Protection of rhesus macaques from SIV infection by immunization with different experimental SIV vaccines

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*The immunogenicity and efficacy of an inactivated whole SIVmac (32H) preparation adjuvanted with muramyl dipeptide (SIV-MDP) and a gp120-enriched SIVmac (32H) ISCOM preparation (SIV-ISCOM), were compared by immunizing four rhesus macaques (*Macaca mulatta*) four times with SIV-MDP and four others in the same way with SIV-ISCOM. Two monkeys immunized with whole inactivated measles virus (MV) adjuvanted with MDP (MV-MDP) and two monkeys immunized with MV-ISCOM served as controls. In the SIV-ISCOM-immunized monkeys higher SIV-specific serum antibody titres were found than in the SIV-MDP-immunized monkeys. In contrast to the MV-immunized monkeys all SIV-MDP- and SIV-ISCOM-immunized monkeys were protected against intravenous challenge 2 weeks after the last immunization with 10 median monkey infectious doses (MID_{50}) of a cell-free SIVmac (32H) challenge stock propagated in the human T-cell line C8166. After 43 weeks the protected monkeys were reboosted and 2 weeks later rechallenged with 10 MID_{50} of the same virus produced in peripheral blood mononuclear cells (PBMC) from a rhesus macaque. None of these animals proved to be protected against this challenge. In a parallel experiment in which the same numbers of monkeys were immunized in the same way, the animals were challenged intravenously with 10 MID_{50} of PBMC from an SIVmac (32H)-infected rhesus macaque. Two out of four SIV-MDP- and two out of four SIV-ISCOM-immunized monkeys proved to be protected from SIV infection.*

Keywords: SIV; ISCOM; protection

The SIV and HIV-2 macaque models have been used extensively for the evaluation of strategies for the development of candidate vaccines against HIV infection in humans[1,2]. Although protective immunity to SIV and HIV-2 infection has been induced by several experimental immunization strategies, including live attenuated and inactivated virus preparations[3-15], in only a few cases was it possible to show that the protection induced was the result of SIV-specific immunity. It has been shown recently that live attenuated SIV with a deletion in the *nef* gene induced protection against a high dose of SIV[3]. The only other successful immunization strategy was based on a combined approach of infection with recombinant vaccinia virus expressing the SIVmac *env* protein and a subsequent boost with purified recombinant *env* protein[7]. In all other experiments the protection induced with inactivated whole or subunit SIV preparations could be attributed to immunity directed to cellular proteins present in the vaccine preparations used[16-19].

It may be speculated that for the induction of protective immunity to lentivirus infections, both humoral and cell-mediated immune responses play a crucial role. In our attempts to develop candidate subunit vaccines against viruses, we have explored the potential of the ISCOM for antigen presentation. This mode of presentation has proven to be highly effective in inducing

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both biologically active antibodies, CD4+ and CD8+ T-cell responses and protection from many viruses including retroviruses[20]. It has been shown that ISCOMs containing HIV-1 gp160 were efficient in inducing cross-reactive virus neutralizing (VN) antibodies[21] and MHC class I-restricted CD8+ T-cell responses[22], both of which are considered important in the induction of HIV-1-specific protective immunity. In a recent experiment, HIV-2 ISCOMs, virtually devoid of viral glycoprotein, failed to induce protection to HIV-2 infection in monkeys[11]. In the present study we have made a SIVmac (32H) ISCOM preparation enriched for gp120 (SIV-ISCOM), and have compared its immunogenicity in rhesus macaques with an inactivated whole SIVmac (32H) preparation adjuvanted with muramyl dipeptide (SIV-MDP). Immunized monkeys were challenged with either cell-free or cell-associated homologous SIV preparations to assess the potential of both approaches for the development of candidate vaccines against lentivirus infections.

MATERIALS AND METHODS

Viruses

SIV for vaccine production. The 32H isolate of SIVmac strain 251 (pool 11/88) was propagated in the human T-cell line C8166. Virus was purified from culture supernatant by ultracentrifugation and gel-exclusion chromatography as previously described and provided for this vaccine trial by programme EVA[14,17]. For the preparation of gp120-enriched ISCOMs, SIVmac (32H) was propagated in C8166 cells (both obtained from MRC (ADP 151 and ADP 013 respectively)). SIV-infected cells were mixed with three times the number of uninfected C8166 cells every 3–4 days, and cultured in 2% fetal calf serum (FCS) in RPMI-GPSM for the production of cell-free virus and SIV-infected cells. Cell-free virus was concentrated from clarified culture supernatant of SIV-infected C8166 cells by a two-phase extraction system as previously described for HIV-1[23]. Briefly, clarified culture supernatant was mixed wih 0.3% (w/v) Dextran T500 (Pharmacia LKB, Uppsala, Sweden) and 8% (w/v) polyethlyene glycol 6000 (Merck, Darmstadt, Germany). After 3 h incubation at room temperature (RT), the mixture was centrifuged for 10 min at 1200 rev min^{-1}. The interphase and bottom phase containing 40- to 50-fold concentrated virus were collected and subsequently treated with 2% Triton X-100; the lysate was clarified by centrifugation (15 min at 2000 rev min^{-1}).

SIV challenge stocks. The titrated homologous cell-free challenge virus (SIVmac (32H) pool 11/88) propagated by short-term culture in C8166 cells was kindly provided by Dr M.P. Cranage and has been described previously[24,25]. An *in vitro* derived cell-associated challenge stock was obtained from an infected rhesus macaque (1XC), injected intravenously with 50 MID_{50} of the SIVmac (32H) stock 11 months postinfection. When the animal showed early-stage symptoms of terminal disease, a large stock of uncultured peripheral blood mononuclear cells (PBMC) was prepared and frozen in aliquots[26]. A dose of 20000 PBMC was found to be equivalent to 1 MID_{50} (H. Niphuis, personal communication). Cell-free rhesus PBMC-grown SIV challenge stock 5 was produced by propagating the SIVmac (32H) pool 11/88 stock in rhesus PBMC *in vitro* and has a titre of 5000 MID_{50} ml^{-1} *in vivo* (S. Norley, personal communication).

