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Priming-Boosting Vaccination with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin and a Nonreplicating Vaccinia Virus Recombinant Leads to Long-Lasting and Effective Immunity

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Virus-specific T-cell responses can limit immunodeficiency virus type 1 (HIV-1) transmission and prevent disease progression and so could serve as the basis for an affordable, safe, and effective vaccine in humans. To assess their potential for a vaccine, we used *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-Tokyo and a replication-deficient vaccinia virus strain (DIs) as vectors to express full-length *gag* from simian immunodeficiency viruses (SIVs) (rBCG-SIVgag and rDIsSIVgag). Cynomolgus macaques were vaccinated with either rBCG-SIVgag dermally as a single modality or in combination with rDIsSIVgag intravenously. When cynomologus macaques were primed with rBCG-SIVgag and then boosted with rDIsSIVgag, high levels of gamma interferon (IFN-γ) spot-forming cells specific for SIV Gag were induced. This combination regimen elicited effective protective immunity against mucosal challenge with pathogenic simian-human immunodeficiency virus for the 1 year the macaques were under observation. Antigen-specific intracellular IFN-γ activity was similarly induced in each of the macaques with the priming-boosting regimen. Other groups receiving the opposite combination or the single-modality vaccines were not effectively protected. These results suggest that a recombinant *M. bovis* BCG-based vector may have potential as an HIV/AIDS vaccine when administered in combination with a replication-deficient vaccinia virus DIs vector in a priming-boosting strategy.

As the rate of new infections with human immunodeficiency virus type 1 (HIV-1) continues to increase globally, an effective preventive vaccine is urgently needed to stem further spread of the virus (24). Because long-term survival in humans has been observed when HIV-1 replication is controlled by protective immunity (12, 29), targeted experimental immunogens have been designed to closely mimic the long-lasting protective immunity induced in long-term human survivors by the natural infection (8, 25). Recently, various vaccine modalities, including live viral vectors and DNA, have been used to elicit protective immunity in nonhuman primate models (9). However, before an HIV-1 vaccine regimen can be considered promising, it must be shown to be not only effective at inducing protective immunity, but also safe, affordable, and compatible with other vaccines (2, 32).

When it comes to safety, traditional live vaccines, which have been administered safely to both the healthy and the infected, may be the vectors of choice for HIV-1 vaccines. In order to fully take advantage of the potential benefits of traditional live vectors in HIV-1 vaccine development, we studied the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrain Tokyo 172 (6) and the replication-deficient vaccinia virus vaccine strain DIs (22, 50), both of which have been shown to be nonpathogenic when inoculated into immunodeficient animals (41, 51, 53) as live recombinant vaccine vehicles (1, 17–19, 46–48). As further evidence of the potential of the live vectors for use in HIV/AIDS vaccines, we noted that a recombinant *M. bovis* BCG vector candidate vaccine for HIV-1-induced positive immune responses in animals (17, 46). Moreover, we found that recombinant vaccinia virus DIs encoding the simian immunodeficiency virus (SIV) gene was effective at eliciting anti-SIV immunity in mice when administered as a booster antigen after priming with SIV DNA (47). In this study, we have developed a new combination regimen, priming with recombinant *M. bovis* BCG-SIV Gag followed by boosting with rDIsSIVgag. This immunization regimen elicited effective positive immunity against an immune deficiency virus in macaques for the 1 year they were under study.

MATERIALS AND METHODS

Animals and virus challenge stocks. All animals used in this study were captive bred and obtained from the Philippines. They were mature, cycling, male cynomologus macaques (*Macaca fascicularis*) from the Tsukuba Primate Center, the National Institute of Infectious Diseases, Japan. Animals used in these studies were free of known simian retroviruses, herpes viruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimenta-

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tion of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals (46) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization. The animals' condition was monitored by analyzing a hemogram parameter as well as absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts with an automated blood analyzer Celltac (Nihon Koden, Tokyo Japan), as described below.

Two thousand 50% tissue culture infectious doses ($TCID_{50}$) of SHIV KS661c, a pathogenic molecular clone, were intrarectally administered as a challenge virus (39). The parent virus, SHIV-C2/1, is an SHIV-89.6 variant isolated by in vivo passage in cynomolgus macaques (40, 42) and the original SHIV-89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (26, 37). SHIV-C2/1 and SHIV KS661c were shown to infect cynomologus macaques by both the intravenous and intrarectal routes (39). Both viruses induced high levels of viremia and marked $CD4^+$ T-cell depletion within 2 and 3 weeks after inoculation, respectively (39, 40, 42). Virus stocks were stored at −125°C and thawed just prior to use.

Production and preparation of recombinant *M. bovis* BCG (rBCG) and vaccinia virus DIs expressing full-length SIV Gag. Detailed methods for plasmid construction were described previously (7, 17, 18, 21). Briefly, a DNA fragment encoding the full-length *gag* sequence of SIVmac239 was cloned downstream of the *hsp60* promoter (52) and then inserted into the multicloning site of the plasmid pSO246 (28). Recombinant *Mycobacterium bovis* BCG substrain Tokyo 172 that stably expressed the inserted DNA fragment (designated rBCG-SIVgag) was then selected and used for all rBCG inoculations. For the Western blot analysis, the transformant of rBCG was grown in 7H9-ADC broth for 2 weeks and a portion of the culture medium was periodically collected, sonicated and blotted using the monoclonal antibody IB6, as described previously (47). Since the recombinant DIs virus (rDIs) encoding the SIVmac239 *gag-pol* open reading frame elicited remarkably high SIV Gag-specific T-cell responses but low polymerase responses in mice (47), confirming the findings of a previous report (20), we named it rDIsSIVgag. The rDIsSIVgag and rDIs encoding β-galactosidase (rDIsLacZ) were prepared with chicken embryo fibroblast (CEF) cells (18, 47). Virus preparations were purified by sucrose density gradient ultracentrifugation and were adjusted to 10^7 PFU/ml. P27 antigen generation in cells was measured by antigen-specific enzyme-linked immunosorbent assay (42).

Virus-specific IFN-γ ELISPOT assays. ELISPOT assays were performed using the method developed by and following the direct instructions of Mothe and Watkins, Wisconsin University Primate Center (31, 46). In brief, 96-well flat-bottomed plates (U-CyTech-BV, Utrecht, Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody MD-1 (U-CyTech-BV). Freshly isolated peripheral blood mononuclear cells (PBMC) were added with either concanavalin A or pooled Gag peptides (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD). The cells were then incubated in anti-IFN-γ-coated plates before lysing with ice-cold deionized water. After plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibody (1 μg per well; U-CyTech-BV) was added. The plates were reacted with gold-labeled antibiotin immunoglobulin G solution by adding 30 μl of the activator mix (U-CyTech-BV) to each of the wells and allowing them to develop for 15 min.

Wells were imaged and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Germany) (31, 46). An SFC was defined as a large black spot with a fuzzy border. To determine significance levels, we established a baseline for each peptide using the average and standard deviation of the number of SFC for each peptide. A threshold significance value corresponding to this average and two standard deviations were then determined. A response was considered positive if the number of SFC exceeded the threshold significance level of the sample with no added peptide.

Detection of intracellular IFN-γ by flow cytometry. Intracellular macaque IFN-γ was detected by intracellular IFN-γ cytokine staining as previously described (30). Briefly, freshly isolated PBMC was incubated with antigen for 16 h at 37°C with 5% CO_2. During the final 6 to 8 h, brefeldin A (Sigma Chemical Co., St. Louis, MO) was added at 10 μg/ml. Antibody to CD28 (1 μg/ml, BD Pharmingen, San Diego, CA) was also added during the incubation as a costimulator molecule. After stimulation, the cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (FN18; Biosource, Camarillo, CA) and peridinin chlorophyll protein-conjugated anti-CD8 antibodies (Leu-2a; Becton Dickinson Biosciences, San Jose, CA). The cells were then sequentially incubated with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) for 10 min and FACS permeabilizing solution (Becton Dickinson) for another 10 min. The cells were washed, stained with phycoerythrin-conjugated anti-human IFN-γ antibody (4S.B3; BD Pharmingen), and fixed with 2% paraformaldehyde. Samples were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Lymphocyte proliferative responses. SIV-specific proliferative responses were measured in freshly isolated PBMC as described by Gauduin et al. and Hel et al. (14, 15). PBMC were cultured in flat-bottomed 96-well plates with either concanavalin A or purified SIVmac251 p27 protein (Advanced BioScience Laboratories, Rockville, MD) (15) for three days before the addition of [^{3}H]thymidine. Cells were harvested 16 h later to determine uptake.

Absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts. An absolute cell count of peripheral blood was measured as previously described (55). Briefly, 50 μl of whole blood was placed in a polypropylene tube and incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource), phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton Dickinson) antibodies at 4°C. After incubation with FACS lysing solution (Becton Dickinson), the cells were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Plasma viral RNA copy numbers. Plasma viral RNA copy numbers were measured using a real-time quantification assay based on the TaqMan system (Applied Biosystems, Foster City, CA) and the Prism 7700 sequence detection system (Applied Biosystems), as reported previously (30, 46). Briefly, viral RNA was extracted and purified from macaque plasma samples using a QIAamp viral RNA mini kit (QIAGEN, Valencia, CA). The RNA was subjected to reverse-transcription and amplification using a TaqMan EZ RT-PCR Kit (Applied Biosystems) with SIV Gag consensus primers SIVmac239-1224F and SIVmac239-1326R, and the SIV Gag consensus Taqman probe FAM-SIV-1272T. To obtain control RNA for quantification, SIVmac239 *gag* RNA was synthesized using T7 RNA polymerase and pKS460, a template plasmid that contains SIVmac239 *gag* under control of the T7 promoter.

To measure the RNA recovery rate, 10^5 copies of SHIV KS661c, in which the viral RNA copy number was previously determined by branched DNA assay (Bayer), were extracted and purified using the same kit as for the sample. Plasma viral load was calculated based on the standard curve of control RNA and the RNA recovery rate. All assays were carried out in duplicate.

Statistical analysis. Data analysis was carried out using the Stat View program (SAS Institute, Cary, NC) and data are expressed as the mean ± standard deviation. A P value of <0.05 was considered significant.

RESULTS

Construction and preparation of recombinant *M. bovis* BCG Tokyo 172 and vaccinia virus DIs expressing whole SIV Gag. In initial studies, we cloned DNA encoding SIVmac239 *gag* downstream of the *hsp60* promoter and the expression unit was inserted into the KpnI restriction site of plasmid pSO246. We also constructed a recombinant *M. bovis* BCG vaccine based on the Tokyo 172 strain expressing the full-length *gag* gene of SIVmac239 (rBCG-SIVgag) (Fig. 1A). The presence of SIV Gag-specific DNA was confirmed in recombinant bacteria by DNA-PCR (42). To determine the in vitro expression of the SIV Gag protein in the cells, we analyzed cell extracts of rBCG-SIVgag bacteria after 2 weeks of culture by Western blot using anti-SIV Gag monoclonal antibody IB6. The rBCG clone produced an SIV Gag recombinant protein that strongly reacted as a single band with the specific monoclonal antibody (Fig. 1B). The concentration of SIV Gag^{p27} protein in transformed bacteria was 28.56 ± 8.30 ng/10^8 CFU of bacilli. In contrast, neither SIV Gag protein nor *gag* DNA was detected in bacteria transformed with rBCG-pSO246, a control construct lacking the SIV *gag* insert and used as a vector control (Fig. 1B).

rDIsSIV*gag* and a control vaccinia virus, rDIsLacZ, were propagated in CEF and adjusted to 10^7 PFU/ml. Using Western blot, we confirmed the expression of each foreign gene in

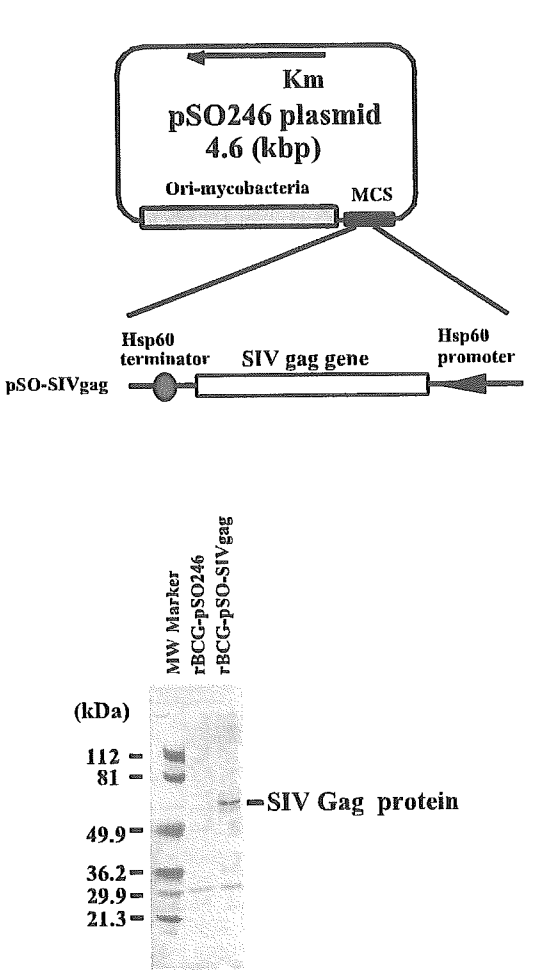

FIG. 1. Vector construction and expression of rBCG-SIVgag. (A) Construction of the expression vector pSO-SIVgag. Full-length DNA of SIVmac239 *gag* was inserted into the multicloning site of pSO246 and expressed in the vaccine strain *M. bovis* BCG Tokyo 172. (B) Detection of SIV Gag protein by Western blot with anti-p27 Gag monoclonal antibody IB6.

the cell extract, and purified virions used as immunogens in this study.

