BUTTERWORTH HEINEMANN

0264-410X(95)00053-4

Vaccine, Vol. 13, No. 13, pp. 1187–1197, 1995

0264–410X/95 $10+0.00

Antigenicity and immunogenicity of recombinant envelope glycoproteins of SIVmac32H with different *in vivo* passage histories

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*Shortly after infection of two rhesus monkeys (*Macaca mulatta*) either with a SIVmac32H challenge stock or with the same virus that had been passaged in another rhesus monkey for 11 months, SIV-envelope genes were cloned from their peripheral blood mononuclear cells and subsequently expressed by recombinant vaccinia viruses. The molecular weights and antigenicities of the thus produced envelope glycoproteins were largely identical to those of the native SIV. The envelope glycoprotein derived from the* in vivo *passaged virus proved to be poorly recognized by virus neutralizing monoclonal antibodies directed against one of the seven antigenic sites for which monoclonal antibodies were available. Immunization studies in rats showed that this protein was also less efficient in inducing antibodies against this antigenic site, and that it induced significantly lower levels of virus neutralizing antibodies than the other SIV-envelope glycoprotein. The immunogenicity of the SIV-envelope glycoprotein incorporated into immune stimulating complexes (iscoms) was compared to that of the same protein presented with Quil A or MDP-tsl.*

Keywords: SIV; SIV-envelope glycoprotein; antigenicity

Simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) are closely related lentiviruses which show similarities both in their genomic organization and in the disease they cause in their respective hosts[1]. Therefore SIV infection of macaques is considered a suitable model for HIV infection in humans to study pathogenesis and development of passive and active immunization strategies.

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The envelope gene encoded surface (SU) and transmembrane (TM) glycoproteins of lentiviruses play a crucial role in virus attachment and fusion to host cells, and serve as targets for virus neutralizing (VN) antibodies and helper and cytotoxic T cells (for review see Ref. 2). Therefore, the inclusion of these envelope glycoproteins in candidate vaccine preparations in a form that stimulates both cellular and humoral immunity is desirable.

In several studies SIV candidate vaccines have been tested for their ability to induce protective immunity against challenge with SIV. Although in many of these studies induction of protective immunity by candidate SIV vaccines was reported, it was shown that in most cases an anti-cell rather than an anti-virus response was at the basis of this protection[3]. We have shown that after vaccination with envelope glycoprotein enriched whole SIVmac, four of the eight vaccinated animals were protected against an intravenous challenge with SIV-infected peripheral blood mononuclear cells (PBMC)[4,5]. So far, in SIV experiments there has been only one report describing protection induced with a recombinant vaccine preparation[6]. In this study priming with live recombinant vaccinia virus expressing the envelope protein followed by boosting with recombinant envelope

protein expressed by baculovirus, resulted in protection of rhesus macaques against a homologous challenge with a molecular clone of SIVmne. In the SIVmac model system, immunization with recombinant SIV preparations led to a reduced virus load[7,8].

Successful vaccines against lentivirus infections should at least provide protective immunity against homologous viruses and related variants. This may be achieved by the induction of immune responses directed to conserved regions of the envelope protein involved in VN, or by the simultaneous induction of immune responses to variable regions on the envelope protein of different variant viruses. In the present study, we describe the analysis of the antigenicity and immunogenicity of the envelope protein of SIVmac32H and its counterpart which had undergone an *in vivo* passage of 11 months. For this purpose, recombinant envelope proteins were obtained from PBMC of two monkeys shortly after infection with the respective viruses, to select for envelope sequences which are related to viruses which actually establish infection.

MATERIALS AND METHODS

Virus

SIV-*env* sequences were analyzed from two rhesus monkeys (*Macaca mulatta*), numbers 8672 and 8789. These animals belonged to control groups of animals in a previous SIV vaccination study[5]. Macaque number 8789 had been inoculated intravenously with 10 MID_{50} of the 32H isolate of SIVmac251 (11/88 pool)[9,10]. The monkey died from AIDS-like symptoms 26 weeks post-infection. Macaque number 8672 was inoculated intravenously with 10 MID_{50} of SIV-infected PBMC derived from rhesus macaque number 1XC. PBMC of number 1XC were isolated 11 months post-infection with an intravenous dose of 50 MID_{50} of the 32H isolate of SIVmac251 (pool 11/88)[11]. Macaque number 8672 died from AIDS-like symptoms 39 weeks post-infection. PBMC were isolated from macaques numbers 8789 and 8672 two and four weeks post-infection, respectively, and used for sequence analysis. At these time points no SIVmac32H specific VN serum antibodies had developed in the respective monkeys[5].

DNA template preparation

PBMC of rhesus macaques numbers 8789 and 8672 were resuspended in a buffer containing 50 mM KCl, 10 mM Tris–Cl (pH 8.4), 1.5 mM $MgCl_2$, 0.5% Tween-20 and 100 $\mu g\ ml^{-1}$ proteinase K at a concentration of 1×10^7 cells ml^{-1} and incubated for 16 h at 54°C, followed by 10 min at 95°C. The resulting cell lysate was used as source of template DNA in subsequent PCR amplifications.

PCR amplification

As the number of infected cells in PBMC of SIV-infected macaques early in infection was expected to be low, a nested primer PCR was developed to obtain sufficient quantities of DNA for cloning. Primers for PCR were chosen which recognized conserved sequences flanking the SIVmac envelope gene sequence (Los Alamos AIDS database). Outer set of primers: 5′-GGC CAACCTGGGGGAGGAAATCC-3′ (6398); 5′-GCA CTGTAATAATCCCTTCCAGTCCCCCC-3′ (9481). Inner set of primers: 5′-*CGC***GTCGAC**GTAAGTAT GGATGTCTTGGGAATCAGC-3′ (6599); 5′-*GAC***CC CGGG**CCCCTGATTGTATTTCTGTCCC-3′ (9264). The positions of the 5′-nucleotide in the SIVmac32H-J5 molecular clone are indicated in parentheses. SmaI and SalI restriction sites (presented in bold) were incorporated near the 5′ ends of the inner primer set to facilitate subsequent cloning. These sites were chosen from a list of "non-cutters" generated after analysis of the SIVmac251 sequence. Ten microlitres of the cell lysate were added to a PCR reaction mixture containing 10 mM Tris–Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 $\mu g\ ml^{-1}$ gelatin, 200 μM of each dATP, dCTP, dGTP and dTTP, 0.1 μM of each primer of one primer set and 2.5 units Taq polymerase (AmpliTaq, Cetus, Emeryville, CA). The total reaction volume of 100 μl was overlaid with mineral oil. DNA was amplified by a 4 min 94°C denaturation step followed by 36 cycles comprising denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min. Reamplification of 10 μl of the first PCR was performed with the inner set of primers under the same reaction conditions for 40 cycles.

