```
MN      CTRPNYNKRKRIHI  GPGRAFYTTKNIIGTIRQAHC
BZ167   -----NKA-R--R-  ----T---G- -V-D----Y-
SF2     ---LSN-T--C-PL  ----V--A-DI- -D------
CI2     ----SN-T-R----  ---------RQ-R-D------
MNp     -----N-R-T----  --------------------

89.6P   -----N-T-E-LS-  -------ARR----D------
SF33    -----N-R-R--TS  ---KVL---GE---D--K-Y-
VI131   -----N-T-QSV--  ---Q---A-GDV--D------
IIIB    -----N-T---KS-QR------V-IGK- -NM-----
```

FIG. 5. Alignment of the amino acid sequences of HIV-1 Env V3 from primary and laboratory isolates. The spaces indicate amino acid deletions; dashes indicate homology. The V3 motif of a neutralization-sensitive HIV-1 strain is enclosed in the shaded rectangle (37).

crease in viral load following challenge, and the levels remained high until the animal was sacrificed. These results demonstrate that vaccination with rBCG Env V3 can induce protective immunity in rhesus macaques against a low-dose challenge with SHIV-MN.

Challenge with high-dose SHIV-MN. The second group of eight macaques (R-09 through R-16) was similarly challenged with a higher dose (200 $TCID_{50}$) of SHIV-MN by intravenous inoculation at 24 weeks p.i. (Fig. 6). Measurements of the viral loads in PBMC and plasma indicated that all the macaques were infected by the high-dose SHIV-MN challenge. However, the level of viremia during the acute phase of viral infection was reduced by 1 to 2 log units in macaques immunized with rBCG Env V3 compared with controls (from 10^6 to 10^7, to $<10^5$ to 10^4 RNA copies/ml) (Fig. 6A). The control macaques developed a transient decrease in $CD4^+$-T-cell counts that rebounded to normal levels ~3 weeks postchallenge (Fig. 6B). In contrast, macaques vaccinated with rBCG Env V3 had little or no change in $CD4^+$-T-cell numbers.

Despite the low levels of V3 peptide-specific IFN-γ ELISPOT activities noted for animals R-09 and R-10 above (Table 2), these animals exhibited a plasma viral load and a rate of $CD4^+$-cell loss after SHIV challenge that was comparable to those seen in the immunized animals designated R-11, -12, and -13. Thus, immunization with rBCG Env V3 generated even low levels of T-cell responses in only 2 animals out of 5 in this group and out of a total of 15 immunized animals. No evidence of higher virus-specific IFN-γ ELISPOT activity was demonstrated in samples obtained 0, 4, or 6 and 24 weeks after vaccination (Table 2), suggesting that few significant cellular anti-SHIV responses were generated and that those few did not affect virus control in this macaque population.

Challenge with pathogenic SHIV-89.6PD. The third group of macaques (R-17 through R-24) was challenged with pathogenic SHIV-89.6PD (20 $TCID_{50}$) 24 weeks postinoculation. The effects of vaccination with rBCG Env V3 on immune induction against the pathogenic virus were followed for 32 weeks, and the macaques were then autopsied. As shown in

TABLE 2. SHIV-MN-specific serum IgG neutralization titers and Env V3-specific ELISPOT responses[a]

Monkey no.	Immunogen	IC_{50} of neutralization serum IgG (μg/ml)[b]			V3-specific IFN-γ SFCs/10^6 cells[c]		
		0 week	4 or 6 weeks[d]	24 weeks[e]	0 week	4 or 6 weeks	24 weeks
R-01	rBCG Env V3	>50	0.5	0.6	<20	30	20
R-02	rBCG Env V3	>50	0.3	0.4	<20	40	40
R-03	rBCG Env V3	>50	0.5	0.6	<20	40	30
R-04	rBCG-Env V3	>50	0.2	0.3	<20	20	40
R-05	rBCG-Env V3	>50	0.08	0.3	<20	30	80
R-06	rBCG-α	>50	>50	>50	<20	<20	<20
R-07	rBCG-α	>50	>50	>50	<20	<20	<20
R-08	rBCG-α	>50	>50	>50	<20	<20	<20
R-09	rBCG-Env V3	>50	0.04	0.3	<20	180	120
R-10	rBCG-Env V3	>50	0.1	0.2	<20	160	110
R-11	rBCG-Env V3	>50	0.05	0.2	<20	20	30
R-12	rBCG-Env V3	>50	0.03	0.4	<20	60	20
R-13	rBCG-Env V3	>50	0.02	0.4	<20	30	30
R-14	rBCG-α	>50	>50	>50	<20	<20	<20
R-15	rBCG-α	>50	>50	>50	<20	<20	<20
R-16	rBCG-α	>50	>50	>50	<20	<20	<20
R-17	rBCG-Env V3	>50	0.2	0.6	<20	40	90
R-18	rBCG-Env V3	>50	0.3	0.3	<20	50	60
R-19	rBCG-Env V3	>50	0.3	0.4	<20	40	30
R-20	rBCG-Env V3	>50	0.5	0.7	<20	20	50
R-21	rBCG-Env V3	>50	0.4	0.5	<20	20	40
R-22	rBCG-α	>50	>50	>50	<20	<20	<20
R-23	rBCG-α	>50	>50	>50	<20	<20	<20
R-24	rBCG-α	>50	>50	>50	<20	<20	<20

[a] Animals were inoculated with either rBCG Env V3 or the vector control. Blood samples were obtained at 0, 4, or 6 and 24 weeks p.i., and antibody inhibitory concentration and the V3-specific IFN-γ ELISPOT activity were compared.
[b] The IC_{50} was derived from the data in Fig. 2 based on neutralization dose-response curves similarly obtained from Fig. 3.
[c] Freshly isolated PBMC were assessed for their ability to produce IFN-γ in response to $HIV\text{-}1_{MN}$ Env V3 peptide.
[d] Mean IC_{50}s: R-01 to R-05, 0.32; R-09 to R-13, 0.05; R-17 to R-21, 0.35.
[e] Mean IC_{50}s: R-01 to R-05, 0.44; R-09 to R-13, 0.30; R-17 to R-21, 0.50.

