Brief Definitive Report

Major Histocompatibility Complex Class I-associated Vaccine Protection from Simian Immunodeficiency Virus-Infected Peripheral Blood Cells

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Summary

To evaluate the effectiveness of vaccine protection from infected cells from another individual of the same species, vaccinated rhesus macaques (*Macaca mulatta*) were challenged with peripheral blood mononuclear cells from another animal diagnosed with acquired immune deficiency syndrome (AIDS). Half of the simian immunodeficiency virus (SIV)-vaccinated animals challenged were protected, whereas unprotected vaccinates progressed as rapidly to AIDS. Protection was unrelated to either total antibody titers to human cells, used in the production of the vaccine, to HLA antibodies or to virus neutralizing activity. However, analysis of the serotype of each animal revealed that all animals protected against cell-associated virus challenge were those which were SIV vaccinated and which shared a particular major histocompatibility complex (MHC) class I allele (Mamu-A26) with the donor of the infected cells. Cytotoxic T lymphocytes (CTL) specific for SIV envelope protein were detected in three of four protected animals vs. one of four unprotected animals, suggesting a possible role of MHC class I-restricted CTL in protection from infected blood cells. These findings have possible implications for the design of vaccines for intracellular pathogens such as human immunodeficiency virus (HIV).

To facilitate the design of effective vaccines for prevention of HIV infection and/or progression to AIDS, an understanding of the immunological mechanisms that elicit protection from infected cells as well as cell free virus are needed (1). Studies in animal models have been important in establishing efficacy of various vaccine preparations of HIV-1 in chimpanzees (*Pan troglodytes*), and of various vaccines prepared from HIV-2 and related SIV strains that can be tested in macaque species. Protection from infection with cell-free homologous virus challenge has been demonstrated in chimpanzees and a correlation between high virus neutralizing titers and protection from cell-free infection appears to be emerging (2–4). Passive immunization studies in both the chimpanzee and macaque models support the role of antibodies in protection from infection from cell-free challenge (5, 6). Homologous protection of chimpanzees from HIV-1 infected cells has been reported (7). However, the mechanism(s) of protection from infected cells in that study were not evident, nor, due to the natural resistance of chimpanzees to AIDS (8) is it possible to evaluate the virulence of the challenge or possible benefit of vaccination on protection from disease progression in that species.

In the SIV macaque model of AIDS, whole inactivated vaccines have been used to study mechanisms of protection, immunisation schedules, doses, and adjuvants in vaccine efficacy studies. Although problems of producing both the whole virus vaccine and the challenge virus on human cell lines while performing the studies in rhesus monkeys have become apparent (9–11), they can be circumvented by challenging with virus stocks propagated on macaque cells. However, to date, independent investigators have failed to achieve protection from SIV infection in macaques from virus preparations propagated on macaque cells.

In the design of vaccines to prevent lentivirus infection, it must be considered that HIV/SIV infection may occur by transmission of an intracellular as well as a cell-free virus (1). We set out to confirm our earlier preliminary observations (12) and to determine if solid long-term protection from primary uncultured cell-associated SIV infection and/or disease could be achieved. Furthermore, by comparing various im-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/08/0769/06 $2.00
Volume 180 August 1994 769–774

munological responses as well as the immunogenetic background of protected and unprotected animals we attempted to gain further insight into the possible mechanisms involved in vaccine protection from PBMCs from another SIV-infected macaque. We report new findings that vaccine protection from infected blood cells was related to sharing of a particular MHC class I allele between the SIV-infected donor and SIV-vaccinated animals protected from infection. These findings suggest the importance of evoking cell-mediated responses in the design of effective HIV vaccine strategies.