SIV-ISCOM preparations

Whole SIV ISCOM. Whole SIV ISCOMs were prepared from 20 mg viable, purified SIVmac (32H), provided by EVA, according to standard procedures previously described for HIV-1 and HIV-2[11,27,28]. Briefly, the proteins were solubilized with 4% β-octylglucoside (Sigma, St Louis, MO, USA) and mixed with 0.1% (w/v) Quil A (Iscotec AB, Luleå, Sweden) and subsequently dialysed. ISCOMs were pelleted through a 10% sucrose cushion and resuspended in phosphate-buffered saline (PBS) at a final concentration of 400 μg protein ml^{-1}.

Gp120-enriched ISCOMs. Gp120 was purified from the lysate of the concentrated SIVmac (32H) preparation by lentil-lectin affinity chromatography according to procedures recommended by the manufacturer (Pharmacia LKB, Uppsala, Sweden). Gp120 was also purified by lentil-lectin affinity chromatography from the solubilized SIV proteins that were not incorporated into the whole SIV ISCOM preparation. The unbound proteins of this protein preparation, a p27-enriched preparation, were stored for the final SIV-ISCOM vaccine formulation. Two methods were used to prepare gp120-enriched ISCOMs. The acidic method has previously been described for gp120 of HIV-1[27]. Briefly, the enriched gp120 preparation (150 μg protein ml^{-1}) in 3% β-octyl-glucoside was mixed with purified lipids, brought to pH 2.5 with 1 M glycine and thereafter mixed with 0.1% Quil A. After sonication and dialysis ISCOMs were pelleted through a 20% sucrose cushion on a 60% sucrose cushion. Gp120-enriched ISCOMs were also prepared by covalent coupling of the protein to a preformed ISCOM matrix, which was prepared with lipids (phosphatidlyethanolamine and cholesterol) and Quil A by dialysis as described previously[29]. The ISCOM matrix was activated by adding *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce, Rockford, USA) and after 30 min of incubation at RT, a reduction step with dithiothreitol (DTT; Sigma). Activated ISCOM matrix was further purified from free SPDP and DTT molecules by gel-filtration chromatography over a PD-10 column (Sephadex G25, Pharmacia LKB). The gp120-enriched protein preparation (1 mg ml^{-1}) was activated by adding SPDP in a molar ratio of 1:25. After incubation for 30 min at RT, free SPDP molecules were removed by gel-exclusion chromatography over a PD-10 column. Activated matrix and gp120 proteins were mixed and stirred for 18 h at RT. This preparation was subsequently layered over a linear (10–50%) sucrose gradient and centrifuged (18 h at 41000 rev min^{-1}, Beckmann SW41). The gradient was fractionated and fractions containing typical ISCOM structures as judged by electron microscopy were pooled[30].

Formulation and analysis of vaccine preparations

The inactivated whole SIV vaccine was prepared by 0.8% formaldehyde treatment of purified SIVmac (32H) in 250 μg aliquots by programme EVA[14]. Before use this preparation was mixed with an equal amount (w/w) of

muramyl dipeptide (MDP; Sigma) and diluted to 250 μg protein ml^{-1} in PBS. For consistency between different experiments carried out in the framework of the EC-concerted action, this formulation was chosen instead of the more frequently used MDP oil-and-water emulsion.

The SIV-ISCOM vaccine preparation consisted of a mixture of whole SIV ISCOMs, gp120-enriched ISCOMs and a p27-enriched protein preparation. Standardization of both SIV vaccine preparations was achieved by incorporating approximately the same amounts of gp120 and p27. The amount of p27 present in each ISCOM preparation was quantified by the Coulter SIV p27 antigen detection kit according to procedures recommended by the manufacturer (Coulter Corp., Hialeah, FL, USA). Quantification of gp120 was carried out by ELISA according to a procedure recommended by Dr B. McBride (PHLS-CAMR, personal communication). Briefly, wells coated with concanavalin A (Con A; Pharmacia LKB) were incubated with twofold dilutions of the vaccine preparations in 1% Triton X-100 in PBS for 2 h at RT. Wells were washed, blocked with 10% FCS and 1% BSA in PBS–Tween and subsequently incubated with a fixed amount of monoclonal antibody KK8 (kindly provided by K. Kent through MRC)[31]. Incubations with conjugates and substrate reaction were carried out as described for the inhibition ELISA. Recombinant gp140 (Repligen Corp., Cambridge, MA, USA) was used as the standard protein for the quantification of gp120. Quantitative analysis of the two SIV vaccine preparations showed that each dose of 250 μg SIV-MDP contained 2 μg gp120 and 10 μg p27, and each dose of SIV-ISCOM (120 μg of protein) contained 6–9 μg gp120, of which 90–95% was incorporated by the acidic method and 2–6% by the conjugation method, and 3 μg p27, of which 96% was derived from the p27-enriched protein preparation.

The SIV vaccine preparations were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 8–25% gradient gel and subsequently Western blot (WB) analysis using PhastSystem™ (Pharmacia LKB) according to procedures recommended by the manufacturer (Application files). For staining of p27, gp32 and gp120 proteins monoclonal antibodies KK33, KK20 and KK8 were used, respectively (*Figure 1*)[31]. It was shown that all these proteins were present in both the SIV-MDP and the SIV-ISCOM preparations. Although all virus preparations were extensively treated with detergent during ISCOM formation, the final SIV-ISCOM preparation was further treated with 1:4000 β-propiolactone (Aldrich, Bornem, Belgium) as described for inactivated rabies virus vaccine[32].

Two measles virus (MV) vaccine preparations served as controls: purified, β-propiolactone-inactivated whole MV (125 μg/dose) mixed with twice this amount (w/w) of MDP and diluted to 1 ml/dose, and a MV-ISCOM preparation (10 μg protein per dose). MV, used for the preparation of these vaccines, was cultured on Vero cells[30,33].