TABLE 3. Comparison of low-dose SHIV-MN infections in macaques vaccinated with either rBCG Env V3 or rBCG-α (control)

Monkey	Immunogen (10 mg)	Efficacy analysis	Results[a]							
			0[b]	2	4	6	8	10	12	16
R-01	rBCG Env V3	Virus isolation	<1	<1	2	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	>500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	20,000	<500	<500	<500	<500	<500
R-02	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-03	rBCG Env V3	Virus isolation	<1	32	<1	<1	2	<1	ND	ND
		Provirus by PCR	<500	>500	<500	<500	>500	<500	ND	ND
		Plasma viral load	<500	310,000	<500	<500	20,000	<500	ND	<500
R-04	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	ND	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	ND	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-05	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-06	rBCG-α	Virus isolation	<1	32	16	<1	2	2	<1	1<
		Provirus by PCR	<500	>500	>500	>500	>500	>500	>500	500
		Plasma viral load	<500	300,000	50,000	20,000	20,000	20,000	20,000	20,000
R-07	rBCG-α	Virus isolation	<1	2	32	<1	<1	<1	<1	ND
		Provirus by PCR	<500	>500	>500	>500	>500	<500	<500	ND
		Plasma viral load	<500	27,000	310,000	350,000	25,000	<500	<500	<500
R-08	rBCG-α	Virus isolation	<1	32	16	<1	2	2	ND	ND
		Provirus by PCR	<500	>500	>500	>500	>500	>500	ND	ND
		Plasma viral load	<500	300,000	50,000	<500	20,000	20,000	ND	<500

[a] Viral loads were determined by either limiting dilution of PBMC or competitive PCR for HIV-1 Env V3 genes, and the results are expressed as the number of infected cells per million PBMC and virus copies per milliliter of blood. Nested PCR for HIV-MN Env V3 was used in all macaques to detect the provirus genome. Naïve macaques were injected intravenously with 20 $TCID_{50}$ of SHIV-MN and used as controls for SHIV infection. The results are expressed as the mean of three different assays; <1, <500, and <500 were the detection limits of virus isolation, proviral copy number, and plasma viral load, respectively. ND, not determined.
[b] Weeks after challenge.

Fig. 7, high levels of plasma viremia were detected in the control macaques, with a viral set point of ~10^6 RNA copies/ml, accompanied by an abrupt decline in $CD4^+$-T-cell counts. Prior vaccination with rBCG Env V3 appeared to have no positive effect on the viral load and $CD4^+$-T-cell counts compared with the control animals.

Association of in vitro neutralization antibody responses following rBCG Env V3 immunization with control of viremia after SHIV challenge. Of the macaques challenged with low doses of homologous SHIV-MN (group 1), the three virus-controlled macaques R-02, -04, and -05 (Table 1) had higher IC_{50}s of SHIV-MN-specific neutralizing antibodies as measured in M8166 cells at 24 weeks p.i. or on the day of challenge, with serum IgG concentrations of 0.4, 0.3, and 0.3 μg/ml, respectively (Table 2). The IC_{50}s of the uncontrolled macaques R-01 and -03 (Table 1) were both 0.6 μg/ml (Table 2).

When the challenge dose was increased 10-fold (Fig. 1), all five animals in group 2 had high neutralizing antibody titers with a mean IC_{50} of 0.30 μg/ml on the day of challenge (Table 2). These animals in group 2 showed partial protection against the same homologous virus challenge (Fig. 6). In contrast, no animals similarly immunized with rBCG elicited any in vivo protection against a low-dose, heterologous viral challenge with SHIV-89.6PD (Table 2 and Fig. 7).

In summary, the rBCG Env V3-elicited NAb response afforded some degree of protection against a homologous viral challenge. However, infection by the heterologous virus SHIV-89.6PD was not controlled by heterologous virus SHIV-MN- or $HIV\text{-}1_{MN}$-specific NAb generated by the recombinant $HIV\text{-}1_{MN}$ Env V3-expressed BCG immunization.

DISCUSSION

First, our study demonstrates the potential of anti-Env V3 NAb induced by immunization of rhesus macaques with rBCG Env V3 to afford protection against homologous challenge with SHIV-MN but not against the heterologous SHIV-89.6PD. With the low-dose homologous SHIV-MN challenge (20 $TCID_{50}$), sterile protection was achieved in three of five immunized animals. These findings correlate well with our in vitro neutralization data for these animals. Protected animals showed higher levels of potent neutralization antibodies than did unprotected animals. Macaques serving as vector and naïve controls experienced high levels of replication of the SHIV-MN challenge virus. With a high-dose challenge, rBCG Env V3 vaccination was effective at reducing viremia during acute infection by ~100-fold. The vaccine consisted of an rBCG vector that expresses a chimeric HIV-1 Env V3 region peptide and the α-antigen of *M. bovis*. The kinetics and magnitude of the HIV-1 Env V3-specific antibody responses elicited in macaques were comparable to those observed in our previous studies using guinea pigs vaccinated with rBCG Env V3 (9, 16).