Molecular cloning and sequence analysis

PCR reaction mixtures were extracted once with phenol/chloroform and concentrated on an Amicon-30 filter (Amicon). DNA was subsequently analyzed on a 0.9% agarose gel, the presumed 2.6 kb envelope fragment was purified and digested with SalI and SmaI and cloned into SalI and SmaI digested cloning vector pBluescriptII SK(+) (Stratagene, La Jolla, CA). Sequencing was carried out using a multiwell microtitre plate DNA sequencing system (Amersham) based on the dideoxynucleotide chain termination sequence reaction. The nucleotide and protein alignments were determined with Lasergene software (DNAstar Inc., London, UK).

Nucleotide sequence accession numbers

Nucleotide sequences of clones 8672-20 and 8789-11 have been submitted to GenBank and are available under accession numbers L35596 and L35597, respectively.

In vitro mutagenesis

In order to mutate the presumed primary and secondary cleavage site of the precursor envelope protein[12], amino acid substitutions were made by site-directed mutagenesis (primary cleavage site: RNKR to RNSA; secondary cleavage site: KR to KS) using three PCR reactions. The pBS^+ constructs containing the respective envelope genes were used as template DNA. Primers 5′-mut (5′-GTGAAGAGcTACACTA CTGGTGGCACCTCAAGAAATAgcgcAGGGG TCT TTGTGC (8135)) and 3′-NcoI (5′-GCATTTGGCCAT GGTACAGTAGTGTGGC-3′ (8478)) were used to obtain fragment 5′-mut/3′-NcoI. Using primer pair 3′-mut (5′-GCACAAAGACCCCTgcgcTATTTCTTG AGGTG CCACCAGTAGTGTAgCTCTTCAC-3′, reverse complementary to 5′-mut) and 5′-ClaI (5′-GGCAGAAC

TATCGATTGGAATTGGG-3' (8059)) fragment 5-'ClaI/3'-mut was obtained. The positions of the 5'-nucleotide in the SIVmac32H (J5) sequence are indicated in parentheses. The lower case letters represent nucleotide changes that were introduced. Subsequently, the 5'-ClaI/3'-mut and 5'-mut/3'-NcoI fragments were used as template in a PCR using primers 5'-ClaI and 3'-NcoI. The PCR-derived 5'-ClaI/3'-NcoI fragment including the nucleotide substitutions was purified by preparative gel electrophoresis, digested with ClaI and NcoI and cloned into ClaI and NcoI digested clones 8672-20 and 8789-11, respectively. The presence of the substitutions was confirmed by sequence analysis. The mutated envelope proteins were designated 8672-m and 8789-m, respectively.

Construction of recombinant vaccinia viruses

Both the wild-type and the mutated 8672-20 and 8789-11 envelope genes were cloned under control of a synthetic early/late vaccinia promoter in the plasmid vector pSC65. Recombinant viruses were made by homologous recombination with vaccinia virus (WR strain) as previously described[13]. A control recombinant vaccinia virus, vSC65, was made by homologous recombination of the plasmid vector pSC65 with no insert[14]. Recombinant vaccinia viruses were plaque purified at least three times and virus stocks were grown on RK13 cells.

Production of recombinant *env* glycoproteins

Baby hamster kidney (BHK) cells were infected with recombinant vaccinia virus (rVV) at a multiplicity of infection of 4 and incubated for 20 h. Cells were collected and the vaccinia virus was inactivated in 1.5% paraformaldehyde for 30 min. Subsequently, cells were incubated in 0.2 M glycine for 1 h. Membrane proteins were solubilized in TN (10 mM Tris–HCl pH 7.4, 0.15 M NaCl) containing 4% RBT (*n*-octylpolyoxyethylene, Rosenbuch-Tenside; Bachem, Bubendorf, Switzerland) at a concentration of 2×10^7 cells ml^{-1} for 90 min at 4°C. The cell lysate was centrifugated for 15 min at 2500 revs min^{-1}. Glycoproteins were allowed to adsorb to lentil lectin sepharose (Pharmacia LKB, Uppsala, Sweden) overnight. Subsequently, the lentil lectin sepharose was washed with buffer A (10 mM Tris–HCl pH 7.4/0.3 M NaCl/0.5% RBT) followed by washing with buffer B (buffer A supplemented with 10 μg ml^{-1} of the lipids cholesterol and phosphatidylcholine (Sigma, St. Louis, USA)). Bound glycoproteins were eluted with buffer C (buffer B containing 0.5 M α-methylmannopyranoside). Quantification of SIV-envelope glycoproteins was performed as described previously[5].

Formulation of antigen preparations and immunization schedule in rats

For the preparation of immune-stimulating complexes (iscoms) lentil lectin enriched 8672-m or 8789-m proteins were mixed with the lipids cholesterol and phosphatidylcholine (Sigma; stock 10 mg ml^{-1} in 10% MEGA-10 (Boehringer Mannheim, Mannheim, Germany)) and Quil A (Spikoside, Iscotec, Luleå, Sweden) at a ratio of 1:1:5 (w/w/w). After ultrasonic treatment for 10 min the mixture was incubated for 1 h at room temperature (RT). Subsequently, the mixture was extensively dialysed against 10 mM Tris–HCl pH 7.4/0.3 M NaCl, layered over a linear (10–60%) sucrose gradient and centrifugated (18 h at 25 000 revs min^{-1}: Beckmann SW28 rotor). The gradient was fractionated and fractions were analyzed for the presence of envelope protein by enzyme-linked immunosorbent assay (ELISA). Fractions containing peak levels of the envelope protein (coinciding with the presence of iscom-like structures, as judged by electron microscopy) were pooled.

For the Quil A and MDP-tsl antigen preparations, 3 μg of lentil lectin enriched SIV-*env* 8789-m was adjuvanted either with 10 μg Quil A (Spikoside, Iscotec) or 0.5 ml MDP-tsl (0.462 ml PBS containing 12.5 μg MDP (muramyl dipeptide) (Sigma), 1 μl Tween-80, 25 μl squalene and 12.5 μl of the plutonic block polymer L101 (Serva, Heidelberg, Germany))[15,16].

Female rats (RIV:tox) were divided into groups of four animals and were immunized intramuscularly (i.m.) with the different antigen preparations and boosted four weeks later with the same material. Each rat received 3 μg of rgp160 per immunization except for the rats immunized with both 8672-m and 8789-m incorporated into iscoms. These received 3 μg of each rgp160 construct per immunization. Serum samples were collected before and 2, 4, 5, 6, 8, and 14 weeks after the first immunization.

Western blotting

Procedures for western blotting were performed as described previously[5].

