Enhanced T- and B-cell responses to simian immunodeficiency virus (SIV)agm, SIVmac and human immunodeficiency virus type 1 Gag DNA immunization and identification of novel T-cell epitopes in mice via codon optimization

Christine S. Siegismund, Oliver Hohn, Reinhard Kurth and Stephen Norley

As a prelude to primate studies, the immunogenicity of wild-type and codon-optimized versions of simian immunodeficiency virus (SIV)agm Gag DNA, with and without co-administered granulocyte–macrophage colony-stimulating factor (GM-CSF) DNA, was directly compared in two strains of mice. Gag-specific T cells in the splenocytes of BALB/c and C57BL/6 mice immunized by gene gun were quantified by ELISpot using panels of overlapping synthetic peptides (15mers) spanning the entire capsid proteins of SIVagm, SIVmac and human immunodeficiency virus type 1. Specific antibodies were measured by ELISA. Codon optimization was shown to significantly increase the immune response to the DNA immunogens, reducing the amount of DNA necessary to induce cellular and antibody responses by one and two orders of magnitude, respectively. Co-administration of murine GM-CSF DNA was necessary for the induction of high level T- and B-cell responses. Finally, it was possible to identify both known and novel T-cell epitopes in the Gag proteins of the three viruses.

Although only identified less than three decades ago (Barre-Sinoussi et al., 1983; Clavel et al., 1986), 33 million people are currently infected with human immunodeficiency virus (HIV) types 1 or 2 and more than 25 million have already died of AIDS (http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/2008_Global_Report.asp). So far, attempts to develop the much-needed AIDS vaccine have failed and anti-retroviral drugs, although highly successful in delaying the onset of disease, are not available to the majority of those infected with HIV. A better understanding of the mechanisms of AIDS induction could lead to the development of ‘single-shot’ immunotherapeutics that would negate the need for daily access to expensive drugs.

HIV-1 and HIV-2 are thought to have crossed the species barrier from chimpanzees (Pan troglodytes) (Gao et al., 1999; Huet et al., 1990) and sooty mangabeys (Cercocebus atys) (Hirsch et al., 1989) sometime in the 20th century, and HIV-1/simian immunodeficiency virus (SIV)cpz appears to be a recent recombinant virus from two other primate species (Keele et al., 2006; Sharp et al., 2005; Takehisa et al., 2007; Van Heuverswyn et al., 2006). Indeed, there are over 30 African primate species now known to be endemically infected in the wild with their own strains of SIV (Kraus et al., 1989; Peeters et al., 2002), each of which also has the potential to cross the species barrier to humans.

Despite having virus loads similar to those seen in HIV-infected humans and SIVmac-infected macaques, these natural hosts maintain infection throughout their entire adult lives without developing AIDS-like symptoms (Hartung et al., 1992; Norley et al., 1999; Norley & Kurth, 2004). One such natural host system is SIVagm infection of African green monkeys (AGMs; Chlorocebus aethiops). Like SIVcpz in humans (i.e. HIV-1) and SIVsm in humans and macaques (i.e. HIV-2 and SIVmac), SIVagm can cause immunodeficiency in a heterologous host species (Johnson et al., 1990; Letvin et al., 1985; Murphey-Corb et al., 1986). As virus loads are similar in the apathogenic natural host and pathogenic heterologous host systems, protection against AIDS appears to be due to the host response rather than a property of the virus itself, and control by the immune response is not the mechanism of protection. Indeed, the lack of hyperimmune activation in the natural hosts (Chakrabarti et al., 2000; Cumont et al., 2008; Hazenberg et al., 2003; Silvestri et al., 2003) that is observed in HIV-infected humans and SIVmac-infected macaques suggests that it may be a lack of an inappropriate immune response that protects the natural hosts from disease.

In this context, it is interesting that SIVagm-infected AGMs do not mount an antibody response to the viral capsid protein (Allen et al., 2001; Norley et al., 1990, 1999; Norley...
sequences (Deml et al., 1999, 2000, 2001; Emau et al., 1991; Hu et al., 2003; Rey-Cuillé et al., 1998; Tsujimoto et al., 1988). In contrast, heterologous hosts infected with SIVagm mount a very strong anti-Gag response (Norley et al., 1990).

Previous experiments indicated that AGMs recognized exogenously administered and endogenously produced Gag protein in different ways (Norley & Kurth, 2004). To address this, it was decided to immunize AGMs with SIVagm Gag DNA before challenge with SIVagm and, as a direct comparison in a pathogenic, heterologous system, to immunize rhesus macaques with SIVmac Gag DNA before challenge with SIVmac.

The degree of protein expression following the successful delivery of plasmid DNA is known, for a wide range of proteins, to be enhanced by codon-optimization for expression in mammalian cells. This is particularly true for genes such as HIV and SIV gag that contain inhibitory sequences (Deml et al., 2001; Kofman et al., 2003). Furthermore, co-delivery of DNA coding for granulocyte–macrophage colony-stimulating factor (GM-CSF) has been shown in other studies to enhance the resulting immune response (Fensterle et al., 1999).