Immune induction after single-modality or combined immunization regimens with vaccine candidates. We examined whether rBCG expressing the full-length *gag* gene of SIVmac239 would be suitable for use in combined prime-boost protocols with the replication-deficient vaccinia virus strain DIs recombinant. The rBCG was intradermally delivered to the inner region of the thigh and rDIs was intravenously administered into the small saphenous vein on the back of the leg. Of the 15 macaques registered in this study, 13 were divided into five groups and immunized using either a single-modality regimen plus vector controls or with a priming-boosting regimen consisting of *M. bovis* BCG and vaccinia virus DIs recombinants (Table 1). The remaining two macaques were inoculated with phosphate-buffered saline and served as naïve controls throughout the experiment.

Group 1 (control group, $n = 3$) served as a vector control group that received rBCG-pSO246 intradermally followed by two inoculations of rDIsLacZ intravenously; group 2 (rBCG group, $n = 2$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsLacZ intravenous, while group 3 (rBCG/rDIs group, $n = 3$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsSIVgag intravenously. Finally, group 4 (rDIs group, $n = 2$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-pSO246 intradermally, while group 5 (rDIs/rBCG group, $n = 3$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-SIVgag intradermally. The 13 immunized and two naive animals were studied for immune induction for 64 weeks before being mucosally challenged with virulent SHIV for a period of 1 year (Table 1).

Antigen-specific T-cell responses in all 15 animals were monitored by SIV Gag peptide-specific IFN-γ-ELISPOT assays (Fig. 2). Fifty weeks postinfection, the rBCG/rDIs group showed the highest SIV Gag-specific IFN-γ-ELISPOT responses; that group's responses peaked at 1,020 ± 360 SFC/10^6 PBMC at 56 weeks postinfection or 2 weeks after the second booster inoculation (Fig. 2A). At 56 weeks postinfection, the ELISPOT responses of the rDIs/rBCG group (380 ± 35 spots per million PBMC, Fig. 2B) were significantly lower than those of the rBCG/rDIs group ($P < 0.05$), as were the ELISPOT

TABLE 1. Immunization and challenge schedule[a]

Group no. (regimen)	Macaque no.	Priming immunization, route, and schedule	Boost immunization, route, and schedule	Mucosal challenge[b]
1 (control)	06, 90, and 91	rBCG-pSO246, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
2 (rBCG)	29 and 93	rBCG-SIV *gag*, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
3 (rBCG/rDIs)	08, 10, and 46	rBCG-SIVgag, 10 mg, i.d., wk 0	rDIsSIVgag, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
4 (rDIs)	01 and 42	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-pSO246, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64
5 (rDIs/rBCG)	85, 36, and 40	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-SIVgag, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64

[a] Vaccines, immunization, and challenge studies for all the macaques are described in the text. Animal studies were simultaneously conducted using cynomolgus macaques. i.d., intradermal inoculation; i.v., intravenous inoculation; i.r., intrarectal inoculation.

[b] All of the animals were mucosally challenged with virulent SHIV KS661c at 64 weeks postimmunization and were observed for at least 1 year or, if they did not survive for a year, until the time of their death.

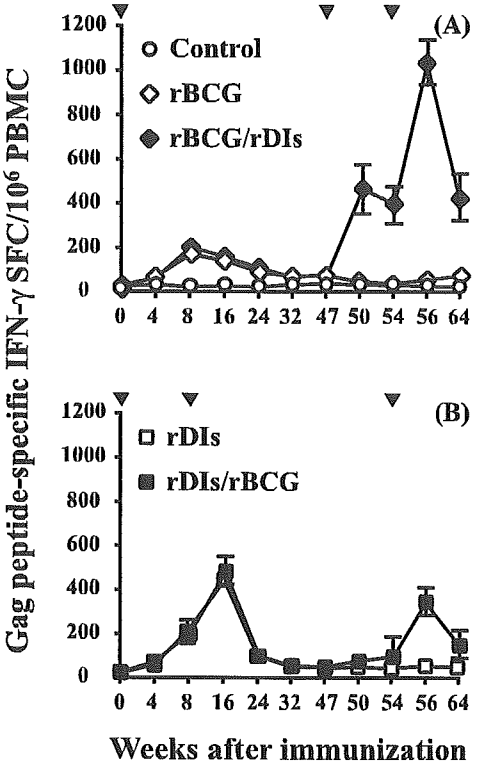

FIG. 2. Kinetics of SIV Gag peptide-specific IFN-γ spot-forming cell responses. PBMC freshly isolated from macaques immunized with either rBCG-SIVgag or rDIsSIVgag alone or with the two in combination were assessed for their ability to produce IFN-γ in response to stimulation by overlapping peptides that span the SIV Gag protein. Arrows indicate inoculation dates of the rBCG/rDIs, rBCG,and control groups, and error bars represent mean ± standard deviation.

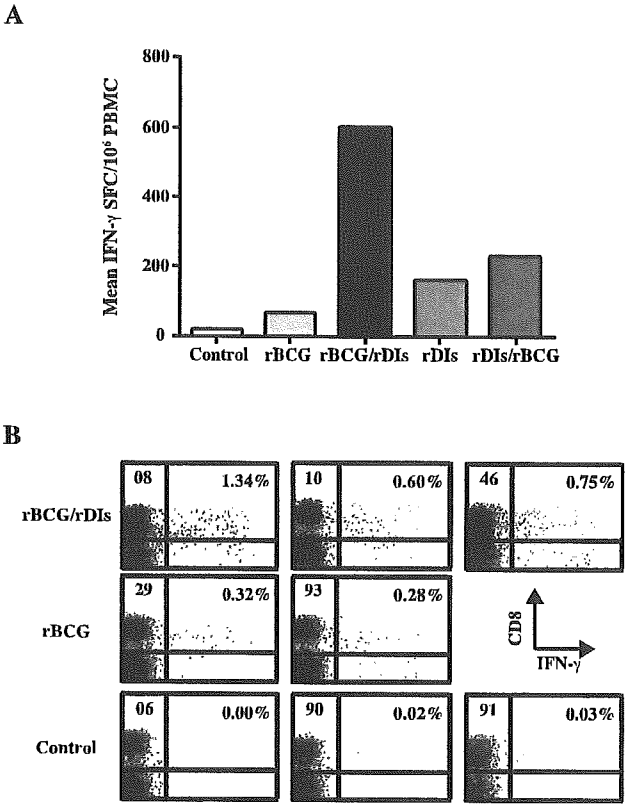

FIG. 3. SIV Gag-specific IFN-γ production in both $CD8^+$ and non-$CD8^+$ T cells in animals immunized with the rBCG/rDIs priming-boosting regimen. (A) SIV Gag protein-specific IFN-γ Elispot responses in immunized monkeys. Monkey PBMC were prepared 2 weeks after final boosting, and 2×10^5 cells were stimulated with 2 μg of recombinant SIV Gag p27 antigen protein. The bars indicate mean values of antigen-specific IFN-γ spot-forming cells per 10^6 PBMC. (B) Flow cytometric analysis of IFN-γ-producing T cells specific for SIV Gag. PBMC from macaques were cultured in vitro with overlapping peptides and stained for intracellular IFN-γ. The percentage of IFN-γ-producing $CD8^+$ T cells in each macaque's PBMC was determined by flow cytometry 2 weeks after final boosting.

responses in other rBCG and rDIs groups, both at 56 weeks postinfection and before mucosal challenge with pathogenic SHIV ($P < 0.05$). Furthermore, the number of SFC in the control and in the two naïve macaque groups did not exceed twenty during the 64-wk immunization period. Thus, the two booster inoculations of rDIs in rBCG-immunized animals effectively induced Gag peptide-specific IFN-γ-ELISPOT responses in peripheral blood, with the booster effect of DIs somewhat resembling that observed in our previous report on DNA/DIs prime-boost immunization in mice (47).

We further studied the induction of SIV Gag-specific IFN-γ ELISPOT by stimulating PBMC with SIV Gag^{p27} protein 56 weeks postinfection (Fig. 3A). The rBCG/rDIs group expressed whole-protein-specific IFN-γ responses of 615 ± 49 cells per million PBMC and the highest peptide-specific ELISPOT responses at 56 weeks postinfection (Fig. 2) of all five groups, with the peptide-specific responses being higher than the protein-specific responses (Fig. 2 and 3A). Other groups exhibited fewer than 200 cells per million PBMC.

To characterize the cellular immune responses in the rBCG/rDIs group, PBMC from the rBCG/rDIs-immunized macaques were compared with those of the rBCG and control groups by staining the surface for CD8 and intracellular SIV Gag-specific IFN-γ expression ($CD8^+IFN\text{-}\gamma^+$ cells) and then performing flow cytometric analysis (Fig. 3B). In vitro stimulation of PBMC with SIV Gag peptides in macaques 08, 10, and 46 of the rBCG/rDIs group generated a higher percentage of $CD8^+$ $IFN\text{-}\gamma^+$ T cells (1.34, 0.60, and 0.75%, respectively) than it did in animals of the rBCG group. Furthermore, non-$CD8^+$ T cells in PBMC from each animal of the rBCG/rDIs group expressed higher levels of SIV Gag-specific IFN-γ activities (macaque 8: 0.42%; macaque 10: 0.29%; macaque 46: 0.55%) than did those of the other two animal groups. The vector control animals had fewer than 0.03% of both $CD8^+$ $IFN\text{-}\gamma^+$ and non-$CD8^+$ $IFN\text{-}\gamma^+$ double-positive cells in PBMC. These findings show that the rBCG/rDIs prime-boost immunization augmented numbers of both IFN-γ-specific intracellular staining-positive cells and ELISPOT in the immunized animals, and that antigen-specific IFN-γ activities were highly induced in $CD8^+$ as well as in non-$CD8^+$ T cells, the latter most likely being $CD4^+$ T cells.

Mucosal challenge study with virulent SHIV KS661c for vaccine efficacy. Ten weeks after the second booster immunization or 64 weeks postinfection, the macaques were chal-

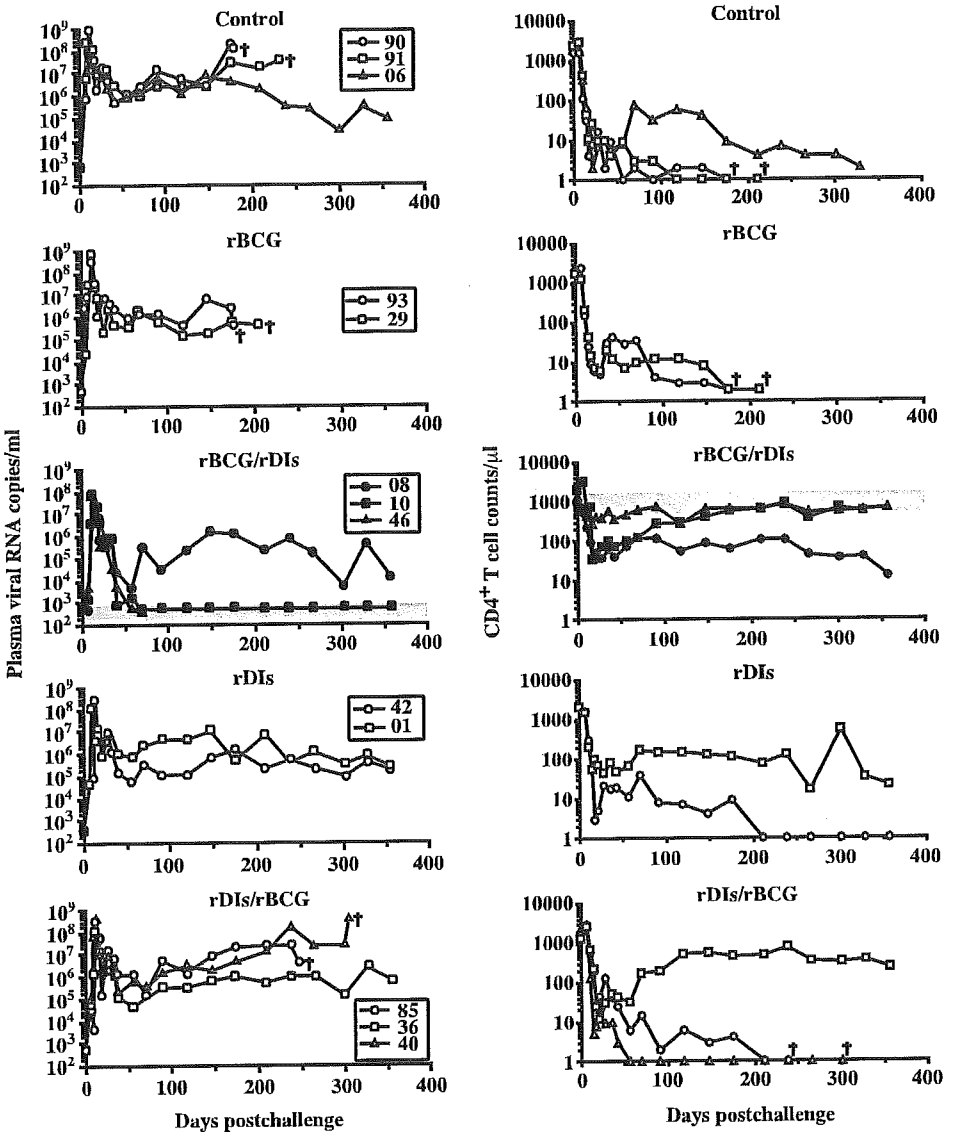

FIG. 4. Plasma viral loads and $CD4^+$ T-cell counts after viral challenge. Postchallenge plasma viral RNA copies and absolute $CD4^+$ T-cell counts in peripheral blood were detected in macaques in each of five groups immunized with a consecutive prime-boost regimen consisting of rBCG-SIVgag and rDIsSIVgag. In the study, 13 macaques were divided into five groups following the experimental designs described in Table 1.

lenged by intrarectal inoculation with 2×10^3 $TCID_{50}$ or 50 50% monkey infectious doses (MID_{50}) of SHIV KS661c, a molecular clone derived from an SHIV-89.6 variant. As shown in Fig. 4, only those macaques in the rBCG/rDIs group first primed with rBCG-SIVgag and then boosted with two inoculations of rDIsSIVgag showed evidence of protective immune responses (rBCG/rDIs). For two animals in this group (macaques 10 and 46), plasma viremia levels remained undetectable (<500 RNA copies/ml, shadow in left panel of rBCG/rDIs in Fig. 4) and $CD4^+$ T-cell counts stayed above 500 cells/μl (shadow in right panel of rBCG/rDIs in Fig. 4) for the entire year of testing. The third animal in this group (macaque 08) had fluctuating levels of viremia that were still significantly lower than those of animals in the other immunization groups. Coincidentally, this animal also had significantly decreased $CD4^+$ T-cell counts.