Binding of monoclonal antibodies (Moabs) to different SIV-envelope preparations

Multiwell plates (Costar, Cambridge, USA) were coated with 100 μl PBS containing 200 μg ConA and then incubated with the different SIV-envelope preparations at a concentration of 10 ng envelope protein in 100 μl PBS containing 1% RBT, for 16 h at RT. Wells were blocked with PBS containing 0.1% Tween-20 and 1% BSA (PTB) supplemented with 10% FCS, and subsequently incubated for 2 h at RT with threefold serial dilutions starting at 1:200 of one of the *env*-specific Moabs (ascitic fluid, kindly provided by Dr K. Kent). Plates were washed and incubated with biotin conjugated goat-anti-mouse IgG (Amersham) and horse radish peroxidase (HRPO) conjugated streptavidine (Amersham). The substrate reaction was carried out with 3,3',5,5'-tetramethyl-benzidine[17]. The antibody titre was defined as the reciprocal of the dilution of the Moab giving 50% of the maximum $O.D._{450}$ obtained for that Moab and was determined from three independent experiments. Values two times that of similarly processed preparations of vSC65-infected BHK cells were considered positive.

Whole SIV ELISA to demonstrate envelope-specific antibodies

ConA coated multiwell plates were incubated with 50 μl of SIV-infected C8166 cell lysate[5] in PBS/1% triton

containing 70 ng ml^{-1} SIV-*env*, for 16 h at RT. (Quantification of the SIV-envelope protein was performed as described previously[5].) Wells were blocked with PTB containing 10% FCS and subsequently incubated with 50 μl of twofold dilutions of rat serum in PTB containing 4% FCS. After 2 h incubation at RT, wells were washed and bound antibody was detected using a rabbit-anti-rat Ig-HRPO antibody preparation (DAKO, Glostrup, Denmark). The substrate reaction was carried out as described above. Endpoint titres were calculated using a cut-off value twofold above the respective dilution of the pre-immune serum at O.D.$_{450}$. Antibodies with specificity for BHK cell components in the immunogen did not react with plate bound C8166 cell components. Comparison of titres between groups was conducted using a one-way analysis of variance (Anova; Minitab Inc., State college, USA). Titres were considered significantly different if $p<0.05$.

SIV-envelope specific Moab inhibition ELISAs

Inhibition ELISAs were carried out as described previously[5] with minor modifications. Briefly, ConA coated multiwell plates were incubated with 100 μl C8166-SIV cell lysate containing 70 ng ml^{-1} SIV-envelope proteins. One hundred microlitres of twofold dilutions of rat serum were incubated for 1 h at RT. Subsequently, 50 μl was taken out and wells were supplemented with 50 μl of Moab in PTB containing 4% FCS. Only Moabs with VN activity were used for these inhibition studies. It has been shown that Moabs KK5 and KK9 of competition group 1, which recognize a conformation dependent epitope, do not interfere with the binding of the SIV envelope protein to sCD4 as demonstrated in immunoprecipitation assays[18,31]. The Moabs were diluted to give an absorbance at O.D.$_{450}$ of 50–70% of the maximum absorbance in an indirect SIV-ELISA. Plates were incubated with a biotin-conjugated goat-anti-mouse IgG antibody preparation (Amersham) in PTB containing 4% FCS and 5% normal rat serum (NRS), which was pre-incubated in this buffer for 1 h at RT, and subsequently with HRPO conjugated streptavidine (Amersham). The anti-mouse biotin IgG antibody preparation did not cross-react with rat Ig in this assay. Inhibition titres were defined as the dilution of rat serum inhibiting 50% of the absorbance measured without competing antibody. The difference in titre between the different immunization groups was evaluated through a student's *t*-test. Differences of $p<0.05$ were considered significant.

SIV neutralization assay

The SIV neutralization assay using SIVmac32H was performed as described previously[5]. Briefly, 10 μl of different dilutions of heat inactivated sera were incubated (four replicates per dilution) with 10 μl of SIVmac32H diluted to give ten infectious particles per 10 μl. After 30 min incubation at 37°C, 200 μl of media containing 2000 C8166 cells was added to each well of the microtitre plate. Plates were incubated for seven days at 37°C after which cells from each well were transferred to poly-L-lysine coated flat bottom microtitre plates, fixed in methanol, and examined for the presence of SIVmac-infected cells using a SIV-specific immunoperoxidase assay. Wells containing no infected cells were scored as negative and the number of negative wells was used to determine the neutralizing dose 50% end-point (ND_{50}) for that serum by the method of Spearmann-Karber. The difference in titre between the different immunization groups was evaluated through a student's *t*-test. Differences of $p<0.05$ were considered significant.

RESULTS

Selection of SIV-envelope glycoproteins and sequence comparison

PBMC were collected from two rhesus macaques, two weeks (monkey number 8789) or four weeks (monkey number 8672) after infection with SIVmac32H. The first animal had been infected with 10 MID_{50} of the cell-free virus (11/88 pool) and the second with 10 MID_{50} of the PBMC from a monkey 11 months after infection with the 11/88 pool[5]. Representative SIV envelope genes were derived from the respective monkeys. SIV envelope clone 8789-11 was selected with a sequence shared by five of six clones in the variable regions 1 and 2 (V1 and V2)[19] and clone 8672-20 was selected from a panel of three clones which showed considerable similarities in this region with the exception of single amino acid differences. The predicted amino acid sequences of clones 8672–20 and 8789-11 are outlined in *Figure 1*, where they are compared with that of the consensus sequence of the envelope glycoprotein present in the 11/88 stock of the 32H isolate of SIVmac251 as identified by Almond *et al.*[19] As expected the 8789-11 sequence showed much more similarity to the consensus sequence of the envelope glycoprotein present in the 32H isolate than the 8672-20 sequence (3 and 19 amino acid differences, respectively). A 98% overall amino acid sequence identity was shown between clones 8672-20 and 8789-11. Most strikingly, in the V1 region of the 8672-20 envelope sequence an extended sequence was found that was rich in serine and threonine residues, leading to a sequence commonly found in heavily O-linked glycosylated proteins[20]. An extra potential N-linked glycosylation site was found in clone 8672-20 directly N-terminal of the start of the V1 region. In addition, two amino acid differences were found in the V2 region, one in the V4 region, four amino acid differences outside the variable regions of the SU and five in the TM part of the SIV-envelope precursor sequence (*Figure 1*).

Expression of SIV-envelope glycoproteins by rVVS

The RNKR sequence in clones 8672-20 and 8789-11, representing the cleavage site between the SU and TM part of the envelope genes, together with a second conserved cluster of basic amino acids which may serve as a secondary cleavage site[12], was modified by site-directed mutagenesis. Subsequently, rVVs were generated from the parental 8672-20 and 8789-11 envelope genes (designated v8672 and v8789, respectively) and from the cleavage site mutated envelope genes (designated v8672-m and v8789-m, respectively).