Materials and Methods

Immunization, Challenge, and Virological Follow-up of Animals. Twelve captive rhesus macaques (*M. mulatta*) derived from an outbred pedigreed MHC-typed colony were used for cell-associated challenge. Eight were immunized with two different whole inactivated SIV vaccine preparations and four controls were immunized with two different corresponding measle virus vaccines as described previously (12). Briefly, the SIV vaccines were prepared from whole SIV virions either inactivated with formalin and mixed with the adjuvant muramyl dipeptide (MDP), or inactivated with β propiolactone and incorporated into immune stimulating complexes (ISCOMs). All twelve animals were challenged intravenously with an in vivo titrated, uncultured stock of PBMC taken directly from a rhesus macaque infected with SIV_{mac32H} and diagnosed with AIDS at the time of euthanasia. Plasma (Pl.) Ag and virus isolation (VI) assays were performed as previously described (12). Blood transfusion to naive recipients was performed from animals that appeared to be protected and from an infected animal as a control.

PCR Assays. For the detection of SIV_{mac32H} provirus, PCR was performed on PBMC DNA at weeks 12 and 21 post challenge at a sensitivity of 1 copy in 1.0–1.5 $\times$ 10^5 cells. Lymph node and bone marrow biopsies were taken at $\sim$1 yr post challenge on all SIV vaccinated animals except for animal 1IM which died at 35 wk post challenge with AIDS (confirmed in all cases by necropsy).

Antibody Responses. Virus neutralization (VN) titers were determined in quadruplicate on plasma taken from the day of challenge and tested in a microtiter neutralization assay. The number of virus negative wells detected by immunoperoxidase staining for virus antigen was then used to calculate the neutralization dose 50% endpoint (ND_{50}) for each sample by the method of Spearmann-Karber. To determine if antibodies to human C8166 cells (used for propagation of SIV used for vaccination) were induced in immunized rhesus monkeys and correlated with vaccine protection, we mixed 10-fold dilutions of postimmunization serum from each monkey with either fresh or formalin-fixed C8166 cells or rhesus PBMCs from various monkey. Endpoints were determined by measuring fluorescence intensity by FACS® analysis (Becton Dickinson & Co., Mountain View, CA). Assays to detect antibodies to HLA class I proteins were performed as described (11). To determine if Mamu class I allele–specific antibodies were induced by SIV vaccination with human cell line (C1866)–propagated whole virus vaccine and correlated with protection, complement-dependent lysis assays were performed (13). Briefly, sera obtained from protected and unprotected animals receiving SIV vaccines were tested for their ability to lyse; (*a*) the human T cell line C8166 used for SIV vaccine production; (*b*) no. 1XC, the rhesus monkey B cell line derived from PBMC used for the vaccine challenge; (*c*) the rhesus monkey B cell line derived from animal no. A12, and; (*d*) SP/20, a mouse B cell line used for control. All prevaccination sera tested were negative, and only in some cases did postvaccination serum give positive results. Wells were scored on a scale from 1 to 6. Scores >5 were considered positive.