Secondly, the levels of neutralizing antibodies generated after injection with a recombinant BCG vector-based vaccine expressing a chimeric protein of HIV-1 Env V3 peptide and α-antigen protein were maintained for at least 24 weeks p.i. with no diminishment in titer. A plausible explanation for the longevity of the neutralizing antibody titers after rBCG immunization is that the carrier protein, α-antigen (also known as MPT59 or antigen 85B), is derived from mycobacteria and has

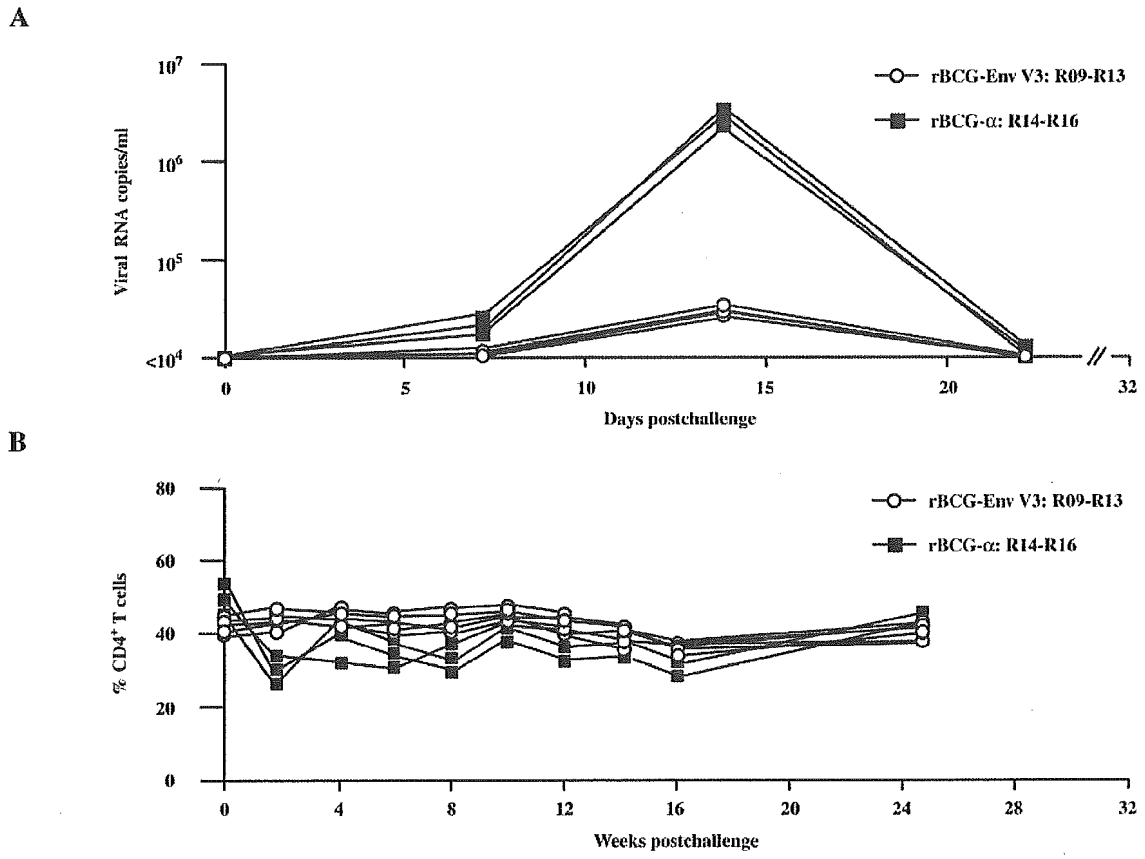

FIG. 6. Comparison of infection kinetics following high-dose (200 $TCID_{50}$) inoculation of SHIV-MN in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Viral RNA copy number per milliliter of serum. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

the ability to elicit potent Th1-type immune responses (24, 43). Our result is consistent with those of other groups, which have shown that BCG immunity is maintained for at least a few years and that the BCG bacillus is effective at increasing NAb responses (40). These characteristics might help to explain the long-lasting enhanced levels of NAb elicited by vaccination with rBCG Env V3.

The concentration of purified macaque IgG in serum was determined to be ~10 mg/ml. By this estimation, 0.5 mg corresponds to a serum dilution of 1:1 in virus neutralization assays. The IC_{50} and IC_{90} values for neutralization of SHIV-MN were 10^3 to 10^4 and 166, respectively (similar values were obtained for neutralization of HIV-1_{MN}). These neutralization titers suggest that antibody responses generated de novo may contribute to a degree of protection against SHIV-MN. The observed relationship of the NAb titer and viral protection is consistent with results obtained by repeated immunization with SHIV-89.6 C4-V3 peptides in guinea pigs and rhesus macaques (6, 27). In this case, NAb titers to homologous SHIV-89.6 were ~10^3 greater than those against heterologous HIV-1_{MN}, while responses to HIV-1 R5 viruses were weak or absent. This suggests that the protection mediated by a C4-V3 peptide vaccine against SHIV-89.6 may be type (or strain) specific. Thus, we assume that the NAb generated by SHIV-89.6 C4-V3 peptide immunization (6) would not mediate protection against a heterologous SHIV-MN challenge.

The present study suggests that the vaccine-elicited antibodies directed against the HIV-1 Env V3 peptide can in some cases confer a degree of neutralization against primary isolates of HIV-1 (26). Following vaccination of rhesus macaques with rBCG Env V3, both binding and NAb responses against this novel construct were clearly evident. At the time of SHIV challenge, immune sera from the vaccinated macaques efficiently neutralized a homologous, type-specific TCLA HIV-1 strain (HIV-1_{MN}) and a related SHIV strain (SHIV-MN) with IC_{90} values of <5 μg/ml. Controls, including preimmune sera and sera from macaques vaccinated with rBCG vector alone, had no neutralizing activity in assays using GHOST cells expressing either CCR5 or CXCR4 or in M8166 cells. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize several primary HIV-1 X4 isolates (HIV-1_{BZ167}, HIV-1_{SF2}, and HIV-1_{CI2}); however, neutralization of an X4-R5 dual-tropic strain (HIV-1_{MNp}) was weak. No neutralization of HIV-1 R5 isolates and primary HIV-1 isolates from different clades was observed. These findings were confirmed in an independent international neutralization trial (conducted by Simon Beddows and Jonathan Weber, Imperial College School of Medicine, Medical Research Council, London, En-