Immunization and challenge of rhesus monkeys (*Macaca mulatta*)

Twenty-four adult, outbred rhesus monkeys, bred in captivity at the TNO primate facility, were all shown to be free from SIV-specific antibodies, as shown in inhibition ELISAs and WB assay. The monkeys were housed under strict isolation conditions at TNO during the immunization–challenge experiment. All immunizations and bleedings were carried out under anaesthesia. At 0, 4 and 10 weeks eight monkeys were immunized intramuscularly with the SIV-MDP preparation, eight monkeys with the SIV-ISCOM preparation, four monkeys with MV-MDP and four monkeys with MV-ISCOM. At 16 weeks half the monkeys in each group were immunized again (group A monkeys) and at 22 weeks the other 12 monkeys were immunized (group B monkeys). Two weeks after the fourth immunization, 12 monkeys (group A1) were challenged intravenously with 10 MID_{50} homologous cell-free SIVmac (32H) challenge virus (2 ml of 10^{-4} dilution in 10% FCS in PBS). The other 12 monkeys (group B) were challenged intravenously with 10 MID_{50} SIV-infected PBMC (200 000 PBMC per 2 ml 10% FCS in RPMI). SIV-immunized monkeys of group A1, which remained SIV-negative for 43 weeks after their first challenge, were boosted with their respective SIV preparations and 2 weeks later re-challenged with cell-free SIV propagated in rhesus PBMC (group A2). Heparinized blood samples were collected at 2-, 3- or 4-weekly intervals during the course of the experiments. Clinical symptoms were recorded and bodyweights and body temperatures determined. Heparinized blood samples were used for haematology, serology and virus detection. PBMC were isolated by Ficoll gradient centrifugation and were used for detection of viraemia.

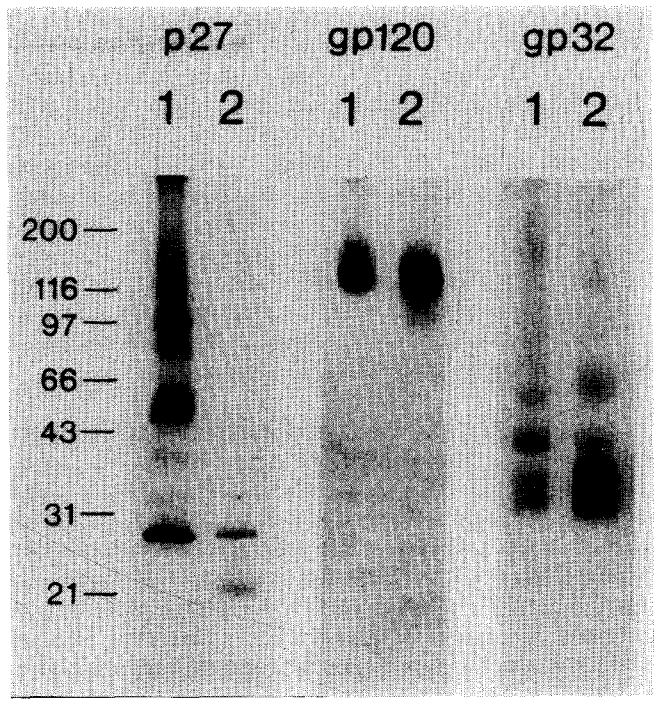

Figure 1 Characterization of the SIV-MDP (lane 1) and the SIV-ISCOM (lane 2) vaccine preparations by WB for the presence of p27, gp120 and gp32. M_r markers are indicated

Serology

WB analysis. SIV antigen was prepared by incubation of 1.7×10^7 SIV-infected C8166 cells ml^{-1} in 0.14 M NaCl, 10 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM DTT, 2 mM phenylmethylsulfonylfluoride and 0.5% Nonidet P-40, for 60 min at 0°C. WB strips were prepared by separation of clarified cell lysate by 12% SDS–PAGE and subsequent

transfer to nitrocellulose filters. Strips were blocked for 1 h at RT with 0.1% Tween-80 in PBS (PBS-T) and thereafter incubated with 1:50 dilution of monkey plasma or 1:500 dilution of monoclonal antibody in PBS-T with 0.1% BSA, 0.5% non-fat dry milk and 0.001% NaN_3 overnight at 4°C. After washing in PBS-T, strips were subsequently incubated with biotin-conjugated goat-anti-human immunoglobulin or goat-anti-murine immunoglobulin (Gibco-BRL, Gaithersburg, USA) for 1.5 h and alkaline phosphatase-conjugated extravidin (Sigma) for 1 h. After washing, staining was performed by addition of *p*-nitrotetrazolium blue and 5-bromo-4-chloro-3-inodolyl phosphate (BioRad, Richmond, USA).

Inhibition ELISA. Ninety-six-well high-binding micro-ELISA plates (Costar, Cambridge, USA) were coated with 100 μg ml^{-1} Con A and then incubated with 100 μl of 1:100 dilution of SIV-infected C8166 cell lysate, as described for SIV-WB. Wells were blocked with dilution buffer (10% FCS in PBS) and subsequently incubated for 1.5 h at 37°C with 50 μl of fourfold dilutions of monkey plasma in dilution buffer. Thereafter, 50 μl of gp120-specific or gp32-specific monoclonal antibodies (KK56, KK7 respectively) in the appropriate dilution was added to the 50 μl of monkey plasma dilution and subsequently incubated for 1 h at 37°C. The appropriate dilution of the monoclonal antibodies was calibrated in an indirect SIV-ELISA to give an absorbance at 450 nm of 30–50% of the maximum absorbance. Plates were incubated with biotin-conjugated goat-anti-murine immunoglobulin and horseradish peroxidase (HRPO)-conjugated streptavidin (Gibco-BRL) subsequently. The biotin anti-murine immunoglobulin conjugate did not crossreact with monkey immunoglobulin in this assay. The substrate reaction was carried out with 3,3',5,5'-tetramethylbenzidine as previously described[30]. Inhibition titres were defined as the dilution of monkey serum that inhibited 50% of the absorbance measured without inhibition. Antibodies with specificity for the cellular components present in the vaccine preparations, did not interfere with the binding of the monoclonal antibodies to gp120 or gp32 (data not shown).

SIV-neutralization assay. Ten μl of plasma dilutions were mixed in U-well microtitre plates (in quadruplicate) with 10 μl of a virus suspension containing 40 infectious SIVmac (32H) particles. The plates were briefly shaken and incubated for 30 min at 37°C in a humidified incubator. Next, 200 μl of a C8166 suspension (2000 cells) were added to each well. After 7 days incubation (37°C, 4% CO_2, 95% humidity) the individual wells were examined microscopically for the presence or absence of infected cells, the contents of each well were then transferred to poly-L-lysine-coated flat-bottomed micro-titre plates and fixed in methanol. The cells were then immunochemically stained for the presence of SIV antigen using IgG from an infected rhesus monkey directly coupled to peroxidase. Wells were again examined and only wells with no stained cells were scored as negative. The number of negative wells was then used to calculate the 'neutralization dose 50 per cent endpoint' (ND_{50}) for each sample by the method of Spearmann-Karber.