Western blot analysis of BHK cells infected with v8672 using a TM-specific Moab, revealed proteins with

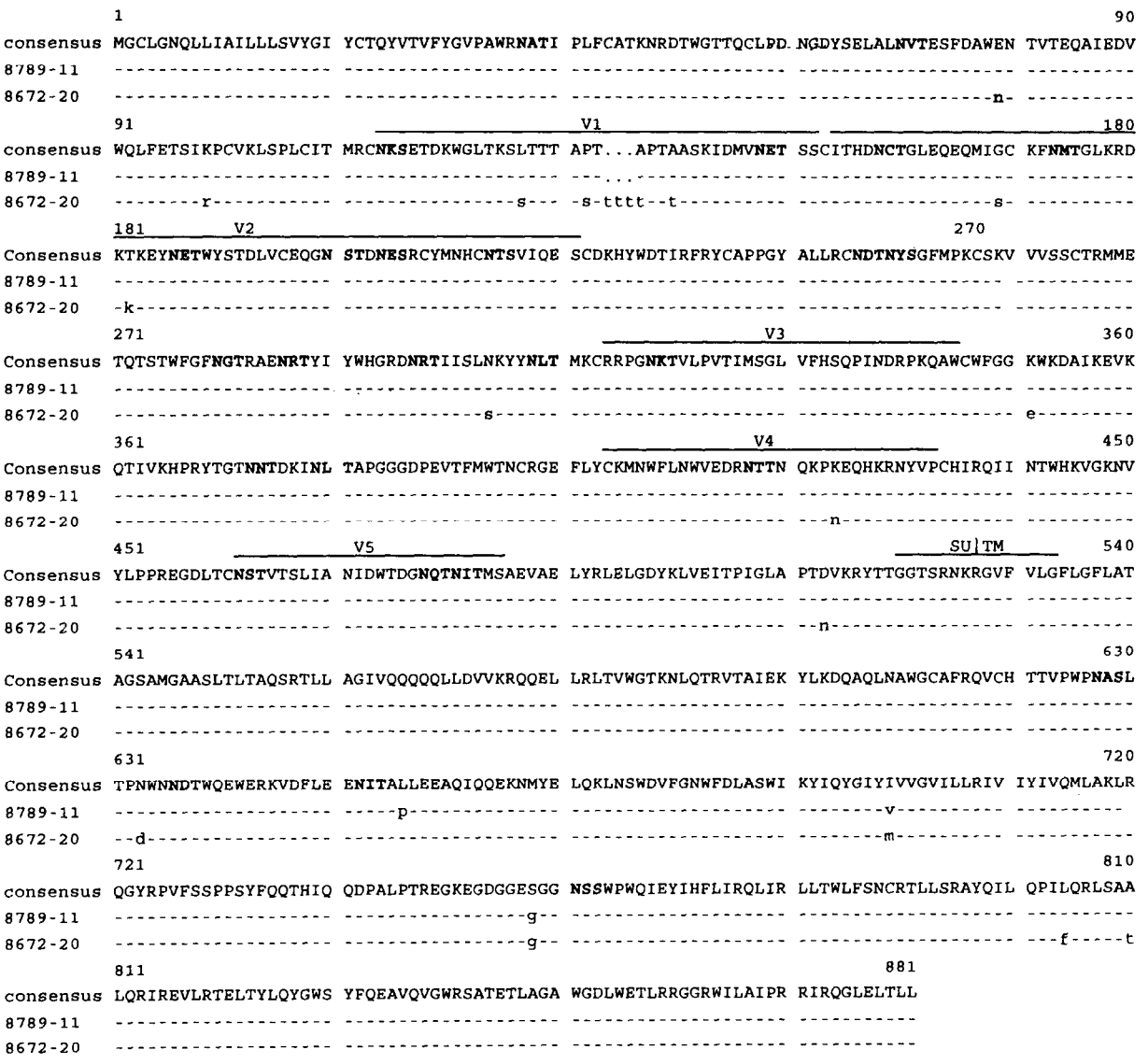

Figure 1 Predicted amino acid sequences of the envelope precursor protein of clones 8672-20 and 8789-11. They are aligned with the consensus sequence for the envelope glycoprotein present in the 32H-isolate of SIVmac251 (11/88 pool) as identified by Almond *et al.*[17] A dash indicates that the amino acid is identical to the consensus sequence; variation in sequence is given in single letter code. Amino acid positions where no equivalent is present are indicated by a dot. The regions of the envelope protein equivalent to the variable regions (V) of HIV-1 are indicated[34]. The predicted cleavage site between the SU and TM part of the envelope protein is indicated. Potential N-linked glycosylation sites (N-X-S or N-X-T) are indicated in bold type

molecular sizes of 160 and 41 kDa (*Figure 2B*, lane 2). The 160 kDa protein was also recognized by the SU membrane specific Moab (*Figure 2A*, lane 2). In the culture medium a protein with a Mr of about 120 kDa was recognized by the gp120-specific Moab (*Figure 2A*). These data indicate that, like in the native protein, a 160 kDa SIV precursor protein is synthesized which is processed to a 41 kDa TM protein and a 120 kDa SU protein which is also released into the medium. In v8789-infected BHK cells the 160 kDa envelope precursor protein was predominant. In the culture medium the SU envelope glycoprotein was detectable. In the culture medium of cells infected with v8672-m and v8789-m containing the mutated envelope genes, no indication for the presence of the SU envelope glycoprotein was found.

Incorporation studies with lentil lectin purified extracts from BHK cells infected with either of the rVVs, showed that iscoms prepared with the cleavage site mutated recombinant envelope proteins contained the gp160 precursor protein (*Figure 3*).

Antigenicity of recombinant envelope glycoproteins 8672, 8672-m, 8789, 8789-m and envelope glycoprotein derived from SIVmac32H

Antigenicity of the envelope proteins expressed by v8672, v8672-m, v8789 and v8789-m incorporated into iscoms was assessed with a panel of 15 SIV-envelope specific Moabs divided over seven competition groups[21,22] (see *Figure 4*). The antibody titre at which 50% of the maximum $O.D._{450}$ was obtained, was similar for all Moabs tested comparing the envelope glycoproteins derived from SIVmac32H-infected C8166 cells and rVV 8789 expressed envelope glycoprotein. Envelope glycoproteins derived from v8672 were poorly recognized by Moabs of competition group 1 compared to envelope glycoproteins derived from v8789 or SIV itself.

