far easier for the natural host species to "switch off" a particular response than it is to develop a whole new protective mechanism. Is there any evidence that the natural host species such as AGMs and sooty mangabeys fail to react immunologically in any particular way compared to the heterologous hosts? The answer is unequivocally "yes".

4.4.1. Hyperactivation

The first, and most closely studied, aspect of the natural host response differing from that of the heterologous host is the activation status of the immune system. Although the precise reasons for the breakdown in the immune system seen in HIV-infected humans and SIV infected Asian macaques is not yet fully understood, it now seems clear that AIDS is anything but an "immunosuppressive" disease in the classical sense. The early assumption that the breakdown of the immune system is simply a direct result of infection and elimination of $CD4^+$ T-cells was soon discarded because there are not enough infected cells to account for the dysfunction. Similarly, the idea that soluble HIV proteins directly suppress the immune response can be excluded because initiation of the extremely vigorous primary anti-HIV immune response occurs at precisely the time of maximum virus load. Indeed, it is now clear that AIDS, if anything, is a disease associated with a general hyperactivation, rather than suppression, of the immune system. Lymphocyte markers associated with activation and proliferation are strongly upregulated in HIV-infected patients and *in vivo* labeling experiments demonstrate a massively increased rate of T-cell turnover (80, 81). It therefore appears that HIV infection causes a profound and sustained hyperactivation of the immune system. This, in association with the continuous and rapid rounds of infection and elimination and the possible induction of anergy and apoptosis in bystander uninfected cells may eventually "exhaust" the reservoir of T cells and lead to the collapse of the immune system.

Activation of T-cells certainly makes sense as far as the virus is concerned for it is precisely these cells that are needed by HIV and SIV to replicate. However, the hyperactivation of the immune system that may eventually lead or at least contribute to AIDS must be viewed as an unfortunate side effect of infection rather than an essential mechanism of virus replication. Whereas T-cell activation and turnover rates are massively increased in SIV-infected Asian macaques, SIVsm infected sooty mangabeys yield values that are not significantly different from those of uninfected animals (82) and yet the virus still replicates to high titers. In addition to (and possibly because of) the normal states of T-cell activation in SIVsm infected sooty mangabeys, the T-cells of this natural host species appear to be resistant to the development of T-cell anergy (83) and bystander cell apoptosis (84).

Such extensive studies of T-cell turnover and bystander immunopathology have yet to be done in the AGM system. A simple analysis of lymphocyte markers fails to reveal any evidence of hyperactivation despite high levels of virus replication. However, the AGM immune system does not remain entirely unaffected by the presence of SIVagm. In addition to expanded populations of $CD8^+CD28^-$ cells - which may or may not be SIVagm-specific CTL - infected AGMs suffer from a significant thrombocytopenia (52).

4.4.2. Immunological Tolerance

It is likely that the African natural host primate species were initially susceptible to disease when they first encountered their respective retroviruses (in the same way that humans and Asian macaques are today) and that natural selection of the host has allowed them to evolve to a state of "indifference". Assuming that these retroviruses entered the primate populations relatively recently (i.e. after development of the different species) then each natural host primate species has independently found a way of coping with SIV infection. There is therefore no guarantee that the mechanism of protection

Table 1. Weak or absent antibody reactivity to SIV Gag protein in a number of SIV/primate natural host systems

Virus	Host	Antibody response to homologous Gag	Reference
SIVagm	African green monkey	Negative (SIVagm Gag ELISA)	34
SIVsm	Sooty mangabey	Weak (immunoblot)	33
SIVmnd	Mandrill	Negative or weak (Immunoblot)	3, 85
SIVsyk	Syke's monkey	Negative (RIPA)	86
SIVrcm	Red-capped mangabey	Negative or weak (RIPA)	87
SIVlhoest	L'Hoest monkey	Negative (SIVmac RIPA)	9
SIVsun	Sun-tailed monkey	Negative (RIPA)	88

from disease is the same in each case – there may be as many ways to overcome the pathogenic effects of SIV infection as there are natural host species. If there is a common mechanism it is likely to be a very simple one, for example, the induction of immunological tolerance.