Cell-ELISA. Cell lysates of uninfected C8166 cells and of a pool of rhesus monkey PBMC, derived from four SIV-free animals, were prepared as described for the WB antigen. Wells were coated with Con A, washed, incubated with a 1:100 dilution of the cell lysate and blocked, as described for the inhibition ELISA. After incubation with serial dilutions of monkey plasma for 1.5 h at 37°C, wells were incubated with biotin-conjugated goat-anti-human IgG and HRPO-conjugated streptavidin. Substrate reaction was carried out as described for the inhibition ELISA.

Detection of SIV and SIV nucleic acid in monkeys

Virus isolation. Virus isolation was carried out by co-cultivation of 10^6 PBMC with 2.5×10^5 C8166 cells in 1 ml 10% FCS RPMI-GPSM in the presence of 50 μg phytohaemagglutinin ml^{-1}. Cells were incubated at 37°C for 3–4 weeks. Culture supernatants were refreshed twice a week and at regular time intervals tested for the presence of p27 protein by either Organon or Coulter p27 antigen detection kits. All positive results were confirmed by running the samples again in the same assay.

Polymerase chain reaction (PCR). PCR reaction was carried out essentially according to the method described by Kitchin *et al.*[34] with minor modifications, as described by Niphuis *et al.* (personal communication).

RESULTS

SIV-specific serum antibody responses after immunization

SIV-MDP-immunized monkeys. The monkeys of groups A and B immunized with SIV-MDP all developed serum antibodies which after the second immunization reacted predominantly to p27 in WB analysis, as is shown for two of these animals in *Figure 2*. With the gp120-inhibition ELISA, serum antibodies to gp120 could also be demonstrated after the second immunization, with titres ranging from 100 to 1600. These titres rapidly declined, but returned to approximately the same levels after the following two immunizations (*Figure 3a*

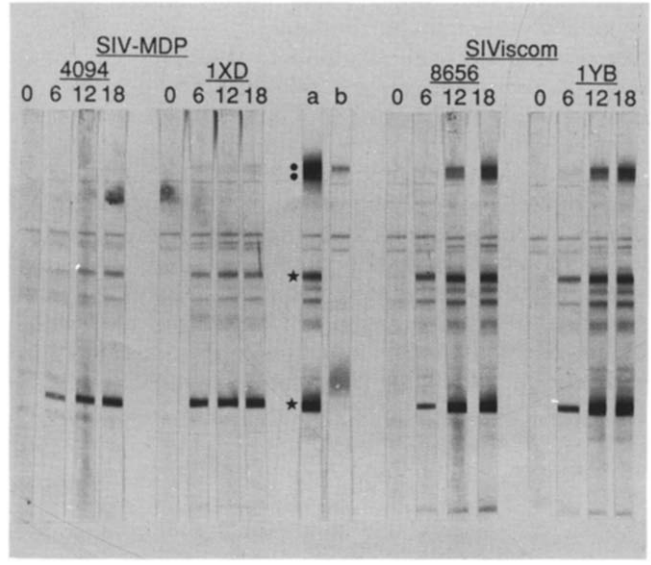

Figure 2 SIV WB analysis of the antibody development in SIV-MDP- and SIV-ISCOM-immunized monkeys at 0, 6, 12 and 18 weeks after the first immunization. Monoclonal antibodies directed against gp160/120 (bands indicated by ●), p55/p27 (bands indicated by *) (pooled in lane a) and gp160/32 (lane b) were used as controls