MHC Analysis and CTL Responses. Mamu-specific allosera were used for MHC typing of the outbred pedigreed *M. mulatta* in this colony (13). Within our rhesus colony, at least 13 Mamu-A alleles can be identified with the following frequencies: Mamu-A2 (0.036), -A11 (0.110), -A13 (0.074), -A14 (0.029), -A17 (0.068), -A18 (0.069), -A20 (0.015), -A24 (0.097), -A25 (0.031), -A26 (0.244), -A29 (0.059), -A31 (0.013), and -A32 (0.155). At least 13 Mamu-B alleles have been identified with the frequencies of Mamu-B1 (0.029), -B3 (0.030), -B5 (0.038), -B6 (0.195), -B9 (0.155), -B10 (0.146), -B19 (0.103), -B21 (0.004), -B22 (0.048), -B23 (0.073), -B27 (0.007), -B28 (0.045), and -B33 (0.029). The B null alleles have a frequency of 0.099. It should be noted that there is no correlation between the nomenclature of the various HLA and Mamu-A and -B alleles since the numbering of both systems is arbitrarily chosen. One-dimensional isoelectric focusing was used to compare MHC class I gene product isoelectric point differences with serotyping (our manuscript in preparation). MHC sequence analyses were performed as described (14). Gp120 directed cytotoxic T cell activity of SIV-challenged macaques was detected as reported (15) with the following modifications. Briefly, CTL activity against three pools of overlapping env peptides was measured prechallenge and at 4–11 wk post challenge. The percent gp120-specific release is shown as percent specific release on gp120-peptide pulsed targets less percent specific release on control medium pulsed targets at an E/T ratio of 30:1. The following peptide pools were used to sensitize target cells: gp120 EVA 774 1-19; gp120 EVA 774 20-25; and gp120 EVA 774 26-49. Peptides were 10 aa overlapping 20 mers based on consensus sequence of the $SIV_{mac251/32H}$ isolate. Peptides 5, 38, and 46 were not available. Effector cells used were cryopreserved macaque PBMC isolated by LSM (Organon Teknika, Oss, The Netherlands) density gradient centrifugation, prepared either by Con A activation (5 μg/ml; Sigma Chemical Co., St. Louis, MO) and IL-2 expansion for 6 d or by cocultivation for 9–13 d with autologous peptide-pulsed feeder cells. Briefly, 5–10 $\times$ 10^6 PBMC were placed in 1 ml of RPMI 1640 containing 10% FCS in 24-well plates together with 10–20 $\times$ 10^6 2,500 rad irradiated 10-d-old peptide-pulsed autologous Con A blasts. Autologous Con A blasts were prepulsed for 2 h with a pool of 46 overlapping gp120 peptides at a concentration of 12.5 μg/ml peptide. On day 3, rIL-2 was added to effector cell cultures at a final concentration of 20 U/ml. Cells were maintained for 6 d (Con A) or 9–13 d (peptide pulse) and placed over Ficoll before the assay. CTL assays were performed using autologous *Herpes papio* immortalized B lymphocyte cell lines or Con A blasts labeled with 0.1 mCi Na_2CrO_4 for 1 h, then pulsed with medium (unpulsed controls) or with pools of peptides at 25 μg/ml per peptide for 1 h followed by a 16-h peptide incubation period at 2.5 μg/ml per peptide. Subsequently, target cells were washed two times and plated at 10^4 cells/well in 96-well U-shaped plates at various effector/target ratios. After 5 h at 37°C supernatants were harvested and counted in a gamma counter. Percentages of specific ^{51}Cr release were calculated as 100$\times$ (experimental release – spontaneous release):(maximum release – spontaneous release). All experimental values were calculated in duplicate while maximum and spontaneous releases were performed in quadruplicate. Responses of 10% or more above specific lysis of control targets, were scored as positive.

Results and Discussion

After challenge, all control animals vaccinated with measles virus became plasma antigen positive at 2 wk post challenge

Table 1. *Vaccine type, VN Titers, Clinical and Virological Status*

Vaccine	Macaque	VN titers[§]	Pl Ag *wk* +	Clinical status	VI PBMC	PCR PBMC	PCR LN	PCR BM	Transfusion recipients* Ab	Transfusion recipients* VI	Transfusion recipients* PCR	Transfusion recipients* CD4
SIV-iscom												
	8653	905	–	Diarrhea	+	+	+	+				
	4097	34	–	AIDS (122w†)	+	+	+	+				
	8668	269	–	Protected	–	–	–	–	–	–	–	–
	8730	453	–	Protected	–	–	–	–	–	–	–	–
SIV-mdp												
	8645	538	–	Protected	–	–	–	–	–	–	–	–
	8649	190	–	Protected	–	–	–	–	–	–	–	–
	1IM	190	–	AIDS (35w†)	+	+			+	+	+	⇓
	KP	80	–	AIDS (54w†)	+	+	+	+				
Controls												
MV-iscom												
	8672	–	2⇒	AIDS (39w†)	+	+						
	8679	–	2, –, 27⇒	AIDS (101w†)	+	+						
MV-mdp												
	2CA	–	2	Asymptomatic	+	+						
	1YH	–	2	AIDS (80w†)	+	+						