As mentioned earlier, during early attempts to quantify the strength of the immune response against individual SIVagm proteins in infected AGMs, a surprising lack of antibodies to the viral p27 Gag protein was noticed (Figure 2). Sera from all naturally infected AGMs, although having high titers of antibodies to whole viral lysates in ELISA failed to react at all with the purified Gag protein (34). This was surprising because sera from HIV infected humans and SIV infected macaques have very strong responses to Gag. As the response to retroviral Gag is usually broadly specific it seemed unlikely that the failure of antibody reactivity could be due to a mismatch between the viruses infecting the animals and the isolate used for preparing the antigen. The high levels of antibodies to the viral envelope glycoprotein (which is relatively strain-specific) made this even more unlikely. To formally rule out this possibility, AGMs were infected with a molecular clone of SIVagm and the immune response to antigens derived from the same virus was tested. As before, no significant serological response to the viral Gag protein was observed, despite high levels of antiviral antibodies in general. More importantly, a pig-tailed macaque (*M. nemestrina*) infected with the same molecular clone developed a strong anti-Gag response. The lack of antibodies to the viral core protein was therefore a feature of the host rather than of the virus.

At that time it was assumed that the failure to respond to this particular viral protein was simply a result of the host's ability to control the virus. It seemed possible that there simply wasn't enough of the antigen being produced to maintain a significant immune response. As it became gradually clear, however, that the virus was not being controlled in the AGMs and that there was as much virus in circulation as in the pathogenic systems, the question of whether the lack of Gag antibodies could be a cause of apathogenicity rather than just an effect thereof was raised. This concept was not really taken seriously (after all, how could antibodies to the viral core protein cause AIDS?) until a review of the literature revealed that in many of the other natural host systems in which the pattern of antibody reactivity has been studied a similar lack or low level of antibody response to Gag has been observed (Table 1). For example, sera from SIVsm infected sooty mangabeys were found to react strongly with the viral envelope glycoprotein but reactivity with the Gag protein was weak or absent (33). Similar patterns of reactivity have also been described for mandrills and red-capped mangabeys naturally infected with SIVmnd and SIVrcm respectively (85-87) and for Syke's monkeys, L'hoest monkeys and sun-tailed monkeys naturally infected with their own SIVs (9, 86, 88).

A failure to detect antibodies in serum could, of course, be simply due to all antibodies being pre-adsorbed by an excess of antigen. However, attempts to detect antibody/Gag immune complexes in circulation in AGMs did not support this scenario. Given the variation between animals and the similarities in virus loads in pathogenic systems and apathogenic natural host systems it seems unlikely that excessive Gag production could account for this general failure to detect antibody. The other possibility is therefore that the antibodies are not produced in the first place, despite prolonged exposure to high levels of antigen – i.e. that the AGMs have a form of selective immunological tolerance to the SIVagm Gag protein. But how could Gag tolerance develop in AGMs (and other natural host species) and how could this be of benefit to the SIV infected animal?

Immunological tolerance usually occurs during ontogeny when exposure to "self" peptides results in elimination of specific cells in a process termed negative selection. The simplest explanation for the apparent tolerance to Gag in AGMs is therefore contact with the protein during embryological development. As there is no evidence that this 'tolerance' is restricted to the offspring of infected AGMs the source of such antigen would have to be endogenous and the obvious candidates are endogenous retroviral genes. Turning on the expression of an SIVagm-related endogenous *gag* gene protein during ontogeny would be a very simple step – perhaps only a single mutation – but would have a profound effect on the subsequent ability of the immune system to raise a response to this or a closely related protein. If such an acquired tolerance did provide a benefit during an early period of epidemic it would gradually spread through the whole population.

4.4.3. A Hypothetical Model

How could tolerance to the viral core protein benefit the infected host? In those host species that succumb to AIDS after prolonged exposure to SIV or HIV a great deal of virus is trapped in the lymph nodes and the gradual degradation of lymph node structure is observed (89). Could the two be linked and could antibodies to the viral Gag protein play a role? The following, highly speculative hypothesis was formulated which appears, at