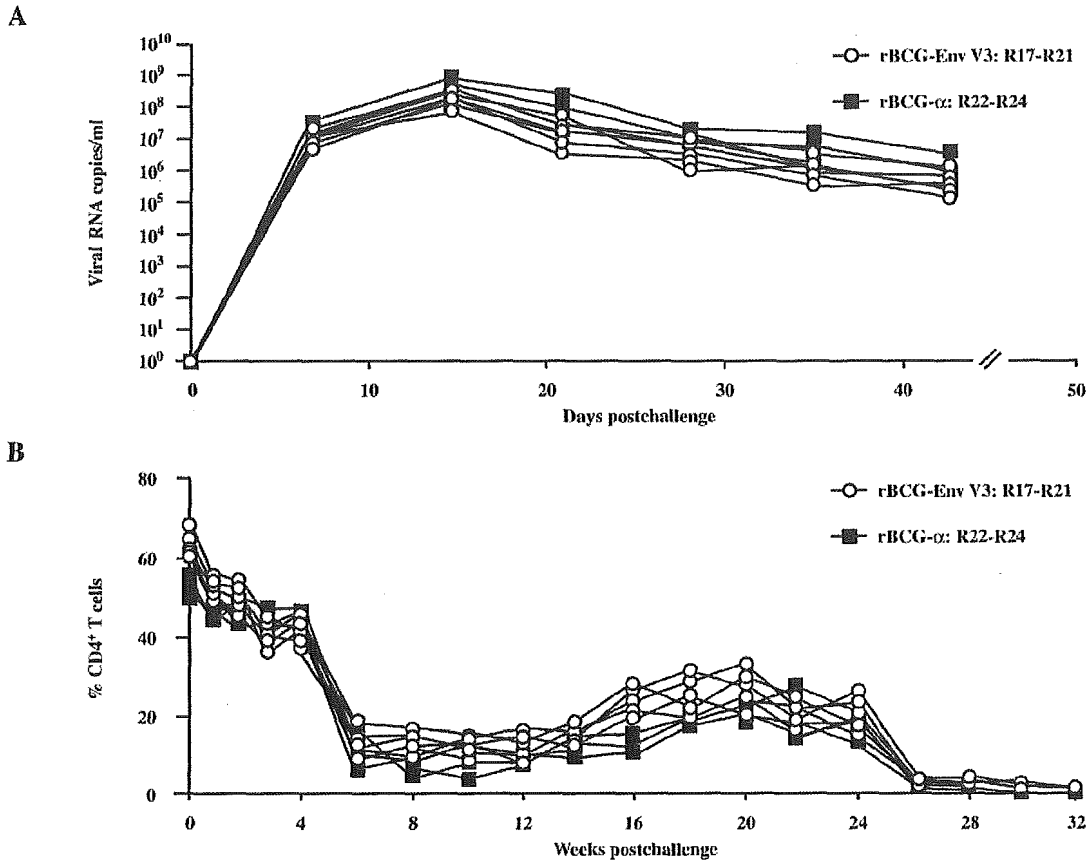

FIG. 7. Comparison of infection kinetics following challenge with pathogenic SHIV-89.6PD in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Plasma viral-RNA copy numbers per milliliter. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

gland, and Pia Scott and Eva-Maria Fenyo at Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden). Preliminary results from this study have had been summarized and reported (11). Despite similarities in the V3 sequence motif, neutralization of the TCLA strain HIV-1_{MN} was found to be 10- to 50-fold more sensitive than neutralization of primary HIV-1 isolates, such as HIV-1_{CI2}, HIV-1_{MNp}, or HIV-$1_{JR\text{-}CSF}$ (11). A reasonable explanation for the relative insensitivity of primary HIV-1 isolates—particularly primary HIV-1 R5 isolates—to neutralization is the presence of cryptic or occluded sites within the virus-associated V3 region (13, 53).

In the Japanese consensus HIV-1 Env V3 expressed in the rBCG construct, the core V3 motif of the neutralization epitope is IHIGPGRAF (39). Although the consensus sequence of the V3 loop differs from the MN-V3 sequence in five amino acid positions, the neutralization epitope of the tip V3 region in the Japanese consensus is identical to that of MN-V3. Some substitutions of amino acids at certain positions within this motif (for example, H to R and A to T in the core motif in BZ167) are tolerated, suggesting that NAbs generated by immunization with rBCG Env V3 are not strictly type specific. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize primary HIV-1 X4 and some HIV-1 X4-R5 dual-tropic isolates, suggesting that the antigenic structure of the chimeric V3 peptide mimics to some extent that of the virus-associated V3 region. Indeed, the chimeric V3–α-antigen protein is estimated to be 38 kDa and contains four cysteine residues, suggesting the possible formation of a new loop structure in the V3 portion of the protein. With regard to the heterologous SHIV-89.6PD challenge in macaques vaccinated with rBCG Env V3, NAbs specific for SHIV-89.6PD were not generated efficiently (IC_{50}, >50 μg of immune serum IgG/ml) and did not provide any protection against the SHIV-89.6PD challenge. The V3 neutralization site of SHIV-89.6PD may differ in sequence or structure or both from that of SHIV-MN or other viral strains, including some of the HIV-1 isolates, making it unrecognizable to antibodies. Such a difference could account for the poor cross-neutralization activity against SHIV-89.6PD.

Thus, our data from the SHIV-macaque models show that the in vitro neutralization titers generated in rBCG-immunized animals correlate with protection. Although a present goal of HIV-1 vaccine development is to reduce the viral set point by eliciting high levels of virus-specific cellular immune responses, induction of cross-reactive NAbs may also contribute to control virus replication in the course of HIV-1 infection and may therefore be useful in the context of a preventive vaccine. Furthermore, although the choice of HIV Env V3 and the

autologous challenge virus SHIV-MN are unlikely to provide information that predicts efficacy in humans, the results presented here demonstrate that recombinant BCG vectors have the potential to deliver a more appropriate immunogen for desirable immune elicitations.