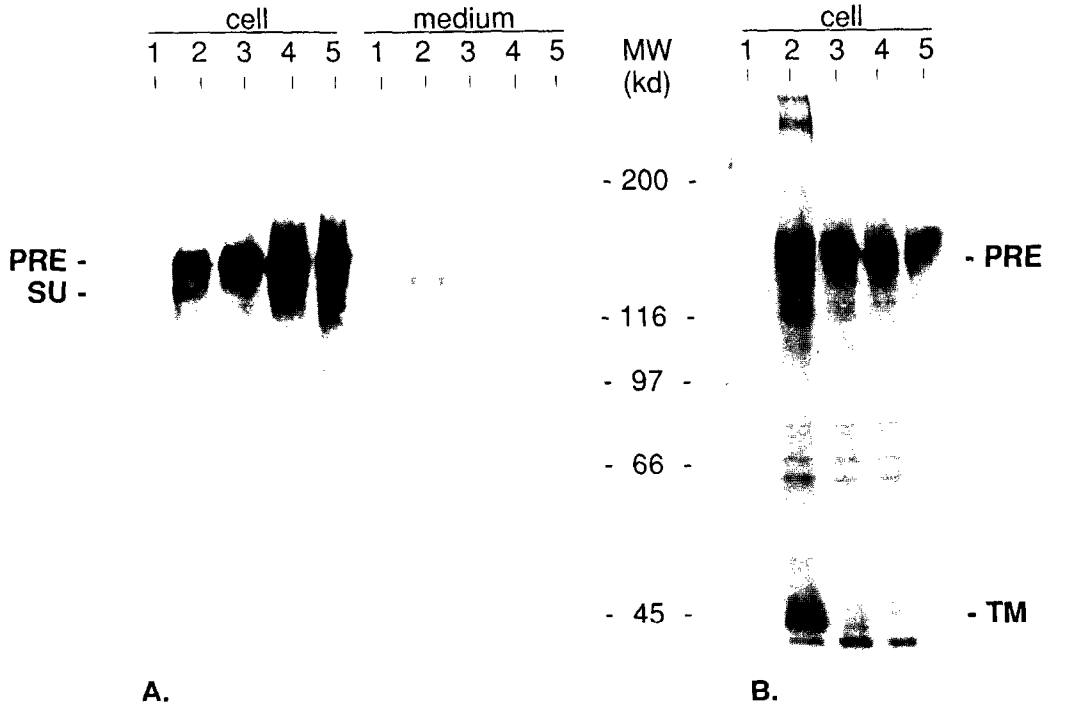

Figure 2 Western blot analysis of wild-type and mutant SIV envelope glycoproteins expressed by rVV in BHK cells using a gp120-specific monoclonal antibody (KK10) (A) or a gp41-specific monoclonal antibody (KK20) (B) with cell lysate (cell) and culture medium (medium). Lane 1, vSC65 (negative control); lane 2, v8672; lane 3, v8672-m; lane 4, v8789; lane 5, v8789-m. The locations of the envelope precursor protein (pre), surface membrane protein (su) and transmembrane protein (tm), as well as the mobilities of molecular weight markers, are indicated

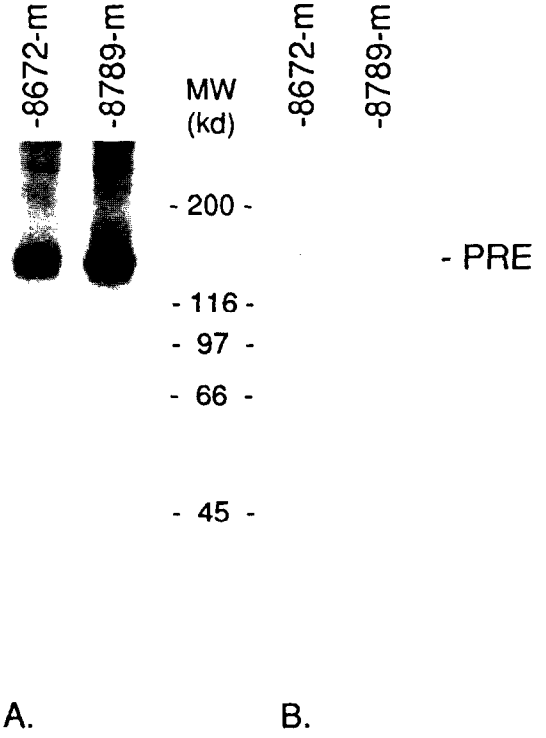

Figure 3 Western blot analysis of iscoms prepared with lentil-lectin purified proteins derived from v8672-m and v8789-m infected BHK cells using a gp120 specific monoclonal antibody (KK13) (A) or a gp41 specific monoclonal antibody (KK20) (B). The location of the envelope precursor protein (pre) as well as the mobilities of molecular weight markers are indicated

Interestingly, all Moabs of this competition group recognize a conformational epitope and have been shown to possess VN activity[21,22].

No differences were found in the binding of the Moabs to the 8672 and 8789 envelope proteins in their native or cleavage site mutated form, except for one of the Moabs of competition group 7, KK16, which hardly recognized the 8789-m envelope protein.

No changes in binding of the Moabs to the recombinant envelope glycoproteins were found as a consequence of either paraformaldehyde fixation, lentil-lectin purification, or incorporation into iscoms (data not shown).

Immunogenicity of recombinant envelope glycoproteins 8672-m and 8789-m

The immunogenicity of the 8672-m and 8789-m envelope glycoproteins incorporated into iscoms was analyzed by immunizing laboratory rats. The overall kinetics and levels of SIV-specific antibody titres induced by immunization with two times 3 μg of one of the envelope glycoproteins proved to be similar when measured by a whole SIV ELISA. Rats immunized with both iscom preparations (3 μg envelope protein of each per immunization) showed a significantly higher antibody response (*Figure 5A*). Inhibition of the Moab KK56 could only be clearly demonstrated with sera from rats immunized with 8789-m envelope glycoproteins (*Figure 5B*). This inhibition pattern of KK56 was representative of all Moabs of competition group 1 (data not shown). Apparently there is a direct relationship between the recognition or presence of certain epitopes and their capacity to induce specific antibodies. VN serum antibody titres of 8789-m iscom immunized rats were also significantly higher than those of 8672-m iscom immunized animals (*Figure 5C*). Similar levels of VN serum antibodies and KK56 inhibition titre were found in rats