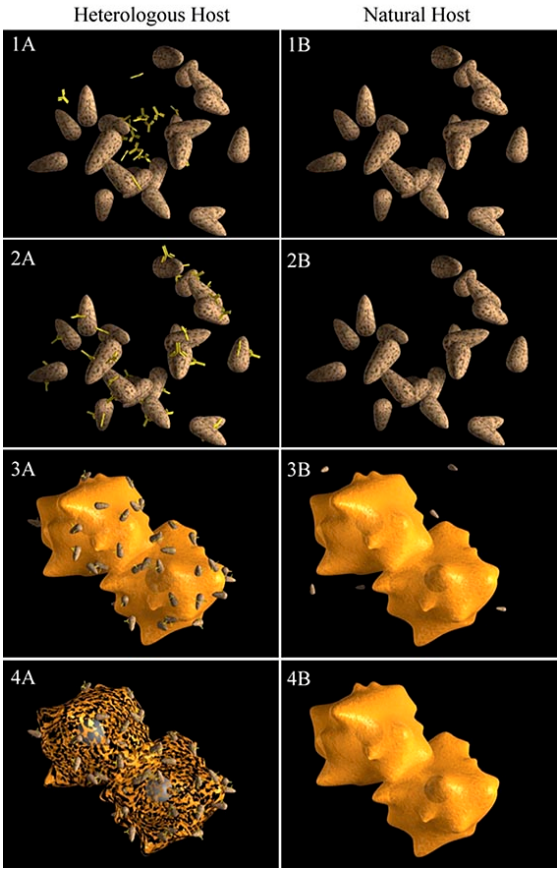

Figure 5. Hypothetical model to explain how tolerance to SIVagm Gag might form the basis for protection from disease progression. In heterologous hosts (e.g. HIV-infected humans) intact core particles released by the death or lysis of infected cells come into contact with Gag-specific antibodies (1A) that form immune complexes (2A). These become trapped at the surface of dendritic cells upon passing through lymph nodes (3A) resulting in death or dysfunction of these cells (4A). In the natural host species lacking anti-Gag antibodies (e.g. SIVagm-infected AGMs) equivalent numbers of core particles are released (1B) but no immune complexes are formed (2B), preventing both trapping in the lymph nodes (3B) and hence lymph node dysfunction (4B).

least, to fit the data (Figure 5): (i) During the prolonged but acute infection with HIV or SIV the majority of particles released into the plasma are non-enveloped viral cores. This could occur as the result of lysis of infected cells by CTL before virion maturation, for example, and could account for the high number of particles detected using assays based on the Gag protein, the viral RNA or the viral reverse transcriptase (all of which form the core particle) compared to the very low levels of infectious virions; (ii) These core particles are immediately opsonized by the abundant Gag-specific antibodies in circulation; (iii) Upon passage through the lymph nodes these immune complexes are trapped by the dendritic cells and are readily detected by immunostaining or *in situ* RT-PCR; (iv) either by inappropriate activation of, for example, the complement cascade or simply by saturation of the cell surface, the dendritic cells are eliminated or rendered inoperative; (v) Years of incessant bombardment of the lymphoid tissue by massive quantities of immune complexes results in the eventual loss of structure, function and ability to mount an effective immune response. In the SIVagm natural host system, where viral particles are produced in equally high quantities, the simple lack of anti-Gag antibodies would prevent the initial formation of immune complexes and no trapping in the lymph nodes would occur.

It should be pointed out that if antibodies binding the viral cores play a role in pathogenesis, it would only be necessary to develop tolerance to those regions of the Gag protein exposed on the surface – antibodies to internal epitopes would not be able to bind viral cores. Evaluation of physiologically relevant antibodies should therefore be carried out using protein in its intact, native form rather than in the denatured form usually used in immunoblots, for example. In this context, a number of African primate natural host species were recently identified by the positive reaction of their sera with commercial immunoblots based on recombinant HIV-1 and HIV-2 proteins (90-92). Some of these sera were indeed found to react strongly with the HIV-1 p24 Gag protein. As the viruses infecting these primates are no more related to HIV-1 than SIVagm, for example, and as the animals have had no exposure to HIV-1, these reactions must be the result of serologic cross-reactivity between HIV-1 p24 and antibodies raised in response to the Gag protein of the particular SIV infecting the monkeys. Although it is possible that the immune systems of these particular natural host species happen to react differently than those described previously, this seems unlikely. It could be that the commercial immunoblots used are exquisitely sensitive and able to detect very low levels of anti-Gag antibodies present in the sera. Another possible explanation, however, is that all infected animals indeed produce antibodies to a variety of Gag epitopes (including some which are conserved in HIV-1 Gag) but that these epitopes are not exposed at the surface of the correctly folded native core particle. This would explain why sera from natural host species sometimes react to Gag proteins in immunoblots in which the secondary structure of the protein is denatured but are negative in assays using native protein such as ELISA and radioimmunoprecipitation. If immunologic tolerance to Gag were indeed of benefit to the host, then tolerance would only have to be induced against those epitopes exposed on the core particle itself. It would be interesting to know the pattern of serologic reactivity of these newly described natural host species to non-denatured proteins from the homologous SIV isolates.