The aim of this study was therefore to evaluate the immunogenicity of the different DNA vaccine constructs in mice, in preparation for the primate study, by comparing the T- and B-cell responses to wild-type (WT) and codon-optimized (CO) versions of the genes and to evaluate the need for co-administration of an ‘adjuvant’ DNA, i.e. GM-CSF DNA.

The CO sequences of SIVagm Gag (1566 bp, GenBank acc. no. M30931), SIVmac239 gag (1533 bp, GenBank acc. no. M33262) and HIV-1 gag (1491 bp, CRF02_AG, provided by Dr C. Kücherer, RKI, Berlin) were determined based on the most frequently used degenerative amino acid triplets in mammalian cells (Kotsopoulou et al., 2000). Each of the CO genes was generated by polymerase chain assembly (Smith et al., 2003) or fusion-PCR of 42 bp oligonucleotides (Invitrogen). Approximately 500 bp sections of these genes were cloned into the pCR4-TOPO-vector (TOPO cloning kit for sequencing, Invitrogen) and electroporated into chemically or electro-competent Escherichia coli TOP10 to allow sequencing. Variations to the intended CO sequence occurring in the separate fragments were corrected by repeated cycles of site-directed mutagenesis. Corrected fragments were then merged by fusion-PCR and recloned into the pCR4-TOPO-vector. Finally, the CO inserts and the WT genes were excised and cloned into the immunization vector pTH (Hanke et al., 1998).

The plasmids with all CO and WT genes were transfected into Chinese hamster ovary cells by PolyFect-Transfection (Qiagen). Expression was monitored by Western blot using anti-V5 horseradish peroxidase antibody (Invitrogen) or the Gag-specific monoclonal antibody AG3.0 (Jonathan S. Allan, San Antonio, USA). A gene product at 57 kDa was visible using both antibodies and it was clear that for each of the three gag genes (SIVagm, SIVmac and HIV-1) the CO version was expressed with a far higher efficiency than the WT form (data not shown).

Mice were bioballistically immunized (Bio-Rad Gene Gun) with plasmid DNA loaded onto 0.1–2 μm gold particles according to the manufacturer’s recommendations. Two non-overlapping shots per mouse were performed into freshly shaved abdominal skin at a discharge pressure of 300 p.s.i. In an initial study, mice receiving one prime and three boosts of SIV Gag DNA in the absence of any co-stimulatory cytokine DNA failed to develop detectable cellular immune responses, although a low-level antibody response was seen (data not shown). However, in a dose escalation study of CO and WT DNA using only a single immunization (two shots) comprising a total of 0.014, 0.14 or 1.4 μg Gag DNA plus a constant 1.4 μg murine pCI-2-IRES-GM-CSF DNA (kindly provided by Dr J. Reimmann, University of Ulm, Germany), it was possible to detect T-cell responses only 9 days after a single immunization with 1.4 μg DNA (Fig. 1). These responses were measured by ELISPOT using plates (Multiscreen-IP MAIP type plates, Millipore) pre-coated with 1.5 μg per well anti-mouse gamma interferon antibody AN18 (Mabtech AB). The assays were performed using the Mabtech protocol with freshly isolated splenocytes or thawed cells and 5 μg Gag peptide ml−1 plus 30 U interleukin-2 ml−1.

There was a difference between not only the levels of response induced using CO or WT genes but also in the amount of DNA necessary to induce a particular response. Indeed, the magnitude of T-cell response induced by CO DNA required an amount of WT DNA that was generally an order of magnitude higher.

Antibody titres were measured by serial dilution of sera in a standard ELISA using microtitre plates (Probind, Nunc) coated with 0.2 μg affinity purified SIVagm Gag protein per well. The difference between the WT and CO DNA with regards to antibodies was even more striking, with responses after immunization with 0.014 μg CO DNA exceeding those seen with 1.4 μg WT DNA (Fig. 2a).

All subsequent immunization studies were performed using co-administration of DNA coding for murine GM-CSF as an ‘adjuvant’ and a simplified prime–boost regime (day 0 prime, day 45 boost) according to the method described by Fensterle et al. (1999). Using such a regime, C57BL/6 mice immunized with CO SIVagm Gag CO DNA developed titres of specific antibodies nearly two orders of magnitude higher than mice immunized with the WT gene (Fig. 2b).