Plasma antigen (Pl Ag) is shown as the week post challenge in which animals had detectable virus antigen in plasma and persistent levels thereafter (indicated as ⇒). VN titers on the day of challenge are shown. VI results on PBMCs at week 6 post challenge are shown, and tests were performed at routine intervals post challenge. All animals positive at week 6 were consistently positive thereafter except for no. 2CA who has become periodically virus isolation negative but remains PCR positive. The absence of infection of protected animals was confirmed by blood transfusion to naive recipients* who remained negative by all criteria, in contrast to the transfusion recipient from one vaccinated but unprotected animal. PCR results from naive recipients 14 wk after blood transfusion from protected vaccinated donors and 1IM, an unprotected animal, are shown. Clinical status is described as protected (uninfected), asymptomatic (infected), and (w†) = week of death post challenge.

and SIV could be isolated from PBMC at 6 wk and time points thereafter (Table 1). Of the eight SIV vaccinates challenged intravenously with SIV-infected rhesus macaque PBMC, animals nos. 8668, 8730, 8645, and 8649 remained negative by all criteria (Table 1). SIV vaccinates nos. 8653, 4097, 1IM, and KP, remained plasma antigen negative, possibly due to vaccine-induced anti-SIV antibodies, but after 6 wk, SIV could be isolated and at 12 wk provirus could be detected in PBMCs. Three of four of these animals progressed to AIDS and died. One of these vaccinates progressed faster to AIDS and died before any of the controls (Table 1). As seen in Table 1, the absence of virus in protected animals was confirmed by blood transfusion to naive recipients who remained negative by all criteria, in contrast to the transfusion recipient from one vaccinated but unprotected animal (Table 1). PCR on lymph node and bone marrow biopsies from protected animals 1 yr post challenge failed to demonstrate evidence of virus infection.

To investigate the mechanism of protection observed, we first analyzed humoral immune responses to both SIV and to human cells. Uninfected C8166 cells have been reported to elicit protection from challenge with human cell grown SIV in a group of immunized cynomolgus macaques. In that study, in contrast to neutralizing antibodies, anticell antibodies were found to correlate with protection (9). As seen in Table 1, no association was found between virus neutralizing activity and protection. Moreover, we were unable to find a correlation between protection and the level of total antibodies to the vaccine substrate (C8166) measured either by ELISA or by fluorescent flow cytometry (data not shown). It has been described that whole inactivated C8166 cell-propagated SIV vaccines induce antibodies that react with HLA class I molecules (17) and in one report these antibodies were found to correlate with vaccine protection from cell-free challenge (16). The presence of MHC proteins bound to lentiviruses propagated on human cell lines has been described (10, 17–19), and antibodies to MHC are reported to inhibit virus infection in vitro (10). The mechanism of protection when both the vaccine and challenge virus are produced on the same xenogenic (human) cell line appear to be due to immune responses to foreign cell components carried by the virus after budding from foreign cells (10, 11). Al-

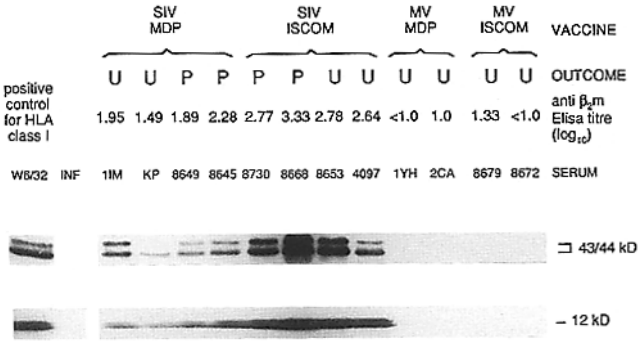

Figure 1. Antibodies to HLA class I proteins with sera from vaccinated macaques. Vaccines were as follows: SIV-ISCOM; formalin-inactivated SIV-MDP (muramyl dipeptide adjuvant); measles virus (MV)-ISCOM; MV-MDP; and from an $SIV_{mac32II}$ infected, unvaccinated macaque (INF). The positive control consisted of a human class I dimer–specific mAb W6/32. (*U*, unprotected, *P*, protected). Titers to β_2-microglobulin are shown above each lane.