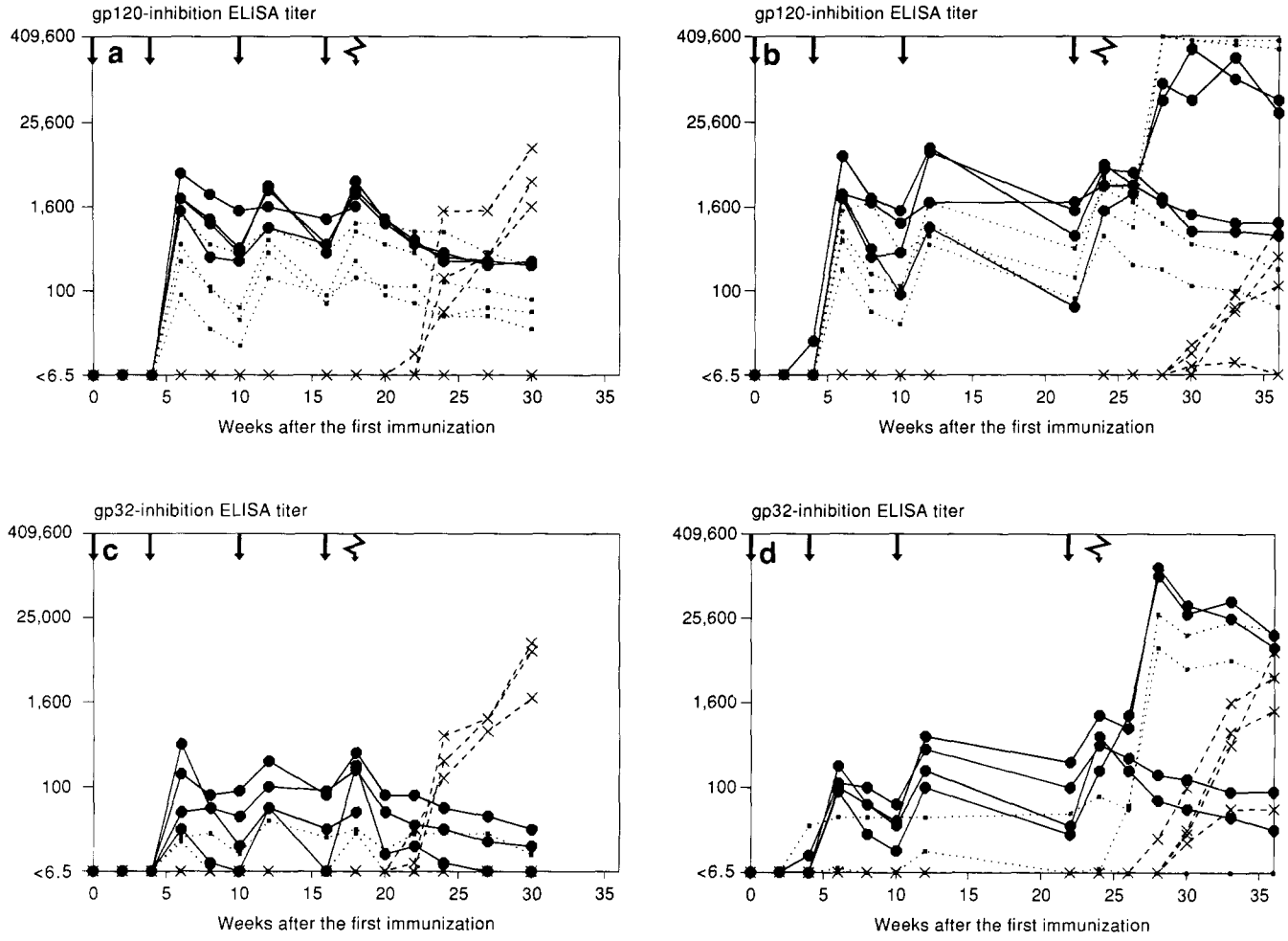

Figure 3 Antibody development in monkeys immunized with MV-MDP and MV-ISCOM (×), SIV-MDP (■) and SIV-ISCOM (●) after immunization and after challenge with cell-free virus (group A; (a) and (c)) or cell-associated virus (group B; (b) and (d)) as measured in gp120-inhibition ((a) and (b)) and gp32-inhibition ((c) and (d)) ELISA. Day of immunization and day of challenge are indicated by arrows

and *b*). Antibodies to gp32 as measured in the gp32-inhibition ELISA, could only be demonstrated in four of the eight immunized animals and the titres remained consistently lower than 100 (*Figure 3c* and *d*). SIV neutralizing serum antibodies were detectable in all the SIV-MDP-immunized monkeys at the day of challenge with titres ranging from 24 to 538 (*Table 1*).

SIV-ISCOM-immunized monkeys. The monkeys of groups A1 and B immunized with SIV-ISCOM all developed serum antibodies to p27 and gp120 detectable in WB analysis after the second immunization, as is shown for two of these animals in *Figure 2*. Serum antibodies to gp120 and gp32 were demonstrated in all these monkeys after the second immunization (*Figure 3a, b, c* and *d*). At week 6 gp120 antibody titres were significantly higher (mean of log titres ± s.d., 3.37 ± 0.25 in group A1 and 3.50 ± 0.28 in group B) than those found in the SIV-MDP-immunized animals (2.53 ± 0.48 in group A1 and 2.75 ± 0.35 in group B). At the day of challenge gp120 antibody titres were higher in group A1 in SIV-ISCOM-immunized monkeys (3.40 ± 0.15) than in SIV-MDP-immunized animals (2.55 ± 0.37). However, in group B only a minor difference between gp120 antibody titres in SIV-ISCOM- and SIV-MDP-immunized monkeys was seen (3.55 ± 0.30 and 3.25 ± 0.37,

Table 1 SIV-VN antibody titres in group A1 and B monkeys at the day of the first challenge and 6 weeks after challenge

	Group A1			Group B		
		Weeks postchallenge			Weeks postchallenge	
Immunized with	Monkey	0	6	Monkey	0	6
SIV-MDP	4083	80	<14	8645	538	48
	4094	20	<14	8649	190	20
	1XD	95	24	1IM	190	320
	3B	24	<14	KP	80	227
SIV-ISCOM	4053	453	80	4097	34	227
	8656[a]	NT	NT	8653	905	538
	1XV	95	<14	8668	269	57
	1YB	160	17	8730	453	40
MV-MDP	4060	<14	2560	1YH	<14	80
	1IH	<14	113	2CA	<14	<14
MV-ISCOM	8789	<14	<14	8672	<14	<14
	8791	<14	2153	8679	<14	33
SIV-infected (1XC)		2153				

NT, not tested
[a] Monkey died during recovery from anaesthesia on the day of challenge

respectively), which most probably can be explained by the different immunization schedules for groups A1 and B. SIV neutralizing serum antibodies were detectable in all the SIV-ISCOM-immunized animals at the day of challenge and the titres in group A1 monkeys, but not in group B monkeys, were generally higher than those found in the SIV-MDP-immunized monkeys (*Table 1*).

Upon immunization none of the MV-immunized monkeys developed SIV-specific serum antibodies detectable in any of the assays used.

SIV-specific serum antibody response after SIV challenge

SIV-MDP-immunized monkeys, challenged with cell-free SIV (group A1). WB analysis of the sera from the SIV-MDP-immunized monkeys challenged with cell-free SIV, did not show changes in the patterns observed after immunization (not shown). Serum antibody titres measured in the gp120- and gp32-inhibition ELISAs gradually declined after challenge (*Figure 3a* and *c*). Also the VN serum antibody titres to SIV had largely disappeared within 6 weeks after challenge (*Table 1*).

SIV-ISCOM-immunized monkeys challenged with cell-free SIV (group A1). As with the SIV-MDP-immunized monkeys, WB analysis of the sera from three SIV-ISCOM-immunized monkeys, challenged with cell-free SIV, did not show changes in the patterns observed after immunization (not shown). Serum antibody titres measured in the gp120 and gp32 ELISAs also declined gradually after challenge (*Figure 3a* and *c*) and the VN serum antibody titres of these monkeys to SIV also declined during 6 weeks after challenge (*Table 1*).

MV-MDP- and MV-ISCOM-immunized monkeys challenged with cell-free SIV (group A1). Three out of four of the MV-immunized monkeys (4060, 8791, 1IH) challenged with cell-free SIV developed p27-specific serum antibodies detectable in WB analysis within 4 weeks after challenge (data not shown). These three monkeys also developed gp120- and gp32-specific serum antibodies detectable in the inhibition ELISAs, within 4 weeks after challenge. The titres in the gp120-inhibition ELISA reached levels similar to those found in the SIV-ISCOM-immunized animals, whereas those found in the gp32-inhibition ELISA largely exceeded those of the SIV-MDP- and SIV-ISCOM-immunized monkeys (*Figure 3a* and *c*). In these three monkeys SIV neutralizing serum antibodies were also detected within 6 weeks after challenge, with titres ranging from 113 to 2560 (*Table 1*). The fourth monkey (8789), which had remained seronegative in all these assays, became viraemic and died soon after challenge (see below).