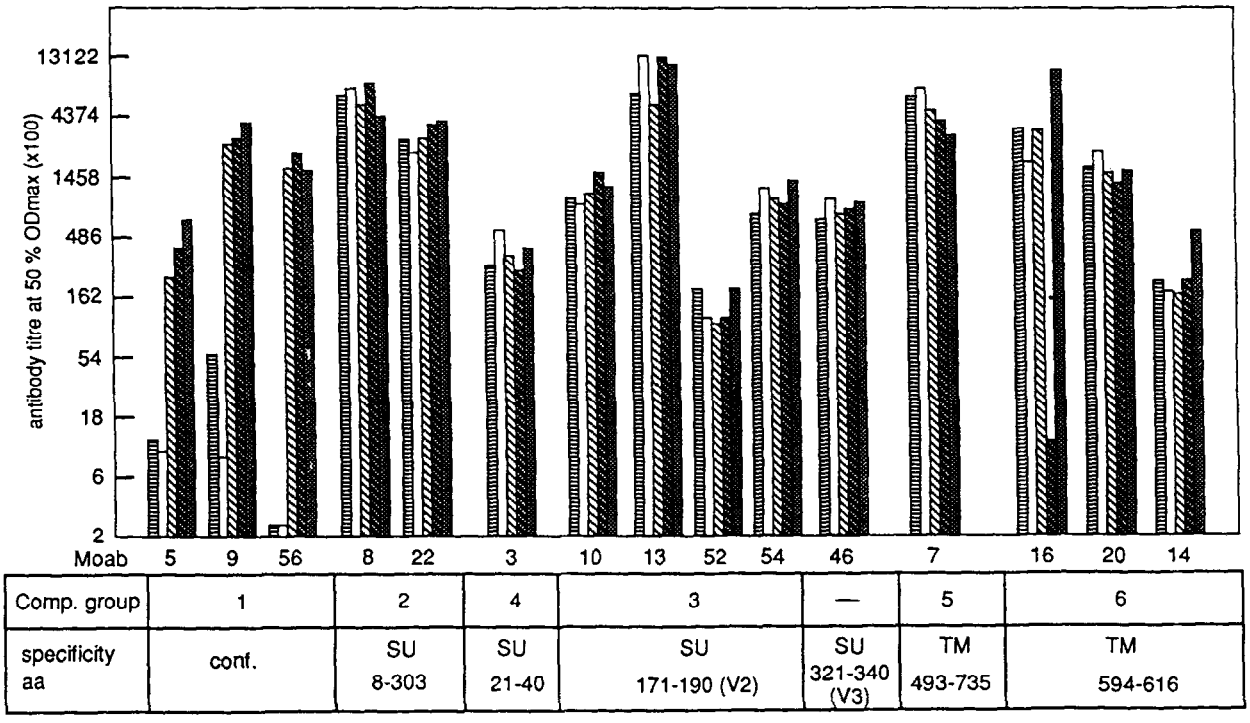

Figure 4 Binding of 15 SIV-envelope-specific monoclonal antibodies to the 8672, 8672-m, 8789 and 8789-m envelope glycoproteins incorporated into iscoms and SIVmac251(32H) produced envelope proteins on C8166 cell[5]. Competition groups are defined in Kent et al.[19,20] aa; amino acid numbers BK28 SIV-envelope protein. Conf.; conformational epitope. Bars represent the dilution of the monoclonal antibody giving 50% of the maximum $O.D._{450}$ obtained using that monoclonal antibody. ▤ =8672, □ =8672-m, ▨ =8789, ▢ =8789-m, ▩ =C8166-SIV

immunized with the 8789-m envelope proteins alone or in combination with the 8672-m envelope protein (*Figure 5B,C*).

Immunogenicity of SIV-envelope glycoprotein 8789-m presented in the context of different adjuvant systems

The effect of different adjuvant systems on the immunogenicity of the 8789-m envelope glycoprotein was studied by immunizing rats twice with 3 μg of this protein using Quil A, MDP-tsl or iscoms as adjuvant systems. Only rats immunized with envelope proteins adjuvanted with MDP-tsl showed a detectable antibody response after one immunization. After boosting, the highest envelope antibody titres measured by ELISA were seen with sera from rats immunized with the 8789-m envelope glycoprotein incorporated into iscoms (p=0.07, *Figure 6A*). There proved to be little decrease in serum antibody titres during the observation period, irrespective of the adjuvant used. The KK56 antibody inhibition titre as well as the VN serum antibody titre of rats immunized with the iscom-adjuvanted envelope glycoprotein were higher than those of rats immunized with envelope protein adjuvanted with Quil A or MDP-tsl, although only the difference in KK56 inhibition titres between sera from rats immunized with iscom and MDP-tsl adjuvanted glycoproteins proved to be significant (*Figure 6B,C*).

DISCUSSION

In the present study, we have shown that the antigenicity and immunogenicity of two envelope glycoproteins derived from the same parent virus, SIVmac32H, with different *in vivo* passage histories differed considerably: the envelope protein derived from PBMC of a monkey infected with a virus which had undergone an extra *in vivo* passage for 11 months was not recognized by a group of VN antibodies able to bind to the envelope protein of the parent virus, SIVmac32H. Upon immunization, this envelope protein failed to induce antibodies which could compete with these monoclonal antibodies for binding to the envelope protein of SIVmac32H. Concurrrently, a lower total VN antibody titre against SIVmac32H was induced in comparison to that induced by the envelope protein derived directly from a SIVmac32H-infected monkey.

There are several indications that conformational epitopes are involved in eliciting SIV-neutralizing antibodies. SIV synthetic peptides proved to be poor inducers of VN antibodies, whereas immunization of macaques with recombinant envelope protein gp110 elicited strong VN antibodies[23]. Furthermore, VN activity in sera from infected macaques could be absorbed out with native gp110, but not with reduced or denatured gp110[23]. Therefore, we chose the eukaryotic rVV expression system for the production of SIV-envelope glycoproteins to be incorporated in SIV candidate vaccines. Vaccinia virus infectivity was eliminated by paraformaldehyde treatment. Although paraformaldehyde is able to alter the antigenic structure of membrane proteins[24], no differences in antigenicity were found in the SIV-envelope protein before and after paraformaldehyde fixation (Hulskotte *et al.*, manuscript in preparation). The SIV 160 kDa envelope precursor proteins, expressed by rVV v8672 and v8789, were similarly cleaved to a 120 kDa SU and 41 kDa TM protein as in SIV-infected cells, although cleavage of especially 8789-*env* proved to be less efficient. No