As initially mentioned, the hypothesis suggesting a role for anti-Gag antibodies in pathogenesis is highly speculative, particularly as it has not been possible to unequivocally demonstrate that the majority of particles in circulation are indeed naked cores. It is, however, testable. If tolerance to Gag plays an important role in protecting AGMs from disease development, then breaking this tolerance prior to infection should abrogate the protection. Groups of AGMs were therefore inoculated with either purified SIVagm p27 Gag protein (prepared from SIVagm itself by affinity chromatography) together with a strong

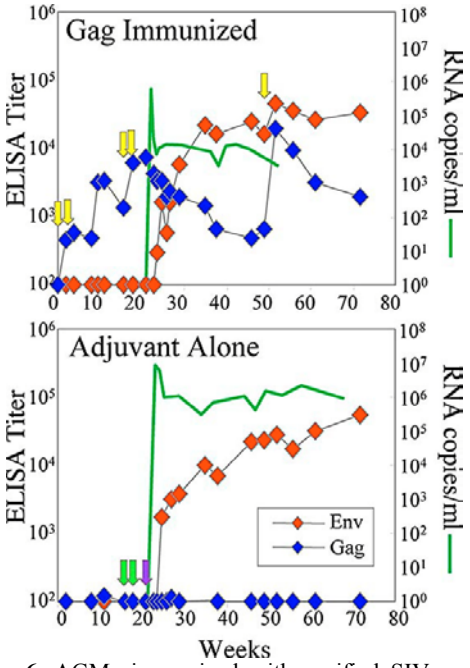

Figure 6. AGMs immunized with purified SIVagm Gag produce specific antibodies but fail to maintain high titers following challenge. Shown are two representative animals from an experiment involving 12 AGMs. Antibody titers against SIVagm Gag (blue) and SIVagm Env (red) were determined in the sera of animals immunized with purified Gag protein (Upper panel, yellow arrows) or adjuvant alone (Lower panel, green arrows) After challenge with SIVagm (purple arrows) the anti-Gag titers unexpectedly declined in the immunized animals and could only be boostered with a further immunization. The animals receiving adjuvant alone failed to develop anti-Gag antibodies following infection but both developed high-titer anti-Env antibodies. Virus loads in plasma are also shown (green).

adjuvant or with the adjuvant alone (as control). The assumption was that repeated administration of high levels of purified protein together with the adjuvant would break the apparent tolerance and that following infection, the continuous exposure to p27 produced by the replicating virus would boost and maintain the response. The aim was to induce a state of high levels of p27 protein and high titer specific antibody over a long period of time, mimicking the situation in heterologous host species. It was therefore surprising that the inoculated AGMs rapidly developed high titers of p27-specific antibody after only 2 or 3 inoculations (Figure 6). Indeed, the kinetics and magnitude of antibody development were similar to those seen following inoculation of rhesus macaques with SIVmac Gag. If the AGMs were truly tolerant to Gag then breaking the state should have been far more difficult. The second surprise was that despite the AGMs experiencing high and sustained virus loads following infection, accompanied by the development of high titers of antibodies specific for the envelope glycoprotein, no boost in the antibody response to Gag was seen. Indeed, the levels of anti-Gag antibodies continued to drop despite the active SIVagm infection and it was only by boosting again with purified Gag protein that high titers of antibodies could be temporarily restored. Although there was some evidence of virus trapping in the lymph nodes at the time of peak anti-Gag antibody response, this was not sustained and no sign of lymph node dysfunction (or disease development) was observed. Basically, the experiment failed to achieve the primary goal – i.e. the prolonged high-level maintenance of both protein and antibodies needed for immune complex formation – but it is curious that the immune system appeared to treat the two forms of Gag (inoculated vs. produced *in vivo*) as if they were completely unrelated proteins. This suggests that the processing and presentation of the protein plays a decisive role in whether the immune system recognizes it or not.

Attention has therefore now shifted to investigating the possibility that the apparent failure to react to Gag is the result of a T-cell, rather than B-cell, tolerance. In initial experiments, SIVmac Gag purified from virus using an affinity column was able to stimulate T-cells from SIVmac infected macaques whereas SIVagm Gag did not stimulate cells from SIVagm infected AGMs.