Immunogenicity testing and identification of T-cell epitopes were performed using panels of overlapping synthetic peptides spanning the entire SIVagm gag gene (Peptides & Elephants), SIVmac239 and HIV-1 clade A/G (NIH Reagent Program) Gag (p27/p24) proteins in the
ELISpot assays. The immunograde peptides generally comprised 15mers with an overlap of 10 aa or, for fine mapping, nonamers overlapping by 8 aa. For mapping the SIVagm Gag epitopes, four pools of overlapping peptides, each corresponding to approximately one quarter of the protein, were tested. In immunized BALB/c mice, the major responses were seen using peptides spanning the middle of the protein (Fig. 3) and it was again clear that both the magnitude and the number of responses obtained with the CO DNA was considerably higher than with the WT: all five mice receiving the CO variant generated Gag-specific T cells compared with only two in the WT group. No responses were seen in the naïve control mice group, as expected. The positive pools were subdivided into smaller pools for retesting and peptide 55 (SIVagm Gag271–285;VGAIYRRWIILGLQK) was shown to be responsible for the reactivity seen in the ELI Spot (data not shown). Fine mapping of this epitope using overlapping nonamers yielded responses to two peptides AIYRRWIIL (SIVagm Gag273–281) and YRRWIILGL (SIVagm Gag275–283). Surprisingly, the central nonamer IYRRWIILG did not induce a positive reaction, indicating the presence of two distinct but overlapping epitopes. It is interesting that an algorithm designed to predict binding affinity to the major histocompatibility complex molecule (www.syfpeithi.de) identified not only those epitopes that showed reactivity in the ELI Spot (data not shown). This was confirmed by testing the individual peptides (data not shown), although the precise epitope could not be mapped using nonamers, despite repeated attempts.

T-cell responses in C57BL/6 mice immunized with SIVmac239 Gag DNA were also evaluated using a peptide matrix containing 15mers spanning the entire SIVmac239 Gag protein. Analysis of the reactive pools indicated the presence of an epitope in the overlapping peptides SIVmac Gag309–323 (QTDAVKNWMTQLL) and SIVmac Gag313–327 (AVKNWMTQLLQLQNA) (data not shown) which is localized in the middle of the SIVmac239 Gag protein. Fine mapping with overlapping nonamers, 10mers and the 11mer of both 15mers resulted in strong responses to the 11mer AVKNWMTQLL (SIVmac Gag313–323) and to the 10mer AVKNWMTQLL (SIVmac Gag313–322) but only weak responses to the nonamers. This may, in retrospect, be due to the fact that the H-2Kb molecule can present epitopes up to 11 aa in length. This epitope corresponds well to the known HIV-1 epitope (QEKNWMTETL) for C57BL/6 mice that has been described previously (http://www.hiv.lanl.gov/content/immunology).

In contrast with the results with SIVagm Gag, BALB/c mice immunized with either WT or CO SIVmac Gag DNA failed to show any T-cell responses to the corresponding SIVmac Gag peptides (data not shown).

Finally, by immunizing BALB/c mice with HIV-1 gag DNA and measuring the resulting T-cell responses using a matrix of overlapping peptides spanning the whole HIV-1 Gag protein, it was possible to confirm the HIV-1 Gag epitope AMQMLKDTI (HIV-1clade A/G Gag197–205) that has been described previously (Jaffray et al., 2004) (data not shown), which also corresponds to the HIV-1 SIV gag313–322 epitope AMQMLKETI described by Mata et al. (1998). The D→E
amino acid exchange at position 7 had no influence on activity (data not shown), probably because the predicted anchor motif indicates that the second and the last amino acids (AMQMLKDTI) are important for binding (www.syfpeithi.de).

Similar studies in C57BL/6 mice failed to detect an HIV-1 Gag epitope for this haplotype. Although one such epitope has been described for the HIV-1 SF2 sequence [i.e. HIV-1SF2 Gag390–398 (SQVTNPANI) (Burshtyn & Barber, 1993; Huang et al., 2007)], this region of the protein is highly variable and the corresponding HIV-1 clade A/G sequence used in this study is highly divergent (SQVQHTNIM).

The studies presented here were carried out in preparation for a comparative immunization–infection experiment to be performed in two species of non-human primates that aims to induce, by genetic immunization, SIV Gag-specific T-cell responses in the absence of responses to other genes. An understanding of this phenomenon may shed light on the lack of disease progression in these natural hosts and hence on the biological basis for AIDS in HIV-infected humans. It was therefore essential for the DNA immunization strategy to be characterized initially and optimized in a small animal model. Taken together, the results of this study confirm that the optimal strategy for the planned primate experiment would be to use gene gun delivery of CO DNA in the presence of DNA coding for GM-CSF. Although the identification of epitopes in SIVs recognized by murine T cells is of little relevance to disease in primates, such information will be useful for studies in which immunogens are pre-screened in mice before use in primates, as it allows the targeted use of individual peptides in place of multiple pools.
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References


Fig. 3. T-cell epitope mapping. BALB/c mice immunized twice with wild-type (WT) or codon-optimized (CO) SIVagm Gag DNA. Results for individual mice (numbered 1–13) are expressed as the number of spot forming units (s.f.u.) above the background, detected by ELISpot after stimulation with four sequential pools of overlapping peptides spanning the SIVagm Gag protein. Pools 1 and 4, shaded bars; pool 2, filled bars; pool 3, empty bars. Controls: mice immunized with empty vector DNA.