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ELSEVIER

Studies on the Most Efficient Vector Systems for Gene Transduction Into Dendritic Cells

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ABSTRACT

Specialized antigen-presenting cells (APC), known as dendritic cells (DC), play a pivotal role in initiating primary immune responses. Several vector systems, including adenoviral vectors, retroviral vectors, hemagglutinating virus of Japan-related vectors, and the electroporation, have been shown to transduce genes into mouse and human but not rat DC. However, there is no direct evidence to support the view that the currently used vector systems are able to transduce genes into mature DC. Inasmuch as most, if not all, gene transfer studies investigating DC or DC-related cell populations are performed employing heterogeneous-groups of cells, it is therefore important to determine the extent to which gene transduction occurs in bona fide DC. In this study, we provide evidence that none of these vector systems are able to transfer genes into mature rat DC, which are derived from bone marrow cells (BMC), driven by Flt3/Flk2 ligand and IL-6, and purified with CD161a. Nevertheless, the most efficient gene transduction was observed with developing DC progenitor cells during long-term culture of rat BMC. Successful gene transfer was achieved after 2-week culture with an HIV-based lentiviral vector system.

IT HAS BEEN WELL ESTABLISHED that the specialized antigen presenting cells, dendritic cells (DC), play a pivotal role in initiating a primary immune response. It has been reported that several vector systems, including adenoviral vectors, retroviral vectors, hemagglutinating virus of Japan (HVJ)-related vector, and the electroporation, are able to transduce the genes into the DC into mouse and human, but not rat DC. In addition, it appears that the reported studies did not always employ highly purified DC. To our best knowledge, there has been no direct evidence to support the view that the currently used vector systems are able to effectively transduce genes into DC. Inasmuch as most, if not all, DC or DC-related gene-transfer studies are performed by employing heterogenous cell populations, it is important to determine the extent to which each vector system transduces the gene into bona fide DC.

MATERIALS AND METHODS

Employing human Flt3/Flk2 ligand and IL-6, a rat DC generation system was first established from long-term bone marrow cell culture as previously described.[1] Rat CD161a-positive (CD161a+) DC were highly purified by autoMACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). The DC-enriched cell population was heterogeneous: the percentage of DC was 40% to 60% including DC progenitors and mature DC. The selected CD161a+ DC were almost homogeneous with a purity of more than 95%. They were mature but not always stimulated DC. We used four vectors to treat DCs generated by this culture and separation system: adenoviral vectors, lentiviral vectors, inactivated HVJ-Envelope vectors (Hemagglutinating Virus of Japan-Envelope vector kit: GenomONE, Ishihara Sangyo Kaisha, Osaka, Japan), and nucleoporation (a electroporation method, human dendritic cell kit of the Nucleofector, Amaxa Biosystems, Cologne, Germany), which contains the Enhanced Green Fluorescence Protein (EGFP) gene. The most efficient conditions for successful gene transduction were determined.

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Table 1. Summary of Tranduction Results in Rat Dendritic Cells

Vector System	Target Cell	MOI	Transduction	Percentage
Adenovirus	(1) DC-rich	50, 100	O	9%–16%
	(2) CD161a DC	50, 100	x	—
Lentivirus	(1) DC-rich	2 × 3	O	84%
	(2) CD161a DC	5	x	—
	(3) from BMC	2 × 5	O	83%
	(4) from BMC	2 × 3	O	60%
	(5) from BMC	5 × 2	O	21%
HVJ-Envelope	DC-rich		x	—
Nucleoporation	DC-rich		O	0.01%

Adenoviral vectors are cytotoxic and most of cells died within 7 to 9 days. Lentiviral vectors are less cytotoxic than adenoviral ones. Therefore it was possible that they were recurrently used into the same DC population. The successful exogenous gene expression was observed in DC after 2-week culturing only with this system. The most critical time point was around day 7 from the beginning of fresh BMC culture admixed with lentiviral vectors. HVJ-Envelope: pEGFP & FITC-BSA were not transduced. Nucleoporation (electroporation): pEGFP was transduced in less than $1/10^4$ cells.

Inbred strains of LEW and DA rats were obtained from Harlan Olac (Blackthorn, Bicester, England). Monoclonal antibodies (mAbs), including mouse anti-rat PE-CD4, PE-CD8, PE-ClassII (OX-6), PE-CD80, PE-CD86, PE-CD161a (NKR-P1A, 3.2.3), were purchased from BD Immunocytometry products (BD Pharmingen International, Fujisawa Pharmaceutical, Osaka, Japan), Cytokines that included recombinant human IL-6 were generously supplied by Kirin Brewer Company Ltd., (Maebashi, Gunma, Japan). Recombinant human Flt3/Flk2 ligand was purchased from PeproTech, Inc. USA. We used the recombinant adenovirus Ax1CAegfp, purchased from Rikeri Biosource Center, Tukuba, Japan (Adeno-EGFP: 1×10^9 pfu/mL). The HIV vectors were produced by cotransfecting 293T cells with pHIV-CS-CDF-CG (cPPT-containing SIN vector plasmid encoding EGFP under control of a CMV promoter (Lenti-EGFP: 1×10^9 pfu/mL). Woodchuck hepatitis virus posttranslational regulatory element (WPRE) was ligated at the 3′ end of EGFP, pMDLg/p RRE (encoding packaging protein), pMD.G (encoding VSV-G envelop protein), and pRSV-Rev by calcium phosphate transfection as previously described.[2] Harvested cell suspensions were stained with monoclonal antibodies and analyzed by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif). Statistical analysis was performed using multiple comparison tests (Fisher's Protected Least Significant Difference) with $P < .05$ considered significant.

RESULTS

The efficiency of gene transduction by different vector systems is summarized in Table 1. A DC–enriched population was incubated with Adeno-EGFP at multiplicity of infection (MOI) values of 25 to 100. At 100 MOI, the maximum gene expression in DC was 16% by FACS analysis. Mature DC purified by CD161a, were likewise admixed with Adeno-EGFP at MOI values ranging from 50 to 100. Surprisingly, EGFP gene expression was not observed in the CD161a+ DC. A DC–enriched cell population was incubated with Lenti-EGFP at MOI values between 0.4 and 4. For the 2 MOI and 4 MOI treatments, gene expression was stronger after postoperative day 7. When a DC–enriched cell population was admixed with Lenti-EGFP (2 MOI) once a week for 3 consecutive weeks, the gene transduction rate at postoperative day 21 was high: 84% of DC were positive upon FACS analysis. Nevertheless, when the purified CD161a+ DC were admixed with Lenti-EGFP (5 MOI), EGFP gene expression was not observed, even after day 7. In contrast, when LEW BMC was admixed with Lenti-EGFP (2 MOI) once a week for 5 consecutive weeks during long-term BMC culture, FACS analysis on day 21 revealed 83% of DC were EGFP-positive. During long-term culture of BMC, Lenti-EGFP was admixed at MOI value 2 once a week for 3 consecutive weeks at the beginning of culture period. The highest transduction rate was achieved at day 21; FACS analysis showed 60% of DC to be EGFP positive. In addition, FACS analysis of the class II surface marker was extremely high. Likewise, the levels of CD80, CD4, and CD161a expression were significantly elevated.