SIV-MDP-immunized monkeys challenged with cell-associated SIV (group B). WB analysis of the sera from two out of four (8645 and 8649) SIV-MDP-immunized monkeys challenged with cell-associated SIV, did not show changes in the patterns observed after immunization, whereas the serum samples of the two other monkeys in this group (1IM and KP) showed an increased staining of the gp120 protein after challenge (*Figure 4*). Serum antibody titres measured in the gp120- and gp32-inhibition ELISAs gradually declined after challenge in two monkeys (8645 and 8649) and strongly increased in the two other monkeys (1IM and KP) within 4 weeks after challenge, with titres ranging from 25 600 to 409 600 (*Figure 3b* and *d*). In contrast to this 256- to 512-fold increase in titre, VN serum antibody titres in these two monkeys showed a less than threefold increase (*Table 1*).

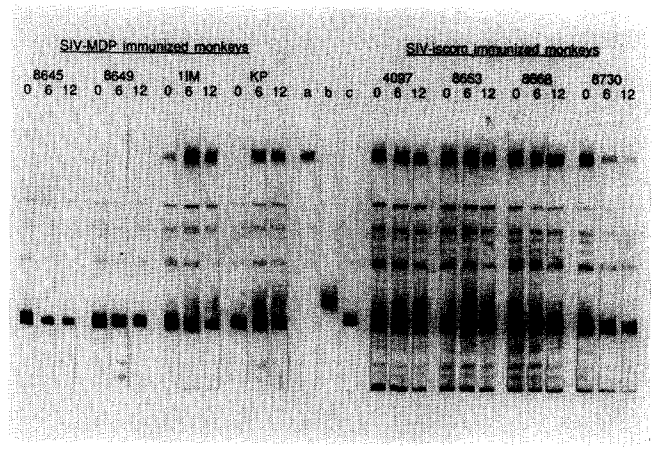

Figure 4 SIV WB analysis of the antibody development in SIV-MDP- and SIV-ISCOM-immunized monkeys at 0, 6 and 12 weeks after challenge with cell-associated virus (group B). Monoclonal antibodies directed against gp160/120 (lane a), gp 160/32 (lane b) and p55/27 (lane c) were used as controls

SIV-ISCOM-immunized monkeys challenged with cell-associated SIV (group B). Although in WB analysis of the sera from SIV-ISCOM-immunized monkeys, challenged with cell-associated SIV, no clear changes in the patterns observed after immunization could be demonstrated (*Figure 4*), serum antibody titres measured in the gp120- and gp32-inhibition ELISAs proved to decline gradually after challenge in two out of four monkeys (8668 and 8730) and strongly increased in the two other monkeys (4097 and 8653) within 4 weeks after challenge, with titres ranging from 25 600 to 409 600 (*Figure 3b* and *d*). In contrast to this 16- to 256-fold increase, VN serum antibody titres in one monkey (4097) showed a sevenfold increase and in another monkey (8653) a decrease during 6 weeks after challenge (*Table 1*).

MV-MDP- and MV-ISCOM-immunized monkeys challenged with cell-associated SIV (group B). All four of the MV-immunized monkeys challenged with cell-associated SIV developed p27-specific serum antibodies detectable in WB-analysis, within 4 weeks after challenge (data not shown). These monkeys also developed gp120- and gp32-specific serum antibodies detectable within 9 or 6 weeks in the respective inhibition ELISAs (*Figure 3*). Twelve weeks after challenge antibody titres measured in the gp120-inhibition ELISA were tenfold lower than antibody titres induced by SIV-ISCOM immunization. Six weeks after challenge low VN antibody titres were detected in two of the four monkeys (8679 and 1YH).

SIV isolation and PCR analysis after challenge

After intravenous challenge with cell-free SIV (group A1), viraemia was not demonstrated in any of the monkeys immunized with SIV-MDP or SIV-ISCOM as measured in SIV isolation and SIV-specific PCR (*Table 2*). In contrast, all four MV-immunized monkeys showed viraemia, since SIV was isolated from PBMC of all these

monkeys at 4, 9 and 12 weeks after challenge and all monkeys were positive in the SIV-specific PCR at 10, 12 and 41 weeks. One monkey (8656) died during recovery from anaesthesia on the day of challenge.

After intravenous challenge with cell-associated SIV (group B), no viraemia was demonstrated in two out of four SIV-MDP (8645 and 8649) and two out of four SIV-ISCOM (8668 and 8730) immunized monkeys, since SIV was not isolated from these monkeys and monkeys were negative in the PCR. After transfer of PBMC of these monkeys to naive monkeys no SIV infection could be detected in the recipients (*Table 2*). In contrast, the other SIV-immunized monkeys and all four MV-immunized monkeys showed viraemia: SIV was isolated from PBMC at 2, 4, 6, 9 and 12 weeks after challenge and all monkeys were positive in SIV-specific PCR at 8, 12 and 52 weeks.

Cell-specific antibody responses at day of first SIV challenge

Since it has been shown that non-virus-specific cellular antigens present in the SIV vaccine preparations may play a role in protection, antibodies directed to C8166 cell antigen or rhesus PBMC were measured in cell protein ELISA at the day of first challenge of monkeys in groups A1 and B. It was shown that all immunized monkeys developed cell-specific antibodies and that the protection found in group B did not correlate with the levels of plasma antibody titres to C8166 cells or rhesus PBMC in the SIV-immunized monkeys (*Figure 5*).