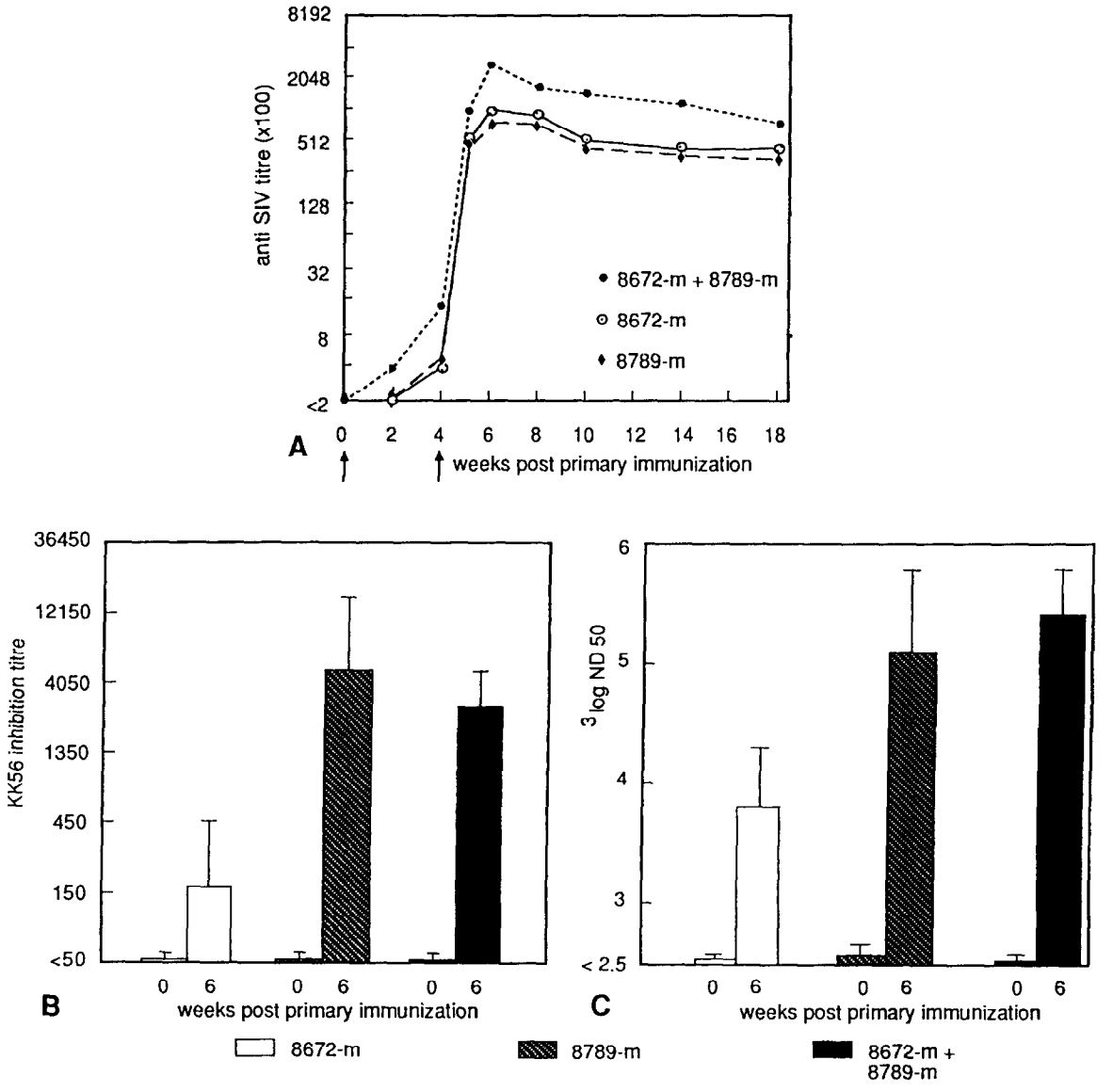

Figure 5 SIV envelope antibody development in rats immunized with 8672-m iscoms (3 μg per immunization), 8789-m iscoms (3 μg per immunization) or both (3 μg each per immunization). Mean titres were calculated from four rats immunized with the same envelope preparation. A. Anti-SIV antibody titre. Weeks of immunization are indicated by arrows. B. Response to a conformational epitope before immunization and at peak level of antibody response (week 6 post primary immunization) as measured by inhibition of binding of monoclonal antibody KK56. The standard error of the means are presented as a vertical line. C. SIV-VN antibody titres before immunization and at peak level of antibody response (week 6 post primary immunization). Serial dilutions of monkey plasma were incubated with SIVmac32H for 30 min at 37°C. Subsequently, C8166 cells were added. The number of negative wells was used to determine the neutralizing 50% endpoint (ND_{50}) for the relative serum. The standard error of the means are presented as a vertical line

differences in antibody reactivities of a panel of 15 Moabs with envelope glycoproteins derived from SIVmac32H-infected C8166 cells and v8789 expressed envelope glycoprotein were found indicating that the rVV expression system is able to produce SIV-envelope glycoproteins in their native conformation.

Comparison of the amino acid sequences of 8672-20 and 8789-11 envelope precursor proteins revealed a 98% amino acid similarity. It is interesting to note that in the V1 region of the 8672-20 sequence—derived from the *in vivo* passaged SIV—a T-rich stretch was inserted compared to the 8789-11 sequence. In SIVmne such a T-rich stretch was observed to be more common late in infection[25,26]. Sequential sequence analysis of the envelope genes derived from these monkeys provided strong evidence that such T-rich stretches emerge later in infection by active selection, indicating expression *in vivo*[26]. The presence of this T-rich sequence in the envelope protein derived from PBMC of a monkey four weeks post-infection with an *in vivo* passaged virus indicates that sequence variation has most likely occurred during the 11 months of *in vivo* passage of SIVmac32H in monkey 1XC. In addition, at four weeks post-infection a homogenous population of envelope sequences has been demonstrated in both HIV and SIV infection[27–30].

The 8672 envelope glycoprotein was poorly recognized by all VN Moabs directed to a conformation dependent epitope on the surface protein (KK5, KK9, KK56). A recent study suggests that the V4 region is involved in the recognition of the SIVmac239 envelope protein by Moabs KK5 and KK9, as demonstrated by an amino acid substitution at position 419 (equivalent position in SIVmac32H sequence, *Figure 1*), or deletions of four amino acids (position 421–424 and