All macaques in the rBCG/rDIs group remained clinically healthy during the one-year observation period. Those in the rDIs/rBCG group maintained antigen-specific immune responses (Fig. 6), but showed no protective immunity against viral challenge, except for macaque 36 who showed fluctuation in the number of $CD4^+$ T cells, with numbers dipping at times below 500 cells/μl (rDIs/rBCG in Fig. 4). macaques in the other three groups all showed high levels of plasma viremia and a loss of $CD4^+$ T cells, suggesting that vaccination with rBCG and rDIs, either alone or as a priming agent, may not be suitable to induce effective, long-term positive immunity against mucosal challenge by virulent virus. By day 170 after

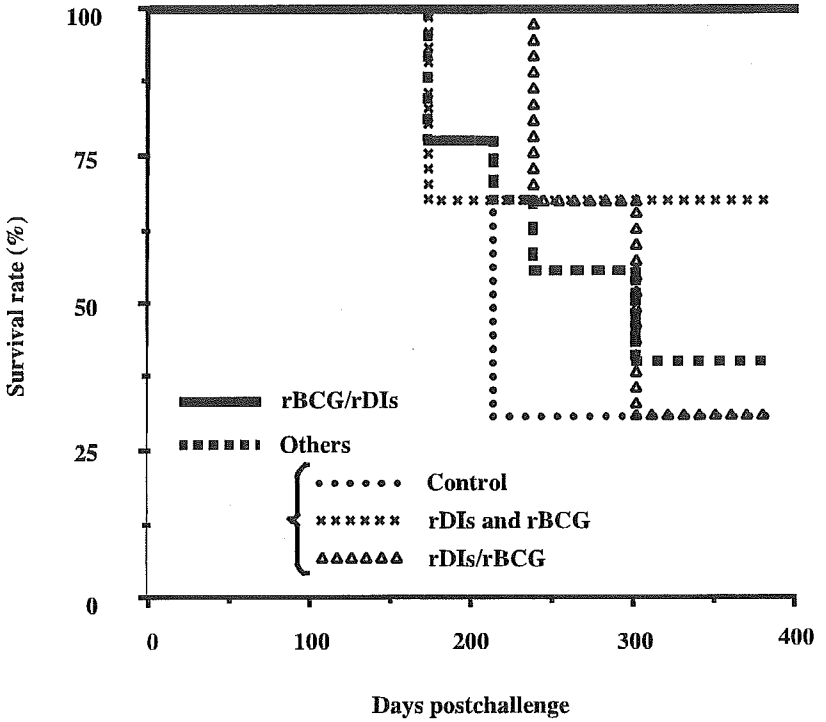

FIG. 5. Survival rates of immunized and control macaques in each of the five immunization groups. A Kaplan-Meier plot of cumulative survival rates at 1 year postchallenge with pathogenic SHIV is shown. The bold line represents group 3 immunized with the rBCG/rDIs priming-boosting regimen; the rectangular broken line represents the mean value for the total number of animals in groups 1, 2, 4, and 5.

challenge, six of the 13 macaques had died with symptoms consistent with simian AIDS: four with interstitial pneumonia, one with neurological disturbances, and one with acquired hemorrhagic diathesis. Analysis of the cumulative survival rate using the Kaplan-Meier plot showed that the rBCG/rDIs group vaccinated with the priming-boosting regimen had a superior survival rate ($P = 0.012$) to the other groups receiving vaccine protocols ($P = 0.548$) and to the control group (Fig. 5). These findings demonstrate that a prime-boost immunization with rBCG-SIVgag/rDIsSIVgag controlled virulent immunodeficiency virus infection in macaques for at least 1 year and more significantly improved survival rates than did other vaccine protocols.

Immune correlates of protection after viral challenge. In order to study virus-specific immune enhancement by SHIV challenge, we followed the postchallenge expansion of the virus-specific IFN-γ-positive cells in each animal by comparing the virus-specific IFN-γ-positive cell numbers pre- and postchallenge (Fig. 6). In all of the challenged animals of the rBCG/rDIs group, the mean number of IFN-γ-positive cells expanded from 369 ± 73 at the time of viral challenge to 629 ± 41 cells per 10^6 PBMC at 7 days after viral challenge, the sharpest increase noted with any of the animal groups. The animals of the rDIs/rBCG group showed much less enhancement, from a mean of 108 ± 46 cells per 10^6 PBMC before challenge to 224 ± 64 postchallenge, demonstrating that cellular immune responses are enhanced by viral challenge in the initial viral infection period in animals. Although in the rBCG/rDIs group high levels of IFN-γ production were observed in both $CD8^+$ and non-$CD8^+$ T cells in all three monkeys, macaque 10 and macaque 46 maintained undetectable setpoint levels of plasma viral load and normal numbers of $CD4^+$ lymphocytes, while macaque 08 did not. The macaques showed no clinical sign of weight loss, lymphoadenopathy, splenomegaly, anemia, or thrombocytopenia in the 1-year observation period. Furthermore, macaques in the rDIs group survived under low

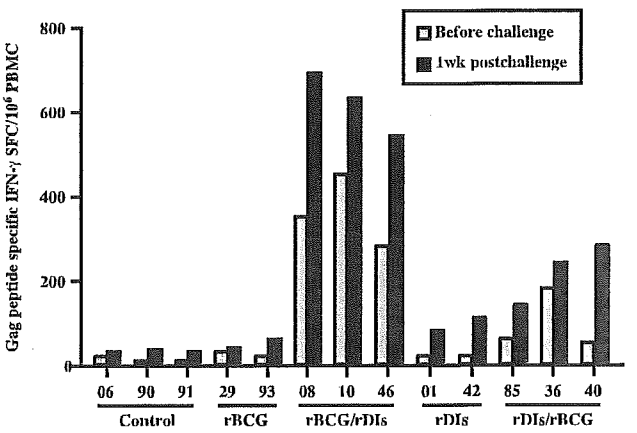

FIG. 6. Virus challenge enhances the SIV Gag peptide-specific IFN-γ ELISPOTs in PBMC from immunized macaques. PBMC from immunized animals were tested with pools of peptides spanning all the proteins from SIVmac239. Results show the production of IFN-γ to pooled peptides in $CD8^+$ and non-$CD8^+$ T lymphocytes.

immune induction but exhibited $CD4^+$ T-cell loss and plasma viremia. Notably, all macaques in the control group exhibited very low levels of immune induction by viral challenge and showed no viral control.

DISCUSSION

In the current study, we initially produced rBCG expressing SIV whole Gag. Second, by introducing a priming-boosting regimen combining rBCG-SIVgag with a nonreplicating rDIsSIVgag, we found that the rBCG/rDIs vaccination induced a long-lasting and effective immunity that was able to control a highly pathogenic virus after mucosal challenge in macaques. Third, elicitation of virus-specific immunity was observed to be important in exerting viral control in the animals immunized with the prime-boost vaccine regimen. Further investigation using larger groups of animals will be needed to determine whether high levels of immune induction correlate with increased efficacy. In this study, the macaques in the rBCG/rDIs group developed high levels of cellular immunity and were protected against the loss of $CD4^+$ T lymphocytes and the increase of viral RNA levels induced by viral challenge. Furthermore, the rBCG/rDIs group showed no evidence of clinical diseases or mortality after viral challenge during the 1-year period of observation.

The rBCG/rDIs prime-boost vaccine controlled the infection efficiently for the duration of the one-year observation period, reducing viral loads to below the threshold level for RNA copies in peripheral blood and maintaining the $CD4^+$ cell numbers above 500 cells per microliter of peripheral blood in two of the three animals in group 3. The remaining animal in the group showed fluctuations in the two parameters. Viral loads and $CD4^+$ cell numbers were not significantly affected in animal groups following the other vaccine regimens. The level of vaccine efficacy for the rBCG/rDIs group seems to be comparable to that observed in previous studies with DNA/fusion protein of interleukin-2 and immunoglobulin G (5), DNA/MVA (3), DNA/recombinant adenovirus type 5 (Ad5) (45), and MVA/recombinant vesicular stomatitis virus (36); that is, effective control of pathogenic SHIV 89.6P infection was achieved in macaques for 6 to 8 months.

SHIV KS661c, which was used as a mucosal challenge virus in this study, is a highly pathogenic molecular clone of a variant of SHIV-89.6 possessing a tropism of CXCR-4. In our preliminary study, the SHIV virus infected GHOST-X4 cells in vitro and the virus challenge eliminated the naïve $CD4^+$ T-cell population in the peripheral blood in macaques, findings which confirmed those by Nishimura et al. (33, 34). In conjunction with CCR5-tropic pathogenic SIVsmE660, Ourmanov et al. obtained similar results with the partial control of homologous viremia by the recombinant MVA vaccination (35). Furthermore, the potential of the DNA vaccination to induce a broad spectrum of mucosal protection against heterologous SIV/DeltaB670 has been demonstrated (13).

Although the virus-specific immune elicitation by DNA/Ad5 vaccination was extremely high in immunized animals (45), the efficacy results for a DNA/Ad5 study with an SIVmac239 were not comparable to those for SHIV 89.6 (43). These discrepancies in vaccine efficacy by challenge viruses suggest that SIVmac239 might be a difficult virus to control by the active immunization of various vaccine candidates. Since DNA/Ad5 is expected to elicit higher levels of immunity than either MVA or DNA alone (43–45), it might be possible to obtain vaccine efficacy in conjunction with different CCR5-tropic SIV or SHIV from SIVmac239. Alternatively, a multicomponent DNA/Ad5 might elicit broad-spectrum immunity as well as protection against SIV or CCR5-SHIV. Recently, a DNA/Sendai virus vaccination (27) proved to be as effective at controlling SIVmac239 as an attenuated live SIV vaccine (10, 11), opening the possibility for studies comparing the protective immunity elicited by ordinary vaccination to that induced by attenuated live SIV vaccination.

Because the lack of an exact HIV-1 macaque model significantly limits our ability to study and calibrate vaccine efficacy, we may need to rely on parameters such as the control of viremia, the loss of $CD4^+$ cells, and the absence of mortality to establish the efficacy of a tested vaccine against an immunodeficiency virus. Certainly, such parameters would represent a more realistic goal for the development of a preventive vaccine in the macaque model. They may also play a key role in the evaluation of vaccine efficacy in human trials I/II using the vaccine modalities developed in the macaque model.

It was recently reported that the AIDS vaccine failed in rhesus macaques approximately six months post-virus challenge, with viral avoidance of cytotoxic T-lymphocyte recognition posing a major limitation to cytotoxic T-lymphocyte-based AIDS vaccines (4). In contrast, the rBCG/rDIs prime-boost vaccine was shown in this study to control viral load throughout the 1-year observation period, suggesting that it may improve the prospects for a vaccine regimen capable of providing long-term protection against HIV-1 replication and disease progression (38, 49). Work is under way to determine whether this rBCG/rDIs vaccine will fail to control the plasma viral load in the macaque model, a failure associated with the viral escape of antigen-specific cytotoxic T lymphocytes.

The route of recombinant DIs administration will be key to effectively inducing immunity in humans. In the preliminary study to determine cellular immune induction, hundred times more rDIs was needed to achieve SIV Gag antigen-specific immunity in macaques by the intradermal (10^8 PFU/ml) than by the intravenous (10^6 PFU/ml) route (K. Someya et al., unpublished data). These findings may suggest that replication-defective vaccinia virus DIs is effective at eliciting antigen-specific immunity by intravenous administration. In addition, they suggest that the intravenous inoculation of rDIs may more effectively induce specific immunity than intradermal inoculation, although intravenous inoculation is not practical for use in human.