As discussed earlier, the evidence that AIDS is the result of prolonged, unrelenting hyperactivation of the immune system is becoming increasingly strong. The role played by the antibody response to Gag may therefore be small or non-existent. Indeed, the failure to develop such antibodies may be, as originally assumed, an unimportant but intriguing by-product of the natural host system. There is, however, the distinct possibility that the two phenomena – lack of hyperactivation and lack of anti-Gag antibodies - are linked. We know from SIVmac vaccine studies that the T-cell response to SIVmac Gag can be extraordinarily strong, with up to 1:20 total PBMC or 25% of all $CD8^+$ T-cells recognizing a single Gag epitope (45) and similar data have been generated for HIV-1 infected humans. A large part of the T-cell immune response in heterologous hosts infected with immunodeficiency viruses can therefore be focused on epitopes within the viral Gag protein. Could this be the basis of the hyperactivation seen in pathogenic systems and could tolerance to Gag at the level of $CD4^+$ helper T-cells be the underlying mechanism of protection from disease progression? Certainly more work needs to be done in this direction but we already know that (i) SIVagm infected AGMs do not appear to mount a T-helper response to SIVagm Gag; (ii) virus specific CTLs have been difficult to demonstrate in both SIVsm infected mangabeys and SIVagm infected AGMs; (iii) many natural host species fail to mount an anti-Gag antibody response. There is no evidence that Gag itself is able to hyperactivate the immune system but it may, in it's abundance, provide the necessary specific trigger to T-helper cells "primed" by other viral proteins or by the cytokine dysfunction caused by infection.

If AGMs have indeed developed a state of resistance to disease development by the induction of immunological tolerance to some critical epitopes, then it is possible that long-term, high level infection with an unrelated SIV expressing a different Gag protein might result in disease. So far, infection of AGMs with, for

example, SIVmac has not been documented but there is one meeting report (http://www.iac2000.org/abdetail.asp?ID=WePpA1294) of AGMs developing signs of disease following infection with a pathogenic SIV/HIV hybrid virus (SHIV). Whether or not these AGMs developed anti-SIV Gag antibodies, showed virus trapping in the lymph nodes and had elevated levels of T-cell activation is unfortunately not known. To address the question more precisely, we are attempting to produce hybrids of SIVagm expressing the HIV Gag protein to allow direct side-by-side comparisons to be made.

In addition to the possibility of natural primate hosts of SIV being susceptible to disease when infected with heterologous viruses, it should be noted that there are indications that even the homologous SIV strains can cause immune dysfunction if the period of infection is long enough. Two mandrills infected for over 15 years were found to exhibit symptoms of progression to AIDS, including rising virus loads and loss of $CD4^+$ T-cells (93). In both sooty mangabeys and AGMs, the natural loss of peripheral $CD4^+$ T-cells with advancing age is accelerated in animals infected with SIVsm (94) and SIVagm (E. Holznagel, personal communication) respectively. Finally, infection of chimpanzees with HIV-1 (closely related to SIVcpz) can result in the development of AIDS (95) although both the geographical distribution and mosaic nature of SIVcpz (96) indicate that SIVcpz is a relatively recent infection of chimpanzees, making the 'natural host' definition of our closest living primate relatives questionable. It therefore seems possible that the various SIVs present in the African natural host species are not strictly apathogenic but that the period between infection and onset of symptoms is longer than the natural life of the primates in the wild – which of course in terms of natural selection amounts to the same thing.

5. SUMMARY AND PERSPECTIVE

In this review we have attempted to summarize the data relating to the role of the immune response in one of the most closely studied natural hosts of SIV, paying particular attention to the ups and downs of our own work. We have shown how the initial assumption that African green monkeys posses a mechanism for suppressing virus replication to apathogenic levels was negated by the observation that SIVagm replicates to levels equivalent to those seen in pathogenic systems such as HIV-1 infection of humans and SIVmac infection of Asian macaques. As a result, attention was given to the possibility that AGMs *lack* a harmful immune response rather than possess an especially helpful one. Observing an apparent lack of antibodies to the viral core Gag protein in AGMs and other natural host species, a speculative hypothesis was proposed which linked anti-Gag antibodies to the formation of immune complexes, virus trapping in the lymph nodes, destruction of lymph node structure and function and the eventual demise of the immune system. However, actual experiments in the animal model to support or refute the hypothesis yielded equivocal results and it is still not clear whether the apparent tolerance to Gag is a major cause or just an unimportant by-product of the infection of the natural host. Finally, we suggested that tolerance to Gag at the level of the T-cell response might be involved in the ability of the natural host species to support high-level replication of SIV without developing the state of hyperactivation and bystander cell dysfunction characteristic of heterologous systems progressing to disease.

Although such hypotheses are speculative and may be wide of the mark, it is important that the natural host systems continue to be studied in detail. There are now dozens of species known to be infected in the wild and each has developed ways to support infection with impunity. By identifying the precise mechanism(s) by which this protection from disease progression is achieved it may be possible not only to finally understand the enigma that is AIDS but also to use this knowledge to protect those infected now and in the future from developing this most awful of diseases.

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