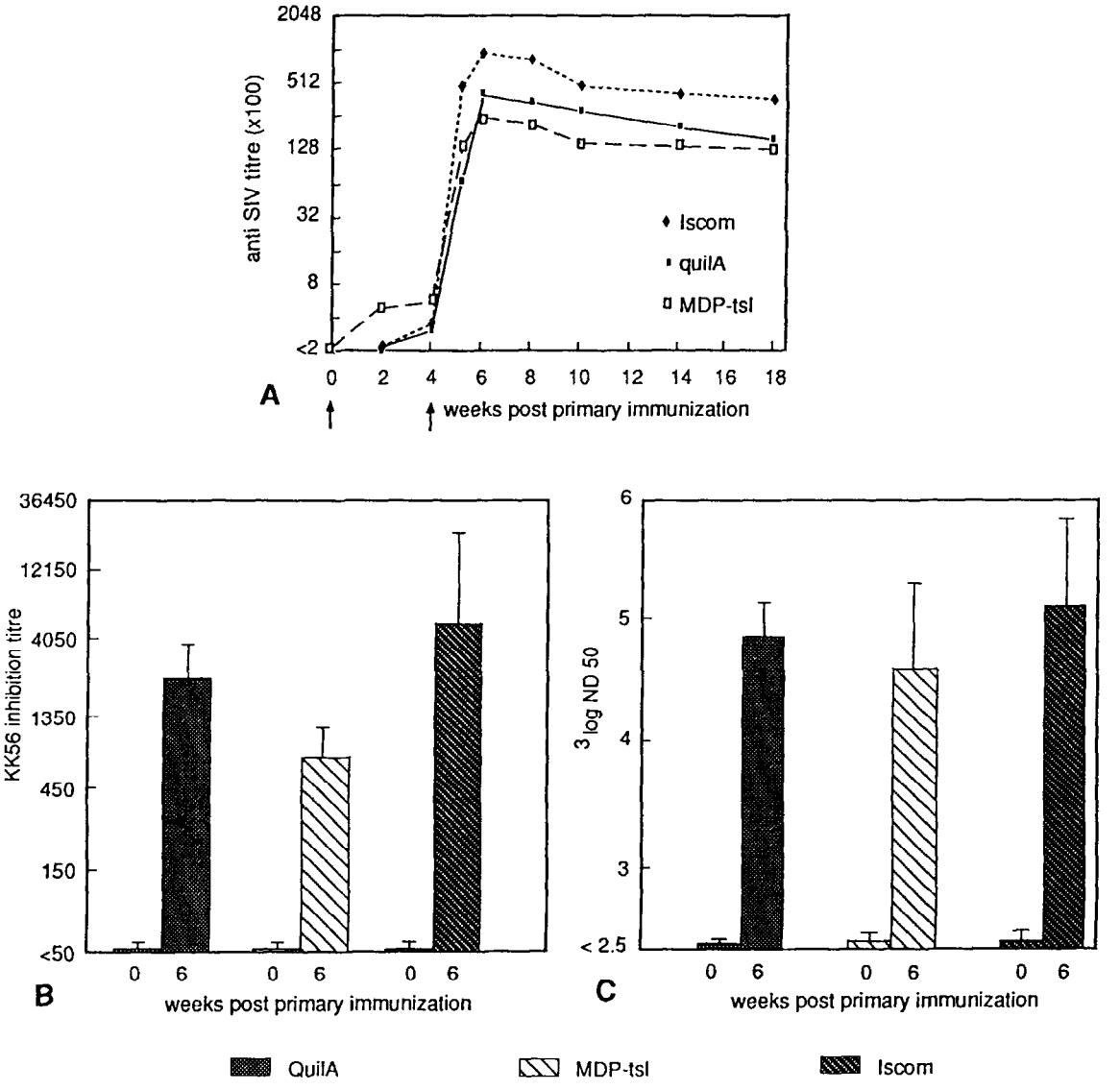

Figure 6 SIV-envelope antibody development in rats immunized with 8789-m envelope glycoproteins using different adjuvant systems. Mean titres were calculated from four rats immunized with the same adjuvant. Legends to *Figure 6A, B* and *C* are as for *Figure 5A, B* and *C*, respectively

position 423–427) in this region[31]. We also found a difference in the amino acid sequence between the 8789 and 8672 envelope proteins in the same region in which the deletions were made[31] (K to N at position 423). However, a possible role for the other amino acid differences found between clone 8672 and 8789 in the recognition by the respective Moabs cannot be excluded and may be implicated in an escape mechanism from neutralization by antibodies which had developed in the macaque which donated the challenge material for monkey number 8672.

No difference between the two envelope proteins was observed in binding of the other Moabs, including the VN antibodies KK10 and KK54[21,22]. However, in contrast to the VN antibodies of competition group 1, binding of the VN Moabs KK10 and KK54 could neither be inhibited by the respective rat sera, nor by sera from monkeys immunized with the envelope glycoproteins 8672-m and 8789-m incorporated into iscoms, nor by sera from SIVmac-infected monkeys (data not shown). However, it has been shown by others that sera from some SIVmac-infected macaques[32,33] and some macaques immunized with virus-derived gp130 in incomplete Freund's adjuvant with or without prior priming with live rVV expressing the SIVmacBK28 envelope protein, react with the peptide epitopes of these Moabs[33].

To facilitate incorporation of the hydrophilic SU protein into iscoms, cleavage site mutated envelope proteins were expressed by rVVs. As mentioned above, the 8789 envelope glycoprotein was inefficiently cleaved exhibiting most of the envelope glycoprotein as the uncleaved precursor. However, we decided to also mutate this construct to obtain a uniform and well defined product. Indeed, the cleavage site mutated envelope glycoproteins, 8789-m and 8672-m, were efficiently incorporated into iscoms, which has also been demonstrated for the cleavage site mutated precursor envelope glycoprotein of FIV[14].

No differences in antibody reactivity of a panel of 15 Moabs were found between the wild type and cleavage site mutated 8672 and 8789 envelope glycoproteins, except for the abrogated recognition of the 8789-m protein by Moab KK16. Sequence analysis showed that

during PCR used for mutation of the cleavage site, a mutation had been introduced in the region where the reactivity of KK16 was mapped[21] (position 619, C to G mutation). This did not influence the recognition of all the other Moabs, including those that recognize a conformation-dependent epitope, indicating that the overall conformational integrity of 8789-m had remained intact.

The capacity of different adjuvants to enhance the antibody response was compared using the 8789-m envelope glycoprotein. This protein was selected on basis of its reactivity with KK5, KK9 and KK56 and its ability to induce high VN titres (*Figures 4* and *5*). The highest titres of SIV-specific antibodies were found in rats immunized with the 8789-m iscoms. This is in agreement with previous studies in which monkeys immunized with SIV proteins also showed a higher envelope antibody response with SIV-envelope proteins incorporated into iscoms than adjuvanted with MDP[5]. However, in rabbits the HIV-1 envelope protein adjuvanted with MDP-tsl was shown to induce a higher antibody response than HIV-1 envelope protein incorporated into the iscom matrix using a multiple immunization schedule[16]. This discrepancy may be due to the quality of the respective preparations, the animal species or to differences in the immunization schedule used.

In conclusion, our results indicate that sequence changes occurring during *in vivo* passage, result in major changes both in antigenicity and immunogenicity of the envelope glycoproteins of SIV. The availability of envelope proteins with distinct variable regions may offer the possibility to study their protective value *in vitro* and *in vivo* against a wider range of virus variants, but may also offer the possibility of sequential immunization to prime and boost for an antibody response directed against conserved regions in the absence of a secondary immune response to variable regions.

ACKNOWLEDGEMENTS

Dr K. Kent and the MRC AIDS directed programme are kindly acknowledged for the gift of the Moabs. We thank Dr B. Moss and Dr S. Chakrabarti for the gift of the pSC65 plasmid vector. Professor Dr B. Währen is kindly acknowledged, for the gift of the MDP-tsl adjuvant. J.S. Teppema is acknowledged for the electron microscopy on the iscom preparations and J. Groen for helpful technical discussions. We thank R. van Kinderen and K. van Essen for biotechnical assistance. Ms W. Witkamp and Ms C. Kruyssen are acknowledged for assistance in preparing the manuscript. This investigation was supported by the Dutch Council for Health Research (RGO) and the Praeventiefonds (grant no. 90.052 and 28-2128, respectively).

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