This study did not show a clear correlation between levels of virus-specific cellular immunity induced by booster inoculations with rDIs to rBCG-primed animals and protection against a highly virulent immunodeficiency virus after mucosal challenge. The levels of both virus-specific IFN-γ ELISPOT and gamma interferon cytokine staining responses in peripheral blood from animals in the rBCG/rDIs group were the highest of the five groups studied. Why did the prime-boost vaccination of animals of the rBCG/rDIs group prove more effective than the vaccine protocols used with the other groups? We speculate that rBCG priming, which occurs at the skin region of the thigh near the inguinal and iliac lymph nodes

draining the genitorectal mucosa, may elicit mucosal immunity in the region (23). Furthermore, we showed that the two booster intravenous inoculations with rDIs help induce a level of protective immunity sufficient to control a mucosal viral challenge in the immunized animals. Although the two intravenous inoculations with rDIsSIVgag alone proved capable of inducing some virus-specific immunity in peripheral blood after the homologous booster immunization in the immunized animals (DIs group), they appeared to provide no protection against the mucosal viral challenge.

The *M. bovis* BCG/DIs prime-boost vaccination might thus provide the opportunity to study the relationship between protection against mucosal viral challenge and elicitation of systemic or mucosal immunity. Our findings regarding the efficacy of the *M. bovis* BCG/DIs prime-boost vaccine regimen confirm those by Lehner et al. (23) and they further demonstrated a significant association between protection from mucosal rectal infection with SIV and an increase in the levels of CD8 suppressor factor and beta-chemokine. Although we cannot fully explain the differences in vaccine efficacy at this moment, it is likely that the routes of immunization and of challenge, the character of the vaccine vectors and the immunization schedule all play profound roles in eliciting vaccine efficacy in macaques.

Recently, considerable progress has been made in understanding *M. bovis* BCG as a HIV vaccine vector. Our own group demonstrated that recombinant *M. bovis* BCG vectors have the potential to deliver an HIV immunogen for desirable immune elicitation in macaques (46). Furthermore, *M. bovis* BCG vaccine substrain Tokyo 172 was revealed to be avirulent in HIV-infected children (16). The insertion of a full-length SIVmac239 *gag* into the *M. bovis* BCG substrain Tokyo 172 does not affect its toxicity, stability, or efficacy against *Mycobacterium tuberculosis* (54). Furthermore, rBCG has been shown to be nonvirulent in immunodeficient mice (54). These findings highlight the utility of rBCG as a vector for HIV-1 vaccine development.

In summary, our results demonstrate that a prime-boost vaccine regimen using rBCG as the prime and vaccinia virus rDIs as the boost can induce effective immunity against a mucosal infection with a highly virulent immunodeficiency virus for at least a year. Both of the vectors are safe for humans, making them attractive candidates for use in a preventive prime-boost vaccine against HIV-1.

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REFERENCES

1. **Aldovini, A., and R. A. Young.** 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature **351**:479–482.
2. **Amara, R. R., and H. L. Robinson.** 2002. A new generation of HIV vaccines. Trends Mol. Med. **8**:489–495.
3. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science **292**:69–74.
4. **Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature **415**:335–339.
5. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin.** 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science **290**:486–492.
6. **Chaparas, S. D., and S. R. Hedrick.** 1973. Comparison of strains of BCG. I. Antigenic analysis and tuberculin reactivity. Infect. Immun. **7**:777–780.
7. **Chujoh, Y., K. Matsuo, H. Yoshizaki, T. Nakasatomi, K. Someya, Y. Okamoto, S. Naganawa, S. Haga, H. Yoshikura, A. Yamazaki, S. Yamazaki, and M. Honda.** 2001. Cross-clade neutralizing antibody production against human immunodeficiency virus type 1 clade E and B' strains by recombinant *Mycobacterium bovis* BCG-based candidate vaccine. Vaccine **20**:797–804.
8. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science **258**:1938–1941.
9. **Davis, N. L., A. West, E. Reap, G. MacDonald, M. Collier, S. Dryga, M. Maughan, M. Connell, C. Walker, K. McGrath, C. Cecil, L. H. Ping, J. Frelinger, R. Olmsted, P. Keith, R. Swanstrom, C. Williamson, P. Johnson, D. Montefiori, and R. E. Johnston.** 2002. Alphavirus replicon particles as candidate HIV vaccines. IUBMB Life **53**:209–211.
10. **Desrosiers, R. C.** 1992. HIV with multiple gene deletions as a live attenuated vaccine for AIDS. AIDS Res. Hum. Retroviruses **8**:1457.
11. **Desrosiers, R. C., M. S. Wyand, T. Kodama, D. J. Ringler, L. O. Arthur, P. K. Sehgal, N. L. Letvin, N. W. King, and M. D. Daniel.** 1989. Vaccine protection against simian immunodeficiency virus infection. Proc. Natl. Acad. Sci. USA **86**:6353–6357.
12. **Fauci, A. S., S. M. Schnittman, G. Poli, S. Koenig, and G. Pantaleo.** 1991. NIH conference on immunopathogenic mechanisms in human immunodeficiency virus infection. Ann. Intern. Med. **114**:678–693.
13. **Fuller, D. H., P. A. Rajakumar, L. A. Wilson, A. M. Trichel, J. T. Fuller, T. Shipley, M. S. Wu, K. Weis, C. R. Rinaldo, J. R. Haynes, and M. Murphey-Corb.** 2002. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J. Virol. **76**:3309–3317.
14. **Gauduin, M. C., R. L. Glickman, S. Ahmad, T. Yilma, and R. P. Johnson.** 1999. Characterization of SIV-specific $CD4^+$ T-helper proliferative responses in macaques immunized with live-attenuated SIV. J. Med. Primatol. **28**:233–241.
15. **Hel, Z., D. Venzon, M. Poudyal, W. P. Tsai, L. Giuliani, R. Woodward, C. Chougnet, G. Shearer, J. D. Altman, D. Watkins, N. Bischofberger, A. Abimiku, P. Markham, J. Tartaglia, and G. Franchini.** 2000. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. Nat. Med. **6**:1140–1146.
16. **Hesseling, A. C., H. S. Schaaf, W. A. Hanekom, N. Beyers, M. F. Cotton, R. P. Gie, B. J. Marais, P. van Helden, and R. M. Warren.** 2003. Danish bacille Calmette-Guerin vaccine-induced disease in human immunodeficiency virus-infected children. Clin. Infect. Dis. **37**:1226–1233.
17. **Honda, M., K. Matsuo, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Kitamura, W. Sugiura, K. Watanabe, Y. Fukushima, S. Haga, Y. Katsura, K. Kitamura, H. Tasaka, K. Komuro, T. Yamada, T. Asano, A. Yamazaki, and S. Yamazaki.** 1995. Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. Proc. Natl. Acad. Sci. USA **92**:10693–10697.
18. **Ishii, K., Y. Ueda, K. Matsuo, Y. Matsuura, T. Kitamura, K. Kato, Y. Izumi, K. Someya, T. Ohsu, M. Honda, and T. Miyamura.** 2002. Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector. Virology **302**:433–444.
19. **Jacobs, W. R., Jr., M. Tuckman, and B. R. Bloom.** 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature **327**:532–535.
20. **Karacostas, V., E. J. Wolffe, K. Nagashima, M. A. Gonda, and B. Moss.** 1993. Over expression of the HIV-1 gag-pol polyprotein results in intracellular

activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. Virology **193**:661–671.

21. **Kawahara, M., K. Matsuo, T. Nakasone, T. Hiroi, H. Kiyono, S. Matsumoto, T. Yamada, N. Yamamoto, and M. Honda.** 2002. Combined intrarectal/intradermal inoculation of recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) induces enhanced immune responses against the inserted HIV-1 V3 antigen. Vaccine **21**:158–166.
22. **Kitamura, T., Y. Kitamura, and I. Tagaya.** 1967. Immunogenicity of an attenuated strain of vaccinia virus on rabbits and monkeys. Nature **215**:1187–1188.
23. **Lehner, T., Y. Wang, M. Cranage, L. A. Bergmeier, E. Mitchell, L. Tao, G. Hall, M. Dennis, N. Cook, R. Brookes, L. Klavinskis, I. Jones, C. Doyle, and R. Ward.** 1996. Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques. Nat. Med. **2**:767–775.
24. **Letvin, N. L., D. H. Barouch, and D. C. Montefiori.** 2002. Prospects for vaccine protection against HIV-1 infection and AIDS. Annu. Rev. Immunol. **20**:73–99.
25. **Lifson, J. D., M. Piatak, Jr., J. L. Rossio, J. Bess, Jr., E. Chertova, D. Schneider, R. Kiser, V. Coalter, B. Poore, R. Imming, R. C. Desrosiers, L. E. Henderson, and L. O. Arthur.** 2002. Whole inactivated SIV virion vaccines with functional envelope glycoproteins: safety, immunogenicity, and activity against intrarectal challenge. J. Med. Primatol. **31**:205–216.
26. **Lu, Y., M. S. Salvato, C. D. Pauza, J. Li, J. Sodroski, K. Manson, M. Wyand, N. Letvin, S. Jenkins, N. Touzjian, C. Chutkowski, N. Kushner, M. LeFaile, L. G. Payne, and B. Roberts.** 1996. Utility of SHIV for testing HIV-1 vaccine candidates in macaques. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **12**:99–106.
27. **Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai.** 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. **199**:1709–1718.
28. **Matsumoto, S., M. Tamaki, H. Yukitake, T. Matsuo, N. Mariko, H. Teraoka, and T. Yamada.** 1996. A stable *Escherichia coli*-mycobacteria shuttle vector pSO246 in *Mycobacterium bovis* BCG. FEMS Microbiol. Lett. **135**:237–243.
29. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983–2003. Nat. Med. **9**:874–880.
30. **Mori, K., Y. Yasutomi, S. Ohgimoto, T. Nakasone, S. Takamura, T. Shioda, and Y. Nagai.** 2001. Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. J. Virol. **75**:4023–4028.
31. **Mothe, B. R., H. Horton, D. K. Carter, T. M. Allen, M. E. Liebl, P. Skinner, T. U. Vogel, S. Fuenger, K. Vielhuber, W. Rehrauer, N. Wilson, G. Franchini, J. D. Altman, A. Haase, L. J. Picker, D. B. Allison, and D. I. Watkins.** 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. J. Virol. **76**:875–884.
32. **Nabel, G., W. Makgoba, and J. Esparza.** 2002. HIV-1 diversity and vaccine development. Science **296**:2335.
33. **Nishimura, Y., C. R. Brown, J. J. Mattapallil, T. Igarashi, A. Buckler-White, B. A. Lafont, V. M. Hirsch, M. Roederer, and M. A. Martin.** 2005. Resting naive CD4+ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. Proc. Natl. Acad. Sci. USA **102**:8000–8005.
34. **Nishimura, Y., T. Igarashi, O. K. Donau, A. Buckler-White, C. Buckler, B. A. Lafont, R. M. Goeken, S. Goldstein, V. M. Hirsch, and M. A. Martin.** 2004. Highly pathogenic SHIVs and SIVs target different CD4+ T-cell subsets in rhesus monkeys, explaining their divergent clinical courses. Proc. Natl. Acad. Sci. USA **101**:12324–12329.
35. **Ourmanov, I., C. R. Brown, B. Moss, M. Carroll, L. Wyatt, L. Pletneva, S. Goldstein, D. Venzon, and V. M. Hirsch.** 2000. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. J. Virol. **74**:2740–2751.
36. **Ramsburg, E., N. F. Rose, P. A. Marx, M. Mefford, D. F. Nixon, W. J. Moretto, D. Montefiori, P. Earl, B. Moss, and J. K. Rose.** 2004. Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. J. Virol. **78**:3930–3940.
37. **Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, R. G. Collman, J. Sodroski, and N. L. Letvin.** 1996. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J. Virol. **70**:3198–3206.
38. **Ruprecht, R. M., T. W. Baba, R. Rasmussen, Y. Hu, and P. L. Sharma.** 1996. Murine and simian retrovirus models: the threshold hypothesis. AIDS **10**(Suppl. A):S33–40.
39. **Sakai, K., K. Shinohara, E. Takahashi, Y. Izumi, Y. Ami, Y. Sasaki, T. Nakasone, and M. Honda.** 2001. Molecular cloning of a pathogenic simian-human immunodeficiency virus for HIV/AIDS monkey model. Sixth International Congress on AIDS in Asia and the Pacific, 5–10 October 2001, Melbourne, Australia, abstract p. 84.
40. **Sasaki, Y., Y. Ami, T. Nakasone, K. Shinohara, E. Takahashi, S. Ando, K. Someya, Y. Suzaki, and M. Honda.** 2000. Induction of CD95 ligand expression on T lymphocytes and B lymphocytes and its contribution to apoptosis of CD95-up-regulated $CD4^+$ T lymphocytes in macaques by infection with a pathogenic simian/human immunodeficiency virus. Clin. Exp. Immunol. **122**:381–389.
41. **Sher, N. A., S. D. Chaparas, L. E. Greenberg, and S. Bernard.** 1975. Effects of BCG, *Corynebacterium parvum*, and methanol extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infections in immunosuppressed mice. Infect. Immun. **12**:1325–1330.
42. **Shinohara, K., K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, M. Kaizu, Y. Lu, and M. Honda.** 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. **80**:1231–1240.
43. **Shiver, J. W.** 2004. Development of an HIV-1 vaccine based on replication-defective adenovirus. Proc. Keystone Symp. **2004**:12.
44. **Shiver, J. W., and E. A. Emini.** 2004. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. Annu. Rev. Med. **55**:355–372.
45. **Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415**:331–335.
46. **Someya, K., D. Cecilia, Y. Ami, T. Nakasone, K. Matsuo, S. Burda, H. Yamamoto, N. Yoshino, M. Kaizu, S. Ando, K. Okuda, S. Zolla-Pazner, S. Yamazaki, N. Yamamoto, and M. Honda.** 2005. Vaccination of rhesus macaques with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. J. Virol. **79**:1452–1462.
47. **Someya, K., K. Q. Xin, K. Matsuo, K. Okuda, N. Yamamoto, and M. Honda.** 2004. A consecutive priming-boosting vaccination of mice with simian immunodeficiency virus (SIV) *gag/pol* DNA and recombinant vaccinia virus strain DIs elicits effective anti-SIV immunity. J. Virol. **78**:9842–9853.
48. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351**:456–460.
49. **Strathdee, S. A., K. J. Craib, R. S. Hogg, M. V. O'Shaughnessy, J. S. Montaner, and M. T. Schechter.** 1995. Long-term non-progression in HIV infection. Lancet **346**:1372.
50. **Tagaya, I., T. Kitamura, and Y. Sano.** 1961. A new mutant of dermovaccinia virus. Nature **192**:381–382.
51. **Takeya, K., K. Nomoto, S. Muraoka, S. Shimotori, T. Taniguchi, and T. Miyake.** 1977. Growth of two strains of *Mycobacterium bovis* (BCG) in a thymic mice. J. Gen. Microbiol. **100**:403–405.
52. **Thole, J. E., W. J. Keulen, J. De Bruyn, A. H. Kolk, D. G. Groothuis, L. G. Berwald, R.H. Tiesjema, and J. D. van Embden.** 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. Infect. Immun. **55**:1466–1475.
53. **Ueda, K., S. Yamazaki, J. Saegusa, and S. Someya.** 1978. Progressive and fetal infection with attenuated *Mycobacterium bovis* (BCG) in nude mice. Jpn. J. Exp. Med. **48**:533–543.
54. **Yamamoto, S., T. Yamamoto, S. Haga, and M. Honda.** 2003. Presented at the Tuberculosis Leprosy Research Conference U.S.-Japan Co. Med. Sci.
55. **Yoshino, N., Y. Ami, K. Terao, F. Tashiro, and M. Honda.** 2000. Upgrading of flow cytometric analysis for absolute counts, cytokines and other antigenic molecules of cynomolgus monkeys (*Macaca fascicularis*) by using anti-human cross-reactive antibodies. Exp. Anim. **49**:97–110.