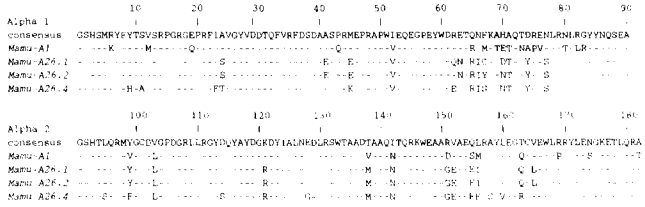

Figure 2. Deduced amino acid sequences of the α 1 and 2 domains of rhesus macaque MHC class I A locus molecules. Identity to the consensus displayed on top is indicated by a dash. Amino acids are depicted by their one letter code. The Mamu-A26.1 and -A26.2 are isolated from A26 positive animals from Indian origin, whereas the Mamu-A26.4 allele was isolated from animal 4097, which has an A26 positive serotyping but is from Burma. Sequence analyses have been performed as described (14).

though this problem was circumvented by using an in vivo-derived rhesus monkey PBMC challenge, we wished to rule out that antibodies to these foreign cell components including foreign HLA and other cellular antigens found on human cells, were not mediating protection as reported in experiments in which the challenge virus was propagated on human cells (9, 11, 16).

To determine if the cell-associated vaccine protection was related to anti-MHC class I antibodies, we performed immunoprecipitation analysis for MHC proteins using lysed C8166 cells with sera from protected and unprotected macaques taken on the day of challenge (Fig. 1). Additionally, antibody titers to β_2-microglobulin were determined by ELISA. As can be seen, no correlation with protection from infected cells with β_2-microglobulin antibody titers or the ability of sera to immunoprecipitate HLA class I proteins in general was found. Subsequently, we considered whether vaccination with SIV whole virus vaccines propagated on the C8166 human cell line had induced an allospecific humoral response capable of recognizing the 1XC cells used for challenge. Although vaccination induced allospecific antibody responses developed in some of the macaques, the presence of such antibodies did not correlate with protection from challenge with PBMC from macaque no. 1XC (data not shown).

We next asked whether MHC-restricted cellular immunity played a role in vaccine protection. The MHC class I and II types of the challenge donor and of the vaccinated recipients were determined and compared. The MHC system of the rhesus macaque has been designated *MhcMamu*, and by using alloantisera, a high number of Mamu-A, -B, and

Table 2. *MHC Serotype and gp120-Directed cytotoxic T Cell Activity of SIV-vaccinated Macaques between 4 and 11 wk Post Challenge*

Macaque	No.	Vaccine	MhcMamu- A	MhcMamu- B	MhcMamu- DR	SIV gp120-directed CTL responses env peptides		
Challenge donor	1XC		**26**, 14	10, 10	3, 3			
						1–19	*20–25*	*26–49*
Protected	8668	SIV-iscom	**26**, 11	10, 1	3, 1	9	8	22
	8730	SIV-iscom	**26**, 25	19, 6	1, 3	0	0	0*
	8645	SIV-mdp	**26**, 18	23, 1	5, 4	11	31	7
	8649	SIV-mdp	**26**, **26**	19, 6	8, 1	9	42	34
Unprotected	8653	SIV-iscom	13, 14	6, 23	5, 3	0	0	0
	4097	SIV-iscom	35, 26‡	10, –	8, 101	0	0	0*
	KP	SIV-mdp	24, 35	10, 19	3, 4	3	0	51*
	1IM	SIV-mdp	24, 24	6, 23	3, 2	0	0	7

* Con A blasts; underlined responses were those 10% or more above specific lysis of control targets and scored as positive.
‡ Macaque no. 4097 was the only Mamu-A26 positive animal originating from another geographically isolated population possessing a Mamu-A26 subtype.