Outcome of rechallenge of protected monkeys of group A with cell-free SIV propagated in rhesus PBMC

To study whether the protection observed in the SIV-immunized monkeys of group A was conferred by SIV-specific immunity, all protected monkeys and two control animals were reimmunized with the respective SIV antigens and subsequently rechallenged with cell-free SIVmac (32H) propagated in rhesus PBMC (group A2). All animals developed SIV-specific antibody titres, as measured in inhibition ELISAs, approximately similar to those measured at the day of the first SIV challenge (*Figures 3* and *6*). Low SIV-VN antibody titres ranging from 17 to 227 were observed at the day of rechallenge. Two weeks after rechallenge all seven SIV-immunized monkeys showed a strong increase of antibody titres

Table 2 SIV detection by virus isolation (VI) from monkeys PBMC and PCR of PBMC and development of clinical disease in group A1 and B monkeys after challenge

Monkey: Group A1	Immunized with	VI[a] at weeks postchallenge					Transfer of PBMC[b]	PCR[a] at weeks postchallenge			Clinical disease[c]
		2	4	6	9	12		6	12	41	
4083	SIV-MDP	ND	–	ND	–	–	ND	–	–	–	–
4094		ND	–	ND	–	–	ND	–	–	–	–
1XD		ND	–	ND	–	–	ND	–	–	–	–
3B		ND	–	ND	–	–	ND	–	–	–	–
4053	SIV-ISCOM	ND	–	ND	–	–	ND	–	–	–	–
8656[d]											
1XV		ND	–	ND	–	–	ND	–	–	–	–
1YB		ND	–	ND	–	–	ND	–	–	–	–
4060	MV-MDP	ND	+	ND	+	+	ND	+	+	+	–
1IH		ND	+	ND	+	+	ND	+	+	+	AIDS (w79)
8789	MV-ISCOM	ND	+	ND	+	+	ND	+	+	+	AIDS (w26)
8791		ND	+	ND	+	+	ND	+	+	+	–

Group B								weeks postchallenge			at w85
								8	12	52	
8645	SIV-MDP	–	–	–	–	–	–	–	–	–	–
8649		–	–	–	–	–	–	–	–	–	–
1IM		+	+	+	+	+	+	+	+	+	AIDS (w35)
KP		+	+	+	+	+	ND	+	+	+	AIDS (w54)
4097	SIV-ISCOM	+	+	+	+	+	ND	+	+	+	–
8653		+	+	+	+	+	ND	+	+	+	–
8668		–	–	–	–	–	–	–	–	–	–
8730		–	–	–	–	–	–	–	–	–	–
1YH	MV-MDP	+	+	+	+	+	ND	+	+	+	AIDS (w85)
2CA		+	+	+	+	+	ND	+	+	+	–
8672	MV-ISCOM	+	+	+	+	+	ND	+	+	+	AIDS (w39)
8679		+	+	+	+	+	ND	+	+	+	CD4↓

ND, not done

[a]Presence or absence of SIV is indicated by + or – respectively

[b]PBMC transfer (taken from Ref. 35)

[c]Development of clinical symptoms during 91 (w91) and 85 weeks (w85) postchallenge infection in monkeys of group A1 and B, respectively, is indicated by: –, no clinical symptoms observed; AIDS: monkey died from AIDS at indicated week postchallenge; CD4↓: reduced number of circulating CD4+ T cells observed (taken from Ref. 35)

[d]Monkey died during recovery from anaesthesia at day of challenge

measured in gp32-inhibition ELISA and within 4 weeks in gp120-inhibition ELISA (*Figure 6*). SIV was isolated from all seven SIV-immunized and from the two MV-ISCOM-immunized control monkeys (3268 and 3980) (*Table 3*).

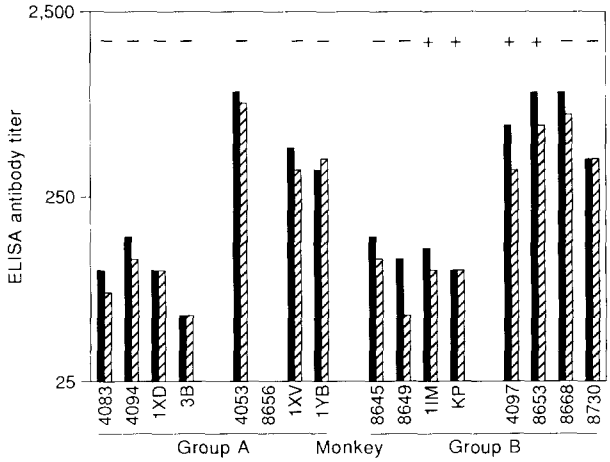

Figure 5 C8166 cell-specific (■) and rhesus PBMC-specific (▨) antibody titre of SIV-MDP- and SIV-ISCOM-immunized monkeys at the day of the first challenge. Protected (−) and unprotected (+) monkeys are indicated

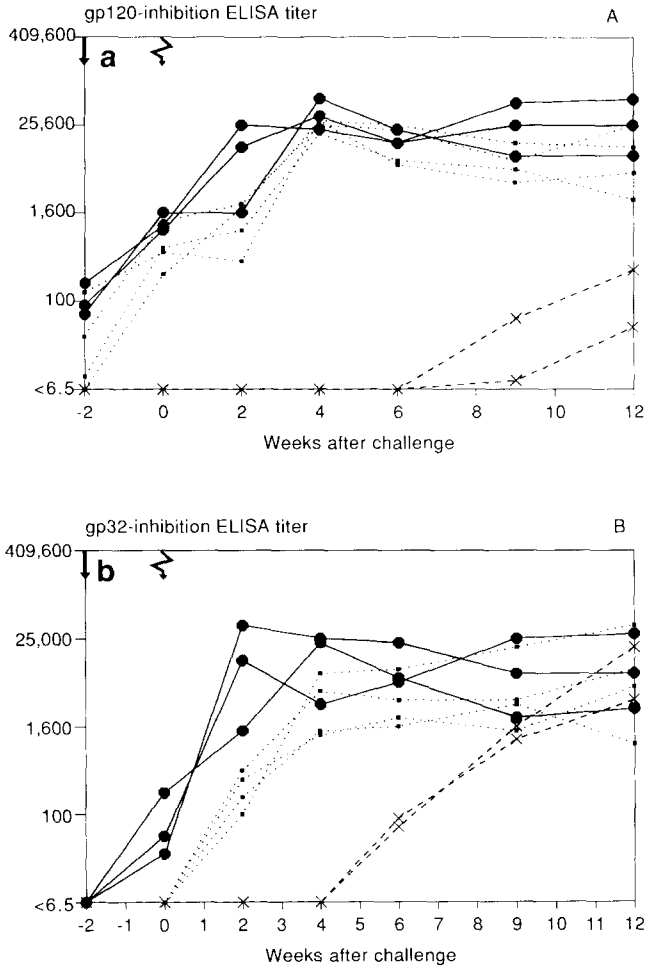

Figure 6 Antibody development in monkeys immunized with MV-ISCOM (×), SIV-MDP (■) and SIV-ISCOM (●) after boosting and after rechallenge with cell-free virus propagated in rhesus PBMC (group A2) as measured in (a) gp120-inhibition and (b) gp32-inhibition ELISAs. Day of immunization and day of challenge are indicated by arrows