Lentiviral vectors were found to be less cytotoxic than adenoviral vectors. Thus, it is likely that lentiviral vector-treated cell populations could be successfully used for DC culture and gene transduction. However, it should be noted that successful exogenous gene expression was only observed in the bona fide DC after 2 weeks of culture with this lentiviral vector system. Fresh BMC were divided into eight groups with or without 1 MOI of Lenti-EGFP at the beginning of culture period, and then with or without 5 MOI of Lenti-EGFP once a week for 3 consecutive weeks. Flow cytometric analysis on day 21 showed that the groups that had been treated with Lenti-EGFP vector at 5 MOI on day 7 exhibited the highest gene transduction rate (20%, $P < .01$). These groups also demonstrated a higher cell recovery than the other groups ($P < .05$). Thus, these results indicate that the most critical time point for gene transduction during long-term BMC culture occurs after 7 days. HVJ-Envelope vector systems[3] that contain either EGFP-encoded plasmids or FITC-tagged bovine serum albumin were investigated for EGFP gene transduction and FITC-bovine serum albumin into DC–enriched cell populations using an HVJ-E vector kit (GenomONE). FITC and EGFP were not expressed on postoperative days 1, 2, and 4. Employing the nucleoporation system,[4] EGFP genes were transduced into DC–enriched cell population using a human DC kit in combination with the Amaxa Nucleofector system. Although some bona fide DC expressed the exog-

enous EGFP, the efficiency of EGFP gene expression was low (around 0.01%).

DISCUSSION

The lentiviral vector system was the most effective method for gene transduction in rat DC. The most efficient transduction of the EGFP gene was observed in DC progenitor cells during long-term culture of rat BMC. Exogenous gene expression was also observed in bona fide DC following 2 weeks of culture using an HIV-based lentiviral vector system. The most critical time was around the day 7 from the beginning of long-term BMC culture admixed with lentiviral vectors. In this study, we demonstrated that none of the vector systems that are currently available are able to transfer the EGFP gene into a subset of mature DC purified by CD161a (NKRP1A, a C-type lectin family).

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A20 Is a Negative Regulator of IFN Regulatory Factor 3 Signaling[1]

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IFN regulatory factor 3 (IRF-3) is a critical transcription factor that regulates an establishment of innate immune status following detection of viral pathogens. Recent studies have revealed that two IκB kinase (IKK)-like kinases, NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 and IKK-*i*/IKKε, are responsible for activation of IRF-3, but the regulatory mechanism of the IRF-3 signaling pathway has not been fully understood. In this study, we report that IRF-3 activation is suppressed by A20, which was initially identified as an inhibitor of apoptosis and inducibly expressed by dsRNA. A20 physically interacts with NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 and IKK-*i*/IKKε, and inhibits dimerization of IRF-3 following engagement of TLR3 by dsRNA or Newcastle disease virus infection, leading to suppression of the IFN stimulation response element- and IFN-β promoter-dependent transcription. Importantly, knocking down of A20 expression by RNA interference results in enhanced IRF-3-dependent transcription triggered by the stimulation of TLR3 or virus infection. Our study thus demonstrates that A20 is a candidate negative regulator of the signaling cascade to IRF-3 activation in the innate antiviral response. *The Journal of Immunology,* **2005, 174: 1507–1512.**

The innate immune system is an important, evolutionarily conserved mechanism that confers host defense against viral and microbial infection (1–3). IFN regulatory factor 3 (IRF-3)[3] is a ubiquitously expressed transcription factor that regulates primary induction of type I IFN, IFN-αβ, and plays a critical role for establishing innate immune status in response to invasion of pathogens (1–2, 4). Although IRF-3 is retained in the cytoplasm of unstimulated cells, it is phosphorylated and forms a dimer upon viral infection, which then translocates to the nucleus, binds to IFN stimulation response element (ISRE) and enhances the transcription of a set of genes including IFN-β (2, 4, 5). Recent studies revealed that two noncanonical IκB kinase (IKK)-like kinases, NF-κB-activating kinase (NAK)/Traf family member-associated NF-κB activator-binding kinase 1 (TBK1) and IKK-*i*/IKKε, could induce the dimerization of IRF-3 by enhancing phosphorylation of IRF-3 and play essential roles for IRF-3-dependent transcriptional activation (6, 7).

Viral and microbial pathogens can be detected by TLR3 and TLR4, which recognize viral dsRNA and bacterial LPS, respectively (3, 8). Engagement of these TLRs triggers the IRF-3 and NF-κB signaling pathways and confers the rapid induction of IFN-β. Although MyD88 is a common adaptor protein for TLRs and plays important roles for NF-κB activation, TLR3 and TLR4 still mediate both IRF-3 and NF-κB activation in MyD88-deficient mice, indicating that the MyD88-independent signaling pathways for production of IFN-β are triggered by engagement of these TLRs (3, 8). We and others identified adaptor molecules, Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (also called TICAM-1) and TRIF-related adaptor molecule (TRAM; also called TICAM-2 or TIRP) as mediators in the MyD88-independent signaling pathways (8–11). TRAM interacts with TLR4 and is specifically involved in TLR4-induced IRF-3 activation. TRIF, which interacts with TLR3 and TRAM, is involved in both TLR3- and TLR4-mediated signaling pathways and associates with NAK/TBK1 to initiate IRF-3 activation (12).