Transmission of Macrophage-Tropic HIV-1 by Breast-Milk Macrophages via DC-SIGN

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Recent findings suggest that macrophage-tropic human immunodeficiency virus type 1 (HIV-1) produced in colostrum/early breast milk may hold a clue to determine the mechanisms of transmission of HIV-1 via breast-feeding. Here, we show that the majority of $CD4^+$ cells in the colostrum are $CD14^+$ macrophages expressing both chemokine receptors and DC-SIGN, a dendritic cell–specific receptor for HIV-1. The R5-type macrophage-tropic HIV-1 isolate NL(AD8) infected such breast-milk macrophages and caused them to secrete virus particles efficiently; however, the secreted virions showed only a weak transmissibility to their susceptible target, MAGIC-5 cells. When stimulated with interleukin-4, the breast-milk macrophages demonstrated a striking enhancement of expression of DC-SIGN and showed a strong capacity to transmit NL(AD8) virions to MAGIC-5 cells, which was specifically blocked by anti–DC-SIGN–specific antibody. These results suggest that HIV-1 virions captured by DC-SIGN, but not secreted cell-free virions, may be more efficiently transmitted to other compartments, such as the gastrointestinal tract, through acidic gastric juice.

Although the benefits of breast-feeding, including decreased infant morbidity and mortality, are well recognized, it may cause a substantial increase in the risk of transmission of HIV-1 from an infected mother to her child. Indeed, in the absence of prophylactic antiretroviral therapy, approximately one-third to one-half of HIV-1 infections in infants are acquired via breast-feeding [1]. The magnitude of breast-milk infectivity is significantly higher for mothers with a more advanced disease status, as measured by prenatal plasma HIV-1 RNA loads and CD4 cell counts [2, 3], indicating the strong correlation between the risk of transmission via breast-feeding and maternal plasma HIV-1 load. In addition, an association between breast-milk HIV-1 load and mother-to-child transmission of HIV-1 has been reported [4]. Thus, both plasma and breast-milk HIV-1 loads in infected mothers seem to be relevant indicators with which to assess mother-to-child transmission of HIV-1.

The origin of HIV-1 virions in breast milk remains unclear. Although the size of the study [5] was small, it has recently been reported that there is a possible compartmentalization of HIV-1 between blood and breast milk, suggesting that the actual virions for transmission via breast-feeding may be produced within the breast milk itself. Also, it has been reported that HIV-1 can infect human mammary epithelial cells and that HIV-1 virions are productively secreted from them [6]. Such mammary epithelial cell–derived HIV-1 may have selective advantages for the infection of mucosal cells and may determine the HIV-1 tropism for transmissible target cells situated in the gastrointestinal tract when they encounter HIV-1 after breast-feeding. Indeed, apart from macrophages that express CD4 but do not express either CCR5 or CXCR4, resident lamina propria lymphocytes that express CD4 as well as CCR5 and CXCR4 are speculated to be the target mononuclear cells for HIV-1 infection in the intestinal mucosa during early HIV-1 infection [7, 8]. Also, the risk of HIV-1 infection via breast-feeding in infants has been confirmed to be

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influenced by breast-milk HIV-1 load, which is significantly higher in colostrum than in mature breast milk obtained 14 days after delivery [9]. Moreover, as indicated elsewhere [10, 11], the principal HIV-1 variants identified in breast milk were R5-type [5]. Collectively, R5-type macrophage-tropic HIV-1 produced in colostrum/early breast milk may hold a clue to determine the mechanisms of transmission of HIV-1 via breast-feeding. Therefore, we have examined the actual components that organize transmission in colostrum/early breast milk. On the basis of the findings obtained by analyzing cells in the colostrum for their susceptibility to HIV-1, a possible mechanism for mother-to-child transmission of HIV-1 via breast-feeding will be discussed in the present study.

MATERIALS AND METHODS

Isolation of breast milk–derived cells and breast-milk macrophages. Breast milk was collected from healthy women within 3–6 days of delivery, after informed consent had been obtained under a protocol approved by the Institutional Review Board of the Nippon Medical School and in accordance with the human-experimentation guidelines of the US Department of Health and Human Services. Breast-milk cells were isolated from freshly obtained breast milk by Ficoll-Paque (Amersham Pharmacia Biotech) gradient centrifugation methods, as described elsewhere [12]. Breast-milk macrophages were isolated from freshly obtained breast-milk cells, followed by adherence to polystyrene tissue-culture dishes for 1 h at 37°C. The adherent cells were then removed by incubation with 5 mmol/L EDTA for 30 min at 4°C. The obtained adherent cells were confirmed to express homogeneous $CD14^+$ cells at ~95% by use of a FACScan cytometer (Becton Dickinson).

Cytokine treatment of breast-milk macrophages. For the treatment of breast-milk macrophages with either interleukin (IL)–2 or IL-4, cells were plated at 10^6 cells/mL in RPMI 1640 medium–based complete culture medium (CCM) [13] supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES (Invitrogen), 50 μmol/L 2-mercapto-ethanol (Sigma), 2 mmol/L L-glutamine (Sigma), and 100 U of penicillin-streptomycin solution (Sigma) and were cultured for 5 days at 37°C in the presence or absence of either IL-2 (50 U/mL) (Shionogi Pharmaceutical) or IL-4 (1000 U/mL) (Biosource International). The culture medium was changed every 2 days.

Antibodies and flow-cytometric analysis. Fluorescein isothiocyanate–conjugated anti-human monoclonal antibodies (MAbs) to CD3 (UHCT1), CD4 (SK3), and CD14 (M5E2); phycoerythrin (PE)–conjugated anti-human MAbs to CD3 (UHCT1), CCR5 (2D7), and CXCR4 (12G4); biotin-labeled MAb to anti-human CD4 (RPA-T4); and PE-conjugated isotype-matched control antibody (MOPC-21) were all purchased from BD Biosciences. PE-conjugated and unlabeled anti-human DC-SIGN (120507) MAbs were purchased from R&D Systems, and CD4 (OKT4) and control isotype-matched polyclonal antibody for the blocking experiments were purchased from American Type Culture Collection.

Cells were stained with the relevant antibody for 30 min on ice in PBS with 2% FCS and 0.01 mol/L sodium azide (PBS-based medium), were washed twice, and were resuspended in the PBS-based medium. Then, the labeled cells were analyzed by use of a FACScan cytometer with CellQuest software (version 3.1F; BD Biosciences). Live cells were gated on the basis of propidium-iodide gating.

Measurement of virus titer. NL(AD8) infectious viral particles [14] were obtained by transfection of 293T cells with pNL(AD8) (gift from Malcolm A. Martin, National Institute of Allergy and Infectious Diseases, National Institutes of Health [NIH]). Polyethylenimine (25 kDa; Sigma) was used for transfection. The virus titer was determined on the basis of the HIV-1 p24 antigen concentration (picograms per milliliter) in the culture supernatant, by ELISA, as described elsewhere [15]. In brief, Immulon II plates (Dynex Technologies) were coated with anti–HIV-1 p24 MAb (183-H12-5C) [16, 17], and the samples were incubated in the plates for 2 h. After washing, biotinylated human anti-HIV immunoglobulin was added for detection of p24. HIV-1 p25/24 Gag protein (NIH AIDS Research and Reference Reagent Program) [18] was used as a standard.

Infection of cultured breast-milk macrophages with NL(AD8). Breast-milk macrophages cultured for 5 days with or without IL-4 were harvested and added to a flat-bottom 48-well microplate (Corning), at $1–2 \times 10^5$ cells/well, in a total volume of 200 μL of CCM. After incubation for 2 h at 37°C, the media were gently removed, and the remaining cells were further incubated with 200 μL of CCM containing 2 ng/mL HIV-1 p24 of NL(AD8) for 2 h at 37°C. Then, the cells were washed 3 times with RPMI 1640 medium containing 2% FCS and were cultured with CCM (total volume, 400 μL) for an additional 7 days. The culture supernatant was replaced with fresh CCM every other day, and the collected culture supernatant was stored at −80°C for further analysis.

HIV-1 transmission assay. An indicator cell line, named MAGIC-5 [19] (provided by M. Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan), was used to examine the capacity of NL(AD8)-sensitized cells to transmit that isolate. MAGIC-5 cells (10^5 cells/well) were plated in a flat-bottom 96-well microplate (Corning) with CCM the day before coculturing with the target cells. After removal of the medium from each well, 5×10^4 intensively washed NL(AD8)-infected breast-milk macrophages were added to 10^5 preincubated MAGIC-5 cells. After 16 h, the loaded cells were removed by gentle washing with warmed PBS. Then, 50 μg of the substrate X-gal was added, to identify the β-gal–expressing infected cells, by use of a β-gal staining kit (Invitrogen). The stained cells were counted, to estimate transmissibility.

Acid treatment of NL(AD8)-infected cells. The NL(AD8)-infected breast-milk macrophages were washed twice with PBS, incubated with adjusted HCl (pH 3.0) for 1–3 min at room temperature, and then promptly neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Cells were washed 3 times with CCM, and 5×10^4 infected cells were added to the wells seeded with 10^5 MAGIC-5 cells. After 16 h, each well was gently washed 3 times, to remove loaded, infected breast-milk macrophages, and the remaining MAGIC-5 cells were stained with X-gal, as described above. The number of β-gal–positive blue-colored cells was counted, to estimate infectivity.

RESULTS

Flow-cytometric analysis of cells in breast milk. On the basis of recent findings showing that the risk of HIV-1 infection via breast-feeding in infants is influenced by breast-milk HIV-1 load, which is significantly higher in colostrum than in mature breast milk [9], we first analyzed the surface phenotype of the cells in freshly isolated colostrum/early breast milk. We could detect only a few $CD3^+$ cells in the early breast milk (figure 1*A*), and the majority of $CD4^+$ cells did not express CD3 (figure 1*B*) but rather expressed CD14 molecules (figure 1*C*), which we have named "breast-milk macrophages." Thus, we focused on the R5-type macrophage-tropic HIV-1 isolate NL(AD8) [14] for further analysis.

DC-SIGN and expression of chemokine receptors on $CD4^+$ breast-milk macrophages. Next, we examined whether $CD4^+$ breast-milk macrophages could be infected with NL(AD8). Contrary to intestinal macrophages, which lack expression of CCR5 [8], freshly isolated $CD4^+$ breast-milk macrophages did express both CXCR4 and CCR5 (figure 2). Also, they weakly but spontaneously expressed DC-SIGN, a dendritic cell (DC)–specific receptor for HIV-1 [20, 21] that might capture cell-free HIV-1 virions (figure 2). On the basis of previous observations showing that surface expression of DC-SIGN on breast-milk macrophages was markedly enhanced when breast-milk macrophages were cocultured with IL-4 for 5 days [12], we compared the surface expression of chemokine receptors and DC-SIGN on breast-milk macrophages cultured for 5 days either in the absence or in the presence of IL-4. Without IL-4 stimulation, the expression of CXCR4, CCR5, and DC-SIGN on breast-milk macrophages cultured for 5 days was almost unchanged, compared with that in freshly isolated breast-milk macrophages (figure 2). In contrast, when breast-milk macrophages were cocultured with IL-4 for 5 days, a dramatic reduction of expression of both CXCR4 and CCR5, together with strong enhancement of expression of DC-SIGN, was observed (figure 2). However, we did not observe any enhancement of expression of DC-SIGN when breast-milk macrophages were incubated with IL-2 for 5 days (figure 2). Also, as we have reported elsewhere [12], the IL-4–stimulated breast-milk macrophages became CD1a-positive DC-like cells that lost CD14 (data not shown).