Development of clinical disease in viraemic animals of group B

The two SIV-MDP-immunized monkeys (1IM and KP) that became viraemic after challenge with cell-associated SIV, developed AIDS and died at 35 and 54 weeks after infection respectively, whereas the two viraemic SIV-ISCOM-immunized monkeys (4097 and 6853) of this group did not develop clinical signs of AIDS up to 85 weeks after infection (*Table 2*). Two of the MV-immunized monkeys (8672 and 1YH) died at 39 and 85 weeks after challenge, respectively, and one monkey (8679) showed reduced numbers of circulating CD4+ T cells. Monkey 2CA of this group did not show any clinical signs up to 85 weeks after challenge.

DISCUSSION

In the present paper we have shown that immunization with different SIV vaccine preparations may induce protection against either cell-free or cell-associated SIV challenge. It could not be demonstrated that the protection observed in the monkeys of group A1, immunized with SIV-ISCOM or SIV-MDP and challenged with cell-free SIV, was indeed mediated by SIV-specific immunity. None of these monkeys proved to be protected from a rechallenge with SIV propagated in rhesus PBMC (*Figure 6*). This is in line with observations by several other groups, who have shown that protection against human-cell propagated SIV was, at least in part, due to immunity to cellular components[16,19]. From the data presented on the monkeys of group B, we conclude that the protection observed in four out of these eight monkeys, immunized with either SIV-ISCOM or SIV-MDP and challenged with cell-associated SIV, must have been elicited by SIV-specific immunity, since the challenge virus and the vaccine preparation had not been propagated in cells from the same species. Interestingly, our observations in group B also suggested that in contrast to SIV-MDP, SIV-ISCOM protected from disease development after challenge with cell-associated SIV.

To our knowledge this is the first demonstration of vaccine-induced protection against challenge with SIV-infected cells of the same host species. Similar

Table 3 SIV detection in rhesus monkeys after rechallenge with SIV-mac (32H) grown in rhesus PBMC (group A2) by virus isolation (VI) and PCR from monkey PBMC and development of clinical disease[a]

Monkey	Immunized with	VI at weeks postchallenge		PCR at weeks postchallenge	
		2	4	4	8
4083	SIV-MDP	+	+	+	−
4094		+	ND	−	+
1XD		+	+	+	+
3B		+	−	+	−
4053	SIV-ISCOM	+	+	+	+
1XV		+	+	+	+
1YB		+	+	+	+
3268	MV-ISCOM	+	+	+	+
3980		+	+	+	+

[a]See legend to *Table 2*

observations have been made in chimpanzees immunized with HIV-1 vaccine preparations and challenged with HIV-1-infected PBMC[36].

The mechanism of protection observed in group A1 monkeys can at least in part be explained by the presence of anti-C8166 immunity. This was indicated by demonstrating the presence of cell-specific antibodies in monkeys of group A. The lack of protection against cell-free SIV challenge propagated in rhesus PBMC, suggested that the levels of SIV-specific protective immunity induced by both vaccines were not sufficient to impart protection against this challenge. We cannot exclude that during the *in vitro* propagation of this challenge virus in rhesus PBMC, changes in virulence or sequence as compared with the original SIVmac-sequenced 32H pool 11/88 challenge stock may have occurred[24]. Both SIV vaccine preparations must have induced SIV-specific immunity, although not sufficient to protect against this cell-free challenge, since four out of the eight SIV-immunized monkeys proved to be protected against challenge with SIV-infected PBMC (group B). Neither cell-specific nor SIV-specific antibody titres, as measured by ELISA and SIV-VN assay, correlated with the protection found in these four monkeys (*Figures 5* and *6*; *Table 1*). Recently, we found that the protected monkeys share the MHC class I A26 allele with the PBMC donor monkey 1XC. We concluded that the mechanism of protection is most probably based on SIV-specific immunity, since sharing of the MHC class I A26 allele with the donor monkey alone, as is the case for two MV-immunized monkeys (8679 and 2CA), proved not to be sufficient for the induction of protection. Our present studies address the possible mechanisms by which this protection was induced. An attractive speculation would be the induction of SIV-specific MHC class I-restricted cytotoxic T cells, as we have recently suggested[35].

Although comparison of the results obtained with the SIV-MDP and the SIV-ISCOM preparations showed that the ISCOM preparation induced higher antibody titres, no difference between the SIV-MDP and the SIV-ISCOM preparation was observed with respect to the induction of protection to infection. However, the development of disease symptoms after infection seemed to be different in the two groups. The SIV-ISCOM-immunized animals of group B have not yet developed any clinical symptoms during the observation period, whereas most of the other animals of this group did develop evidence of disease progression.

It is known from several studies that the ISCOM, in contrast to other non-replicating antigen-presenting forms, is effective in inducing virus-specific CD8+ T cells, as has been described for HIV-1, measles virus and influenza virus[22,37]. We are currently investigating whether indeed CD8+ T-cell responses were induced upon immunization with the respective vaccine preparations.

The role of SIV-specific antibodies in protection remains unclear. Both SIV preparations induced low VN antibody titres compared with titres that could be measured in some of the SIV-infected monkeys (*Table 1*). This may have been caused by the presence of a denatured form of gp120 after formaldehyde or acid treatment in both vaccine preparations. Therefore, in future experiments recombinant SIV gp160 proteins incorporated into the ISCOM matrix under mild denaturing conditions, as described for HIV-1 gp160 proteins[21], will be tested for their capacity to induce SIV-VN antibodies and protection against challenge virus grown on rhesus PBMC. Further study of the mechanisms leading to the protection from infection and disease development observed in this animal model, may provide clues for the development of a vaccine against HIV.

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