-DR alleles can be detected. Analysis of protected vs. non-protected animals revealed that a MHC class I allele, Mamu-A26, was found to be shared between all protected monkeys and monkey no. 1XC, the SIV-infected challenge donor of the PBMC (Table 2). This suggested that vaccination and sharing of MHC class I alleles could be related to the mechanism of protection. However, one of the four SIV-vaccinated animals that was infected after cell-associated challenge (no. 4097) also shared the Mamu-A26 serotype. Interestingly, animal no. 4097 was the only macaque in this study not originating from India and thus may have a different Mamu-A26 subtype. This would be consistent with the finding that geographically separated populations of humans also may possess different types of MHC class I alleles, due to rapid evolution (20). Sequencing studies indeed showed that Indian and Burmese macaques possess related but different Mamu-A26 alleles that are recognized by the same typing sera (Fig. 2). The correlation between SIV vaccine protection and the Indian Mamu-A26 allele was found to be highly significant ($p < 0.005$, Fisher's exact test). The two measles-vaccinated monkeys, which also expressed the Mamu-A26 allele, were not protected from SIV infection. In addition, we followed these animals for over 2 yr post challenge and studied survival time of SIV-vaccinated animals as compared with measles-vaccinated controls. SIV vaccination did not appear to prolong survival after animals became infected. Of the four SIV vaccinees that became infected after cell-associated challenge, three have developed AIDS, one of which was the Mamu-A26 serotype no. 4097 (the three with AIDS have died). Similarly, three of the four measles-vaccinated controls have developed AIDS and died (Table 1). Hence, there appears to be no beneficial effect of SIV vaccination in preventing progression to AIDS if SIV infection occurs, nor any advantage of having Mamu-A26 serotype in prolonging survival after infection (Tables 1 and 2). However, SIV vaccination and sharing of a particular Mamu-A26 allele (Fig. 2) was an advantage in preventing infection from cell-associated challenge from an infected animal having this same allele.

SIV infected or vaccinated rhesus macaques may develop MHC class I–restricted CTLs (15). Interestingly, gp120-directed responses were only demonstrated in protected animals after and not before challenge (Table 2). Apparently, vaccination-induced SIV-specific CTL precursor levels were boosted either by antigen presenting 1XC cells, or by undetectable limited virus replication. Consequently, it may be speculated that the mechanism of protection observed in animals nos. 8668, 8645, and 8649 (Tables 1 and 2), depends on an MHC-restricted cell-mediated defence to eliminate infectious 1XC cells. The evidence for the role of MHC class I–restricted CTL in the observed vaccine protection from infected cells is suggested from this study. However, no unique subregion of gp120 was identified as target for CTL (Table 2). It is not unlikely therefore, that, besides Mamu-A26, other alleles were involved in the presentation of viral peptides on autologous infected cells. Alternatively, the Mamu-A26 allotype may have possibly played a role through other MHC-mediated mechanisms such as epitope selection (21). As a control experiment to determine if the Mamu-A26 allotype also played a role in protection from cell-free challenge, macaques immunized with the same SIV vaccines and protected from challenge with human cell grown SIV (12), were rechallenged with monkey cell grown cell-free $SIV_{mac251/32H}$ after revaccination. All of these monkeys became infected, three of which were Mamu-A26 positive (data not shown). Hence, it appears that the mechanism is not Mamu-A26 linked resistance to infection in general, but that it is a specific mechanism that involves protection from infected cells sharing this MHC allele.

We are grateful to Drs. N. Letvin, M. Murphy-Corb, and A. Dalgleish for their constructive comments. We would like to thank Corrie Wimmers, H. Wiersema, Dr. P. van Eerd, and members of the biotechnical department at TNO for their assistance and kind care of animals.

The Laboratory of Viral Pathogenesis is sponsored as a European Centralised Facility for AIDS Research (BMHI-CT92-1203). Materials for this study were generously supplied by Programme EVA. This study was performed in concert with the European Community Concerted Action on macaque models for AIDS.

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Received for publication 22 December 1993 and in revised form 12 May 1994.

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