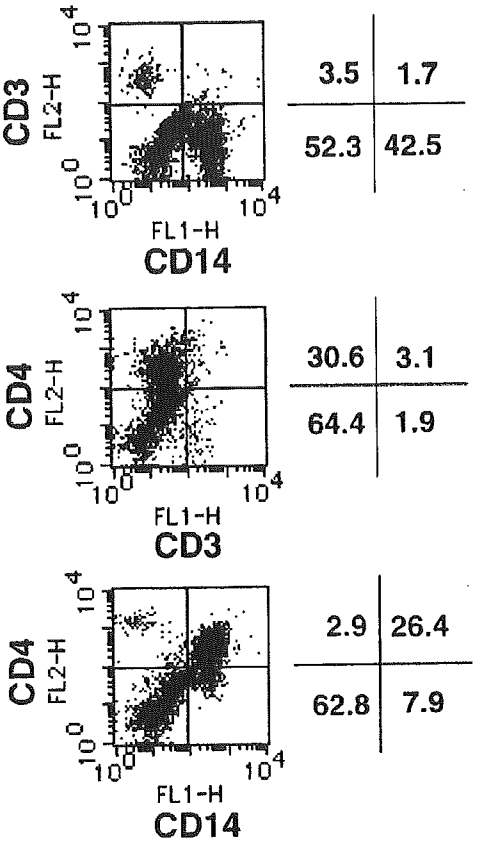

Figure 1. Fluorescence-activated cell sorter analysis of surface markers in freshly isolated colostrum/early breast-milk cells. *A*, The majority of the breast-milk cells were breast-milk macrophages expressing CD14 molecules, and only a few $CD3^+$ T cells were detected. The majority of $CD4^+$ cells did not express CD3 *(B)* but rather expressed CD14 molecules *(C)*. The nos. indicate the percentage of cells in each quadrant. The data shown are representative of 5 distinct experiments.

Analysis of susceptibility of R5-type HIV-1 to cultured breast-milk macrophages. Next, we compared the susceptibility of HIV-1 to breast-milk macrophages cultured for 5 days, using the R5-type macrophage-tropic HIV-1 isolate NL(AD8). Cultured breast-milk macrophages were infected with NL(AD8) (2 ng/mL p24 antigen) for 2 h at 37°C and were washed extensively, to remove cell-free HIV-1 virions, and the quantity of HIV-1 p24 Gag protein in the supernatant of the further-cultured breast-milk macrophages was measured. As expected, a marked increase of production of HIV-1 p24 antigen was observed in the supernatant of NL(AD8)-infected, cultured breast-milk macrophages on days 3 and 5 (figure 3*A*). Unexpectedly, although a striking reduction of expression of CCR5 was mediated by treatment with IL-4, the IL-4–stimulated breast-milk macrophages could also be fairly infected with

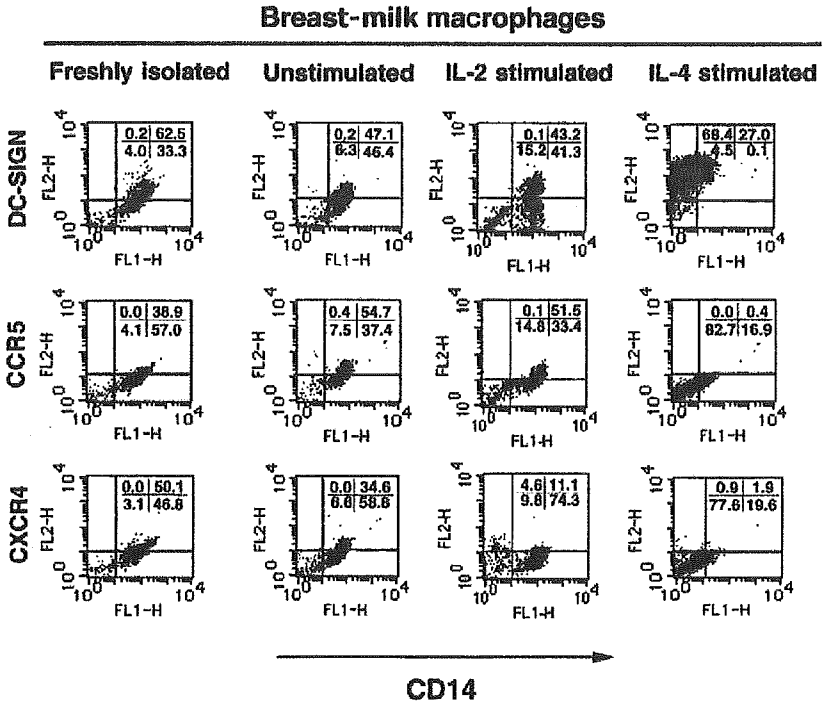

Figure 2. Expression of chemokine receptors and DC-SIGN on $CD14^+$ breast-milk macrophages. Both CXCR4 and CCR5 (left middle and lower panels), as well as DC-SIGN molecules (left upper panel), were expressed on freshly isolated breast-milk macrophages. Without stimulation with interleukin (IL)–4 (second 3 panels from the left) or IL-2 (third 3 panels from the left), surface expression of CXCR4, CCR5, and DC-SIGN molecules on breast-milk macrophages cultured for 5 days was almost unchanged, compared with that on freshly isolated breast-milk macrophages. In contrast, breast-milk macrophages cocultured with IL-4 for 5 days showed a dramatic reduction of expression of both CXCR4 and CCR5, whereas strong enhancement of expression of DC-SIGN was observed (right 3 panels). The nos. indicate the percentage of cells in each quadrant. The data shown are representative of 3 distinct experiments.

NL(AD8), and they secreted good amounts of p24 antigen on day 5 (figure 3*A*). This may be because of the enhancement of virus acquisition induced by the *cis* effect of DC-SIGN, as reported elsewhere [22]. It is important to note that, on day 3, the production of HIV-1 p24 antigen by IL-4–stimulated breast-milk macrophages was much less than that by unstimulated macrophages, indicating the substantial difference of intracellular replication of NL(AD8) between those distinct conditioned breast-milk macrophages at that time point. Nevertheless, on day 3, the ability of IL-4–stimulated breast-milk macrophages to transmit the virions to NL(AD8)-sensitive MAGIC-5 cells was far stronger than that of unstimulated macrophages (figure 3*B*), suggesting that the high transmissibility was mediated through the virions captured via DC-SIGN but not through cell-free virus particles released by those infected cells.

Inhibition of transmission of NL(AD8) by IL-4–stimulated breast-milk macrophages with anti–DC-SIGN antibody but not with isotype-matched antibodies. The above results reveal that DC-SIGN–mediated capture and transmission of HIV-1 virions may be a major pathway for vertical transmission via breast-feeding in infants. Therefore, we asked whether blocking of DC-SIGN with specific antibody would reduce the capacity for transmissibility of HIV-1 virions in IL-4–stimulated breast-milk macrophages. Pretreatment of the IL-4–stimulated breast-milk macrophages with 1–5 μmol/L anti–DC-SIGN MAb for 30 min on ice and subsequent infection with NL(AD8) for 2 h at 37°C, in the presence of the antibody, showed remarkable inhibition of transmission of NL(AD8) to MAGIC-5 cells (figure 4). In contrast, pretreatment with either isotype-matched control IgG or anti-human CD4-specific MAb (OKT4) (5 μmol/L) did not induce significant inhibition (figure 4). Also, the DC-SIGN–specific MAb was not toxic—treatment of control breast-milk macrophages cultured for 5 days with the same or higher concentrations of anti–DC-SIGN MAb did not affect the transmissibility of virus at all (data not shown). Thus, in breast-feeding, DC-SIGN–mediated capture of HIV-1 virions seems to be critical for transmission of cell-associated viral particles to target cells. Moreover, the effect of pretreatment with the anti–DC-SIGN MAb (5 μmol/L) on the secreted virus titer of IL-4–stimulated breast-milk macrophages cultured for 5 days was examined, and ~70% reduction in the amount of p24 was observed (data not shown). This result indicates that DC-

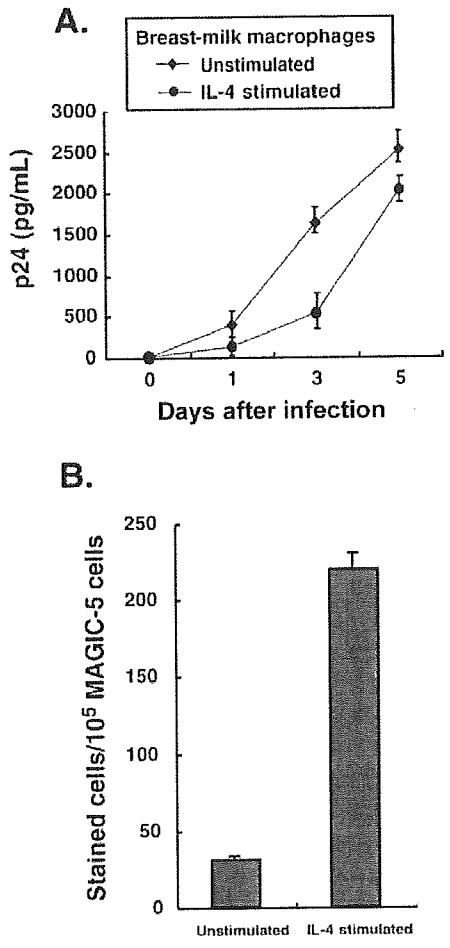

Figure 3. Macrophage-tropic HIV-1 infectivity to breast-milk macrophages cultured for 5 days. Cultured breast-milk macrophages were infected with the R5-type macrophage-tropic HIV-1 isolate NL(AD8) (2 ng/mL p24 antigen) for 2 h at 37°C and washed extensively to remove cell-free HIV-1 virions, and the quantity of HIV-1 p24 Gag protein in the supernatant of further-cultured breast-milk macrophages was measured by ELISA. *A*, Production of HIV-1 p24 antigen in the supernatant of NL(AD8)-infected interleukin (IL)–4–unstimulated breast-milk macrophages (◆) and IL-4–stimulated breast-milk macrophages (●). The data shown are mean ± SD and are representative of 4 independent experiments. *B*, Fifty thousand intensively washed NL(AD8)-infected breast-milk macrophages were added to 10^5 MAGIC-5 cells. After 16 h, the loaded cells were removed by gentle washing 3 times with warmed PBS. Then, 50 μg of the substrate X-gal was added, to identify the β-gal–expressing cells, by use of a β-gal staining kit (Invitrogen), and stained cells were counted. Data shown are mean ± SD and are representative of 3 independent experiments.

SIGN–mediated transcytosis of HIV-1 virions may contribute to acquisition of the virus by IL-4–stimulated breast-milk macrophages.

Effect of acidification on virus infectivity. There are 2 modes of transmission of virus: (1) the cell-free situation and (2) the cell-associated pattern. Breast milk from HIV-1–infected mothers contains both forms, and breast milk with virions, when ingested, must encounter gastric juice with low pH (pH 3.0–4.0 in infants [23, 24]) before meeting with intestinal target cells that have high susceptibility to macrophage-tropic HIV-1. Thus, we first examined the effect of acidification on virus infectivity, using cell-free NL(AD8). In our preliminary data, we observed that 1 min of treatment with medium whose pH was adjusted to <4.0 produced a profound inhibition of virus infectivity (data not shown). Therefore, we treated the virions in the cell-free form with HCl (pH 3.0) for 1 min at 37°C, followed by subsequent neutralization with Tris-buffer (pH 8.0), and found that the infectivity of HIV-1 virions was lost almost completely (figure 5*A*), indicating that most HIV-1 virions may not reach intestinal targets in their cell-free form via breast-feeding. Then, we investigated the effect of acidification on transmission of DC-SIGN–mediated cell-associated HIV-1 virions. To our surprise, at least 10%–20% (mean, ~40%) of the infectivity to MAGIC-5 cells remained when NL(AD8)-infected

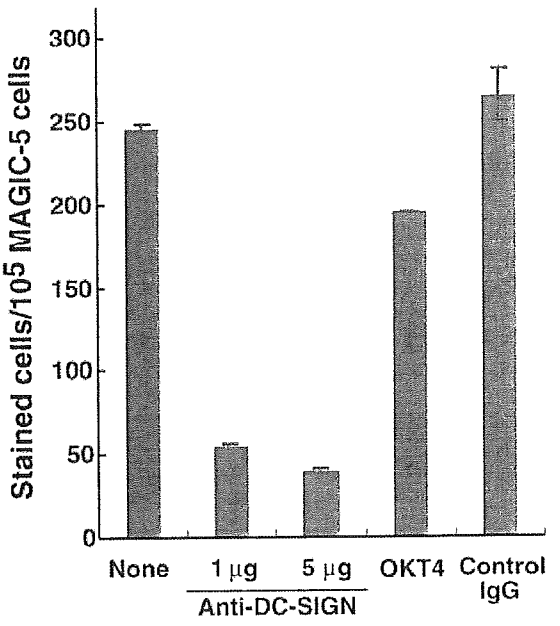

Figure 4. Inhibition of transmission of HIV-1 by NL(AD8)-infected interleukin (IL)–4–stimulated breast-milk macrophages by use of DC-SIGN–specific monoclonal antibody (MAb). IL-4–stimulated breast-milk macrophages were pretreated with 1–5 μmol/L anti–DC-SIGN MAb for 30 min on ice and infected with NL(AD8) for 2 h at 37°C in the presence of the antibody. After being washed 3 times intensively to remove free MAb, 5×10^4 NL(AD8)-infected breast-milk macrophages were added to 10^5 MAGIC-5 cells and further cultured, for an additional 16 h, before X-gal staining. As a control, 5 μmol/L isotype-matched control IgG and anti-human CD4-specific MAb (OKT4) were used for blocking. The data shown are mean ± SD and are representative of 3 independent experiments.