Although recent discoveries identified critical mediators of IRF-3 signaling, regulatory mechanisms of these molecules are not fully understood. Because NAK/TBK1 and IKK-*i*/IKKε are IKK-like molecules, we hypothesized that the regulatory mechanism of IRF-3 kinases might be similar to that of the canonical IKKα/IKKβ/NF-κB essential modulator (NEMO) complex. We therefore examined regulators of the IKK complex activation for participation in IRF-3 signaling pathways and have found that A20 potently inhibits IRF-3 activation. A20 is a 90-kDa protein whose expression is induced by a variety of stimuli including poly(I)·poly(C) (polyIC), LPS, and TNF-α (13, 14). A recent study indicated that viral infection also induced A20 expression through activation of protein kinase R (15). A20 has a deubiquitinase domain at the N terminus and seven repeats of zinc finger domain at the C terminus, although the role of these domains for A20 function is not fully understood (13, 16). A recent study using mice deficient in A20

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[3] Abbreviations used in this paper: IRF, IFN regulatory factor; ISRE, IFN stimulation response element; IKK, IκB kinase; NAK, NF-κB-activating kinase; TBK1, Traf family member-associated NF-κB activator-binding kinase 1; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; TRAM, TRIF-related adaptor molecule; *NEMO*, NF-κB essential modulator; *polyIC*, poly(I)·poly(C); NDV, Newcastle disease virus; siRNA, small inhibitory RNA; TRAF, TNFR-associated factor.

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provided genetic evidence that A20 worked as a negative regulator in NF-κB signaling pathways. Injection of TNF-α in A20-deficient mice induced severe inflammation due to persistent activation of NF-κB (17). These mice exhibited elevated sensitivity to endotoxin shock, suggesting that A20 might negatively regulate TLR-initiated signaling pathways (17, 18). However, involvement of A20 in IRF-3 signaling pathways triggered by engagement of TLR or virus infection has not been reported.

In this study, we demonstrate that A20 interacts with IRF-3 kinases, NAK/TBK1 and IKK-*i*/IKKε, and inhibits TLR3- or virus-induced IRF-3 dimerization and ISRE-dependent transcriptional activation.

Materials and Methods

Reagents

Monoclonal anti-HA Ab (12CA5) was a kind gift from Dr. A. Israël (Institut Pasteur, Paris, France). Monoclonal anti-FLAG Ab (M2) and anti-HA Ab (HA-7) were purchased from Sigma-Aldrich. Anti-actin Ab (C-2) and anti-IRF-3 Ab (FL-425) were purchased from Santa Cruz Biotechnology. Polyclonal anti-human IRF-3 (phospho-Ser^{386}) Ab was described previously (5). PolyIC was purchased from Amersham Biosciences. Newcastle disease virus (NDV) was prepared as described previously (19). All other regents were purchased from Sigma-Aldrich unless otherwise noted.

Plasmids

pcDNA3-HA-A20, pFLAG-CMV-1-hTLR3, pEF1-*lacZ*, and p125-luc were kind gifts from Drs. D. Wallach (The Weizmann Institute of Science, Rehovot, Israel), K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA), S. Memet (Institut Pasteur, Paris, France), and T. Taniguchi (University of Tokyo, Tokyo, Japan), respectively. pcDNA3 and pISRE-luc were purchased from Invitrogen Life Technologies and Stratagene, respectively. pcDNA3-FLAG-NAK, pcDNA3-FLAG-dnNAK (K38A), pEF-FLAG-IKK-*i*, pEF-HA-IRF-3, pEF-HA-dnIRF-3 (58–427), and pEF-p50-IRF-3 5D were described previously (5, 12, 19, 20). Complementary DNAs encoding either the N-terminal 378 aa (1–378) or C-terminal 412 aa (379–790) of human A20 were amplified by PCR using pcDNA3-HA-A20 as a template and inserted into pcDNA3-HA. The resultant plasmids were referred to as pcDNA3-HA-A20-N or pcDNA3-HA-A20-C, respectively. The primer sequences used for PCR are available upon request. pU6 plasmids capable of small inhibitory RNA (siRNA) expression were constructed according to the procedures described previously (21). Sequences inserted immediately downstream of U6 promoter were as follows (only sense sequence is shown): specific to A20, 5′-GGAAACAGACACACGCAAC-3′; the unrelated control, 5′-GTAGCGCGGTGTATTATAC-3′. The resultant plasmids were referred as pU6-A20i or pU6-Ctli, respectively.

Cell culture, transfection, and reporter assay

293/TLR3 cells were described previously (22). The 293, 293/TLR3, and HeLa cells used in this study were all maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. THP-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. 293 and 293/TLR3 cells were transfected by the calcium-phosphate method as described previously (23). HeLa cells were transfected by FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. THP-1 cells were transfected by DMRIE-C transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. 293, 293/TLR3, and HeLa cells were transfected with 250 ng of EF1-*lacZ* and 250 ng of pISRE-luc or p125-luc along with the indicated effector plasmid. THP-1 cells were transfected with 500 ng of EF1-*lacZ* and 500 ng of pISRE-luc along with the indicated effector plasmid. After the transfection, cells were treated as indicated and lysed in lysis buffer (25 mM Tris-HCl, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol). Luciferase activity was normalized based on β-galactosidase activity. All of the experiments were conducted at least six times. The results were essentially reproducible.