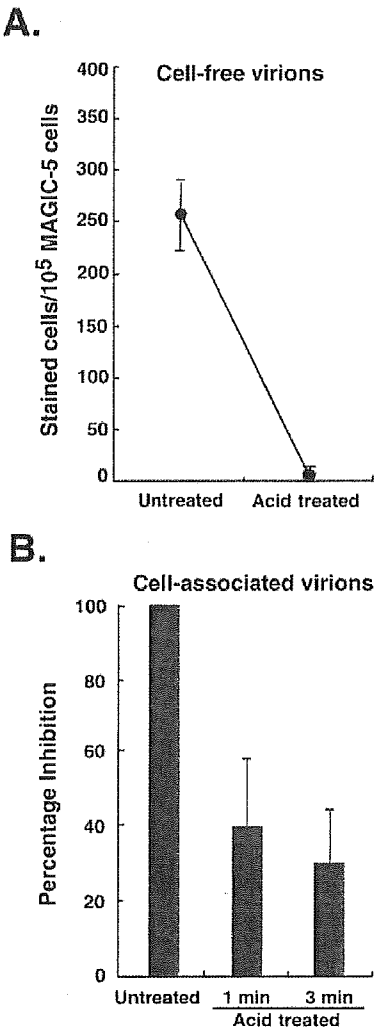

Figure 5. Effect of acidification on transmissibility of cell-free and cell-associated virions. *A*, Cell-free NL(AD8) virions (2 ng/mL p24 antigen) were treated with HCl (pH 3.0) for 1 min at room temperature and immediately neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Then, the acid-treated virions were added to 10^5 MAGIC-5 cells and further cultured, for an additional 16 h, before X-gal staining. The infectivity of the treated virions was evaluated by comparing the no. of blue-stained MAGIC-5 cells with the untreated virions. The data shown are mean ± SD of 3 experiments. *B*, NL(AD8)-infected breast-milk macrophages were washed twice with PBS and incubated with adjusted HCl (pH 3.0) for either 1 or 3 min at room temperature and then promptly neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Cells were washed 3 times with complete culture medium, and then 5×10^4 HIV-1–infected cells, either acid treated or untreated, were added to the wells seeded with 10^5 MAGIC-5 cells. After 16 h, each well was gently washed 3 times, to remove loaded, infected breast-milk macrophages, and the remaining MAGIC-5 cells were stained with X-gal, as described above. The no. of β-gal–positive blue-colored cells was counted, to estimate infectivity. Results are expressed as the percentage change of acid-treated cell no. from each untreated control. The data shown are mean ± SD of 4 experiments, each from a separate donor.

IL-4–stimulated breast-milk macrophages were incubated with HCl (pH 3.0) for 1–3 min at 37°C (figure 5*B*).

DISCUSSION

In the present study, we found that we could detect only a few $CD4^+$ T cells in colostrum/early breast milk and that the majority of $CD3^+$ T cells were $CD14^+$ breast-milk macrophages. Therefore, cells in breast milk can produce and transmit predominantly macrophage-tropic HIV-1, which seems to be a major reason for the dominant spread of R5-type macrophage-tropic virions in vertical transmission of HIV-1 via breast-feeding, if the cellular part contains a critical element for spread of HIV-1. As expected, such $CD4^+$ breast-milk macrophages did express CCR5, were susceptible to NL(AD8), and efficiently secreted the virions at least 3–5 days after infection. In addition to this observation, it has been reported that HIV-1 can infect human mammary epithelial cells that productively release macrophage-tropic HIV-1 virions [6]. Also, possible compartmentalization between blood and breast milk has been shown [5]. Taken together, these findings strongly suggest that R5-type macrophage-tropic HIV-1 might be dominantly produced in the breast milk, and, thus, macrophage-tropic HIV-1 seems to predominantly spread among infants via breast-feeding, probably through breast-milk macrophages.

When breast-milk macrophages were cocultured with IL-4, surface expression of CCR5 was markedly down-regulated, but susceptibility to NL(AD8) was not significantly decreased. This might be because IL-4–stimulated breast-milk macrophages showed a striking augmentation of expression of DC-SIGN, and, thus, the enhancement of virus acquisition would be induced by the *cis* effect of DC-SIGN, as reported elsewhere [22]. However, on day 3, the production of HIV-1 virions by IL-4–stimulated breast-milk macrophages was far less than that by unstimulated macrophages. Nonetheless, on day 3, the transmissibility of the virions to NL(AD8)-sensitive MAGIC-5 cells was dominantly observed in IL-4–stimulated breast-milk macrophages, compared with that in unstimulated macrophages. Therefore, we speculated that high transmissibility was mediated through the virions captured by DC-SIGN but not through cell-free virus particles released by infected cells, although some reports indicate that cell-free HIV-1 in breast milk may contribute to vertical transmission of HIV-1 [25]. Moreover, that cel-free HIV-1 virions may lose their infectivity when treated with HCl (pH 3.0) for 1 min suggests that the transmission of cell-free virions in breast milk may be impeded by gastric acidification, although it may be possible for cell-free virions to infect via oral or esophageal mucosa, which are subjected less to such acidification [26]. In contrast, the infectivity of cell-associated virions captured by DC-SIGN unexpectedly remained after the same acidification procedure. This might be a protective effect mediated by DCs, similar to the previous observation that

HIV-1 captured by follicular DCs is highly infectious, even when attached to neutralizing antibody [27]. Collectively, our present findings strongly suggest that vertical transmission of HIV-1 via breast-feeding may be mediated through cell-associated virions retained by breast-milk macrophages through DC-SIGN, rather than through cell-free virus particles.

That the striking enhancement of expression of DC-SIGN on breast-milk macrophages was induced by stimulation with IL-4 indicates the requirement of local inflammatory changes with Th2 dominancy for the acceleration of transmission of HIV-1 via breast-feeding. Indeed, it has recently been reported that mastitis is linked with a higher breast-milk HIV-1 load, as well as with a greater risk of transmission of HIV-1 via breast-feeding [4]. Thus, DC-SIGN–mediated vertical transmission may be prevented by the establishment and preservation of Th1 dominancy in the breast milk, which would be mediated either by treatment of milk-borne infections with appropriate antibiotics, by direct injection of Th1-type cytokines—such as IL-2, IL-12, or interferon-γ—by manipulating the internal Th1/Th2 balance by use of the polarization of innate NK T cells [28], or by stimulating DCs with poly (I:C) by use of Toll-like receptor (TLR) 3 [29] or with a CpG DNA fragment by use of TLR9 [30]. Those treatments may modify breast-milk macrophages into cells with less ability to transmit HIV-1 virions and with lower-level expression of DC-SIGN. The establishment of methods to reduce expression of DC-SIGN on breast-milk macrophages by maintaining Th1 dominancy in the breast milk may offer another strategy to prevent mother-to-child transmission of HIV-1 via breast-feeding.

References

1. Nduati R, John G, Mbori-Ngacha D, et al. Effect of breastfeeding and formula feeding on transmission of HIV-1: a randomized clinical trial. JAMA **2000**; 283:1167–74.
2. John GC, Nduati RW, Mbori-Ngacha DA, et al. Correlates of mother-to-child human immunodeficiency virus type 1 (HIV-1) transmission: association with maternal plasma HIV-1 RNA load, genital HIV-1 DNA shedding, and breast infections. J Infect Dis **2001**; 183:206–12.
3. Richardson BA, John-Stewart GC, Hughes JP, et al. Breast-milk infectivity in human immunodeficiency virus type 1–infected mothers. J Infect Dis **2003**; 187:736–40.
4. Semba RD, Kumwenda N, Hoover DR, et al. Human immunodeficiency virus load in breast milk, mastitis, and mother-to-child transmission of human immunodeficiency virus type 1. J Infect Dis **1999**; 180:93–8.
5. Becquart P, Chomont N, Roques P, et al. Compartmentalization of HIV-1 between breast milk and blood of HIV-infected mothers. Virology **2002**; 300:109–17.
6. Toniolo A, Serra C, Conaldi PG, Basolo F, Falcone V, Dolei A. Productive HIV-1 infection of normal human mammary epithelial cells. AIDS **1995**; 9:859–66.
7. Smith PD, Meng G, Sellers MT, Rogers TS, Shaw GM. Biological parameters of HIV-1 infection in primary intestinal lymphocytes and macrophages. J Leukoc Biol **2000**; 68:360–5.
8. Meng G, Sellers MT, Mosteller-Barnum M, Rogers TS, Shaw GM, Smith PD. Lamina propria lymphocytes, not macrophages, express CCR5 and CXCR4 and are the likely target cell for human immunodeficiency virus type 1 in the intestinal mucosa. J Infect Dis **2000**; 182:785–91.
9. Rousseau CM, Nduati RW, Richardson BA, et al. Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. J Infect Dis **2003**; 187:741–7.
10. Wolinsky SM, Wike CM, Korber BT, et al. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. Science **1992**; 255:1134–7.
11. van't Wout AB, Kootstra NA, Mulder-Kampinga GA, et al. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. J Clin Invest **1994**; 94:2060–7.
12. Ichikawa M, Sugita M, Takahashi M, et al. Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. Immunology **2003**; 108:189–95.
13. Takeuchi J, Watari E, Shinya E, et al. Down-regulation of Toll-like receptor expression in monocyte-derived Langerhans cell–like cells: implications of low-responsiveness to bacterial components in the epidermal Langerhans cells. Biochem Biophys Res Commun **2003**; 306:674–9.
14. Englund G, Theodore TS, Freed EO, Engleman A, Martin MA. Integration is required for productive infection of monocyte-derived macrophages by human immunodeficiency virus type 1. J Virol **1995**; 69: 3216–9.
15. Shinya E, Hidaka C, Owaki A, et al. Effect of *Nef*-deleted pseudotyped HIV virions bearing an enhanced green fluorescent protein (EGFP) gene in the *env* on HIV-sensitive transformed T cells. Biomed Res **2003**; 24:59–69.
16. Chesebro B, Wehrly K, Nishio J, Perryman S. Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell–tropic isolates: definition of critical amino acids involved in cell tropism. J Virol **1992**; 66:6547–54.
17. Toohey K, Wehrly K, Nishio J, Perryman S, Chesebro B. Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread. Virology **1995**; 213:70–9.
18. Steimer KS, Puma JP, Power MD, et al. Differential antibody responses of individuals infected with AIDS-associated retroviruses surveyed using the viral core antigen p25gag expressed in bacteria. Virology **1986**; 150:283–90.
19. Hachiya A, Aizawa-Matsuoka S, Tanaka M, et al. Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (MAGIC-5). Antimicrob Agents Chemother **2001**; 45:495–501.
20. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell–specific HIV-1–binding protein that enhances trans-infection of T cells. Cell **2000**; 100:587–97.
21. Geijtenbeek TB, van Kooyk Y. DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission. Curr Top Microbiol Immunol **2003**; 276:31–54.
22. Lee B, Leslie G, Soilleux E, et al. *cis* Expression of DC-SIGN allows for more efficient entry of human and simian immunodeficiency viruses via CD4 and a coreceptor. J Virol **2001**; 75:12028–38.
23. Washington N, Spensley PJ, Smith CA, et al. Dual pH probe monitoring versus single pH probe monitoring in infants on milk feeds: the impact on diagnosis. Arch Dis Child **1999**; 81:309–12.
24. Mitchell DJ, McClure BG, Tubman TR. Simultaneous monitoring of gastric and oesophageal pH reveals limitations of conventional oesophageal pH monitoring in milk fed infants. Arch Dis Child **2001**; 84:273–6.
25. Lewis P, Nduati R, Kreiss JK, et al. Cell-free human immunodeficiency virus type 1 in breast milk. J Infect Dis **1998**; 177:34–9.
26. Moore JS, Rahemtulla F, Kent LW, et al. Oral epithelial cells are susceptible to cell-free and cell-associated HIV-1 infection in vitro. Virology **2003**; 313:343–53.

27. Heath SL, Tew JG, Szakal AK, Burton GF. Follicular dendritic cells and human immunodeficiency virus infectivity. Nature **1995**;377:740–4.
28. Boyson JE, Rybalov B, Koopman LA, et al. CD1d and invariant NKT cells at the human maternal-fetal interface. Proc Natl Acad Sci USA **2002**;99:13741–6.
29. Fujimoto C, Nakagawa Y, Ohara K, Takahashi H. Polyriboinosinic polyribocytidylic acid [poly(I:C)]/TLR3 signaling allows class I processing of exogenous protein and induction of HIV-specific $CD8^+$ cytotoxic T lymphocytes. Int Immunol **2004**;16:55–63.
30. Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H. CpG-DNA–mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. J Immunol **2000**;165:1228–35.

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Langerhans Cells Stimulated by Mechanical Stress Are Susceptible to Measles Virus Infection

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Key Words
Measles virus · Langerhans cells · Dendritic cells · CD150 · CD83 · Mechanical stress

Abstract
Objective: Measles virus (MV) first infects the human respiratory tract, but the initial target cells are unknown. We examined whether MV infects Langerhans cell-like dendritic cells (LCs) generated from $CD14^+$ monocytes in the presence of GM-CSF, IL-4, and TGF-β1. ***Methods:*** Cultured LCs were established as described recently [Biochem Biophys Res Commun 2003;306:674–679]. The expression of immunological markers was detected by FACScan. Infection with MV was assessed by syncytia formation, viral-specific fluorescence, and Western blotting. ***Results:*** MV did not infect and replicate the freshly established, unstimulated LCs expressing CD1a, E-cadherin and Langerin but not CD83. Also, CD150, a receptor for MV was not expressed on the surface of the LCs. However, LCs stimulated by mechanical stress such as washing and centrifugation became susceptible to MV infection. ***Conclusion:*** A subset of mechanically stimulated LCs but not unstimulated immature ones became susceptible to MV. The actual role of Langerhans cells in local immunity seems to be to suppress unfavorable reactions initiated by virus intrusion.

Introduction

Measles is a major cause of childhood mortality in developing countries despite the existence of live attenuated vaccines. Such mortality is caused by immune suppression related to the dysfunction of professional antigen-presenting cells such as dendritic cells (DCs) infected with measles virus (MV) [1]. Infection by MV has been exclusively studied in tissue cultures using DCs isolated from peripheral blood [2, 3], Langerhans cells isolated from fresh skin [4], DCs generated in vitro from monocyte precursors or $CD34^+$ stem cells [5], and monocytes [6]. However, it is not known whether subsets of DCs are the targets for MV infection. DCs are a heterogeneous group of professional antigen presenting cells found in most lymphoid and nonlymphoid organs. They exist in different states of activation ranging from immature cells specialized for antigen uptake to mature cells for stimulating T cells [7–9]. Langerhans cells were shown to be a subset of immature DCs different from conventional DCs in their life cycle [10], and route of maturation and migration [11]. Such Langerhans cells dominantly express E-cadherin, Langerin and CLA, but DCs do not [12]. In addition, skin-derived Langerhans cells are expressing CD1a and HLA-DR, but not costimulatory molecules like CD80, CD86 that must be required for T cell activation, or CD83, a marker for mature DCs.

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Recently, it has been reported that freshly isolated Langerhans cells, immature DCs located in skin and mucosal epithelia, were susceptible to MV infection similar to human DCs derived from $CD34^+$ progenitors in the presence of GM-CSF and TNF-α [4]. However, we have recently found that the responsiveness of Langerhans cell-like dendritic cells (LCs) generated from $CD14^+$ monocytes obtained from peripheral blood in the presence of GM-CSF, IL-4, and TGF-β1 to bacterial components was far weaker than that of DCs, indicating that LCs must be downregulated to pathogen invasion in comparison with DCs to prevent local hypersensitivity [12].

Thus, the purpose of the present study was to confirm whether MV actually infects and replicates in peacefully situated immature LCs similar to DCs. Here, we show that MV did not replicate in monocyte-derived LCs expressing CD1a, E-cadherin and Langerin, but not CD83, whereas it did infect LCs stimulated by mechanical stress such as washing and centrifugation (cfgLCs).

Materials and Methods

Media, Reagents, and Antibodies

Cultured cells were grown in RPMI1640 (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 2 m*M* L-glutamine (Gibco BRL, Grand Island, N.Y., USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco BRL), and 10% heat-inactivated fetal calf serum (FCS), referred to as complete medium [13]. Recombinant human GM-CSF was purchased from Pepro Tech (Rocky Hill, N.J., USA), recombinant human IL-4 from Biosource International (Camarillo, Calif., USA), and recombinant human TGF-β1 from R&D Systems (Minneapolis, Minn., USA). Lipopolysaccharide from *Escherichia coli* (026:B6 and 0127:B8) was obtained from Sigma-Aldrich (Buchs SG, Switzerland). Anti-CD1a monoclonal antibody (mAb; HI149), and PE-conjugated anti-CD83 mAb (HB15e) were purchased from BD PharMingen (San Diego, Calif., USA). Anti-E-cadherin mAb (HECD-1) was obtained from Takara (Shiga, Japan), and anti-langerin mAb (DCGM4) was obtained from Immunotech (Marseille, France). Hybridoma clone (1D6) secreting mAb to MV-hemagglutinin was obtained after a fusion of NS-1 cells and spleen cells of a BALB/c mouse immunized with purified MV. MAb were purified by affinity chromatography using Protein A-Sepharose 4 Fast Flow according to the procedure recommended by the manufacturer (Pharmacia Biotech, Wilstroms, Sweden). FITC-conjugated goat anti-mouse IgG antibody was obtained from Immunotech.

Culturing of Peripheral Blood Monocytes

LCs and DCs were prepared as previously described [12, 14]. In brief, peripheral blood mononuclear cells (PBMCs) were freshly isolated with Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) from the peripheral blood of healthy volunteers. $CD14^+$ monocytes were immediately separated from PBMCs using a monocyte isolation kit (Miltenyi Biotec, Gladbach, Germany) containing hapten-conjugated antibodies against CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs and a magnetic cell separator (Miltenyi Biotec) according to the manufacturer's instructions. This procedure resulted in >90% purity of $CD14^+$ cells. These cells were cultured in 24-well culture plates for 6 days in complete medium supplemented with either 200 ng/ml GM-CSF, and 10 ng/ml IL-4 to obtain DCs, or 200 ng/ml GM-CSF, 10 ng/ml IL-4, and 10 ng/ml TGF-β1 to obtain LCs. On days 2 and 4, fresh medium supplemented with the same combination of cytokines was added, respectively. After 6 days of culturing, the cells were resuspended in either 48-well or 96-well culture plates at 5×10^5 cells/ml in complete medium supplemented with the same combination of cytokines. The cells were further incubated for an additional 48–72 h in complete medium supplemented with the same combination of cytokines.

Virus Preparation

Vero cells (Dainihon Seiyaku Co., Osaka, Japan) were grown in Eagles' Minimum Essential Medium (Gibco BRL) supplemented with 10% heat-inactivated FCS and 2 m*M* glutamine. The Edmonton strain Rapp clone (R-5) of MV, which forms giant plaques, was grown in Vero cells [15]. Infective titers were determined by $TCID_{50}$ in Vero cells grown in 96-well cluster plates (Sumitomo Bakelite Co., Tokyo, Japan). 0.1 ml of 6-fold serially diluted samples were added to Vero cells, and the cells were then overlaid with minimum essential medium containing 10% FCS and incubated in 5% CO_2 at 37 °C for 7 days. $TCID_{50}$ was calculated by Reed and Muench's method [16].

MV Infection and Detection

DCs and LCs were infected with 0.1 PFU/cell Vero cell-derived MV R-5 clone on day 6 as previously described [17]. The cells were then cultured for 72 h without washing to remove the unutilized virus. In some cases, LCs were washed three times with phosphate-buffered saline (PBS) by centrifugation at 1,300 rpm (320 *g*) for 10 min (cfgLCs) before infection with MV.

Flow Cytometric Analysis of LCs and DCs

For single-cell flow cytometry analysis, 5×10^4 cells were incubated for 30 min at 4° in PBS containing 2% human AB serum, 0.01 *M* sodium azide and the aimed mAbs at saturating concentrations, or with isotype-matched negative control mAbs at the same concentration. After being washed twice, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibody for 30 min at 4° in the same buffer. Then, the labeled cells (1×10^4) were analyzed with a FACScan (Becton-Dickinson, San Jose, Calif., USA) using Cell Quest software (Becton-Dickinson).

Immunofluorescence Microscopy

Cells were adhered onto glass slides using cytospin, mixed with 2% formaldehyde in PBS, and permeabilized with 0.2% saponin. The permeabilized cells were incubated with the anti-MV-H mAb, followed by FITC-conjugated anti-mouse IgG antibody, and then examined under a fluorescence microscope.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

DCs and LCs (5×10^5) were infected with MV for 72 h at 37° in 5% CO_2. The cells were washed twice with 10 m*M* tris buffer containing 10 m*M* NaCl and 1.5 m*M* $MgCl_2$ (pH 7.4) and solubilized in 300 μl of sodium dodecyl sulfate (SDS) sample buffer containing 2% SDS, 10% glycerol and 5% 2-mercaptoethanol (2-ME). Cell lysates were boiled for 5 min and 20-μl samples were separated by 13% SDS-

polyacrylamide gel electrophoresis according to the method of Laemmli [18]. Samples were run for 90 min at 1 W, and then electrophoretically transferred to nitrocellulose membranes using a Trans Blot Cell (Atto Co., Tokyo, Japan). Transfer was performed at 30 V overnight, in Tris buffer containing 12.4 m*M* Tris, 192 m*M* glycine, and 20% (v/v) methanol.

To perform Western blotting, filters were washed with PBS for 20 min and blocked at room temperature for 2 h with 3% bovine serum albumin. The filters were then rinsed three times with PBS containing 0.05% Tween 20, and incubated for one hour at room temperature with a 1:200 diluted rabbit antimeasles antibody in PBS containing 1% bovine serum albumin. After washing three times with PBS-Tween 20, the filters were incubated for an additional one hour at room temperature with 1:1,000 diluted peroxidase-conjugated goat antirabbit antibody (Jackson Lab., Pa., USA) in PBS containing 0.1% bovine serum albumin. Finally they were washed three times with PBS-Tween 20, and developed with HRP Color Development Reagent (Wako Co., Japan) and H_2O_2.

T Cell Proliferation

Mixed LC-lymphocyte reactions were prepared as previously described with several modifications [19, 20]. Briefly, both LCs and cfgLCs were harvested on day 3 of MV infection, washed three times extensively to remove free virions, and were used as stimulators for allogeneic mixed lymphocyte reactions (allo-MLR). As for responders, nylon column purified, nonadherent T cells obtained from PBMCs of healthy donors were used. The purified T cells (2×10^5/well) were stimulated with 2,500 rad-irradiated various LC-preparations (5×10^3/well) in 200 µl of complete medium in 96-well U-bottom microplates at 37° in a 5% CO_2 incubator. After 3 days, 10 µl of 3-(4.5-dimethylthioazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) solution were added to the culture. Four hours later, 100 µl of acidic isopropanol was added to the wells and the liquid was mixed to dissolve the blue crystals. MTT substrate was cleaved in the mitochondria of living cells to yield a dark-purple formazan product, and its absorbance (595 nm) was measured with an enzyme-linked immunosorbent assay plate reader (Bio-Rad, Hercules, Calif., USA).

Results

Analysis of the Surface Phenotype for the Generated LCs

To examine whether monocyte-derived immature LCs were susceptible to MV, we prepared both LCs and DCs from human PBMCs in the presence of GM-CSF, IL-4 and TGF-β1 or GM-CSF and IL-4, respectively. The generated DCs expressed CD1a uniformly, but only marginal levels of E-cadherin were detected in 10% of the cells (right panels of fig. 1). In contrast, the cultured LCs expressed CD1a as well as langerin, and E-cadherin, but not CD83, a marker for mature DCs (left panels of fig. 1). The generated LCs showed typical dendritic morphology and expressed Birbeck granules observed by electron and immunofluorescence microscopy as reported recently [12]. These LCs were phenotypically similar to the epidermal Langerhans cells. However, it is important to note that the expression of MHC class II, CD83 and CD86 was variable on the LCs among the distinct batches of FCS after 40 h exposure to 10 ng/ml lipopolysaccharide although 95% of monocyte-derived DCs expressed the molecules with similar culturing (data not shown). Thus, selection of the serum batch is critical for generating immature LCs from $CD14^+$ monocytes.

MV Receptor-Expression on the Surface of the Generated DCs and LCs

Next, we examined the expression of critical receptors for MV entry such as CD46 and CD150 on the surface of the generated LCs and DCs. As long as we handled them gently, the freshly isolated LCs did not express CD150 receptor at all (left lower panel of fig. 2A), whereas DCs did express the CD150 at high levels (right lower panel of fig. 2A), although they both expressed CD46, a receptor for MV attaching (upper panels of fig. 2A). In contrast, when the LCs were washed three times with centrifugation (cfgLCs), although the magnitude was small, they turned out to express MV receptor, CD150 (right panel of fig. 2B).

Syncytia Formation of Immature DCs and LCs Infected with MV

To examine whether MV actually replicates in PBMC-derived LCs and DCs to form syncytia, the cultured cells (5×10^5 cells/ml) were infected with MV at MOI 0.1. As represented in figure 3, although the LCs did not show any syncytia formation 3 days after the culture in the presence of GM-CSF, IL-4 and TGF-β1 (A, D), DCs showed syncytia when infected with MV (E) in comparison with uninfected control (B). Thus, the cultured LCs do not seem to be susceptible to MV infection. However, when the LCs were washed twice with centrifugation, the mechanically stimulated LCs (cfgLCs) (C) became susceptible to MV infection to form syncytia (F).

This was confirmed at a single cell level by staining with fluorescein-labeled anti-MV-H mAb. As shown in figure 4, among immature DCs (B and E) and cfgLCs (C, F), MV-H-positive cells were detected as infected cells but at less than 10% the frequency of LCs. The generated DCs and cfgLCs showed susceptibility to infection with MV as demonstrated in figure 4E, F, respectively. However, syncytia formation was not observed by virus-specific fluorescein labeling in immature LCs (A) after incubation with MV for 3 days (D).