Preparation of whole-cell extracts, immunoblotting, and immunoprecipitation

Cells were suspended in lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, and 0.1% Nonidet P-40) supplemented with 1 mM

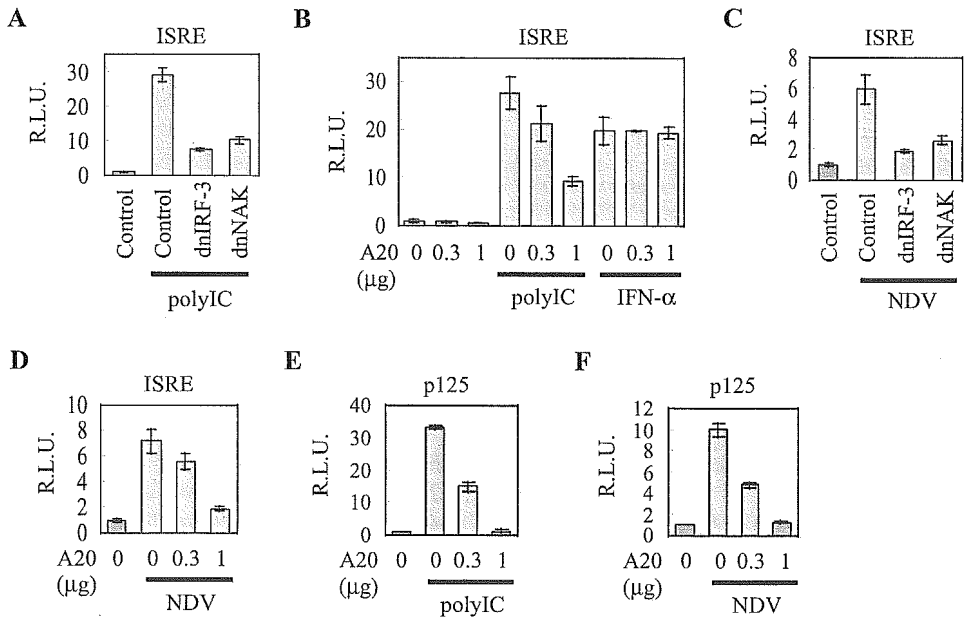

FIGURE 1. A20 suppresses IRF-3-dependent transcription. *A* and *B*, 293/TLR3 cells were transfected with 1 μg of the indicated expression plasmid (*A*) or the indicated amounts of pcDNA3-HA-A20 (*B*) along with pISRE-luc and pEF1-*lacZ*. Total amount of effecter plasmid (1 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, the cells were stimulated with polyIC (10 μg/ml) or IFN-α (2000 U/ml) for 3 h. *C* and *D*, 293 cells were transfected with 1 μg of the indicated expression plasmid (*C*) or the indicated amounts of pcDNA3-HA-A20 (*D*) along with pISRE-luc and pEF1-*lacZ*. Total amount of effecter plasmid (1 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, the cells were infected with NDV for 12 h. *E* and *F*, 293/TLR3 cells or 293 cells were transfected with the indicated amounts of pcDNA3-HA-A20 along with p125-luc (IFN-β promoter) and pEF1-*lacZ*, and then the cells were stimulated with polyIC for 3 h (*E*) or infected with NDV (*F*) for 12 h, respectively. The harvested cells were subjected to luciferase reporter assays. The luciferase activity was normalized based on the β-galactosidase activity. The values shown are means ± SD from three separate transfections. R.L.U., Relative luciferase unit.

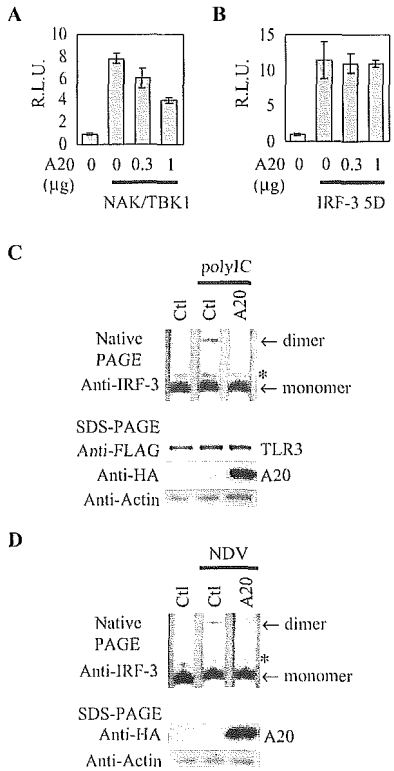

FIGURE 2. A20 inhibits dimerization of IRF-3. *A* and *B*, 293 cells were transfected with 50 ng of pcDNA3-FLAG-NAK (*A*) or 15 ng of pEF-p50-IRF-3 5D (*B*) along with the indicated amounts of pcDNA3-HA-A20, pISRE-luc, and pEF1-*lacZ* for 24 h. The harvested cells were subjected to luciferase reporter assays. *C*, 293 cells were transfected with 1 μg of pcDNA3 or pcDNA3-HA-A20 along with 100 ng of pFLAG-CMV-1-hTLR3. Twenty-four hours after transfection, the cells were stimulated with polyIC for 1 h. *D*, 293 cells were transfected with 1 μg of pcDNA3 or pcDNA3-HA-A20. Twenty-four hours after transfection, the cells were infected with NDV for 12 h. The whole-cell extracts were subjected to native PAGE or SDS-PAGE. The membranes were probed with the indicated Abs. The asterisks indicate nonspecific bands. Positions of IRF-3 monomer and dimer are indicated.

PMSF and 1 mM Na_3VO_4. Extracts were cleared by centrifugation. Immunoprecipitation and immunoblotting were performed as described previously (23). Briefly, cell lysates were incubated with anti-FLAG mAb (M2) for 1 h and then incubated with protein G-Sepharose for 1 h. The beads were washed four times with lysis buffer. Precipitated proteins and whole-cell extracts were fractionated by 8% SDS-PAGE, and transferred onto Immobilon membranes (Millipore), and blots were revealed with an ECL detection system (Amersham Biosciences). All of the experiments were conducted at least twice. The results were essentially reproducible.

Detection of IRF-3 dimer by native PAGE

Cells were suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40) supplemented with 1 mM PMSF and 1 mM Na_3VO_4. Extracts were cleared by centrifugation. Whole-cell extracts (10 μg) were fractionated by 7.5% native PAGE, and transferred onto Immobilon membranes, and blots were revealed with an ECL detection system. All of the experiments were conducted at least twice. The results were essentially reproducible.

Results

A20 suppresses IRF-3 activation

We examined involvement of proteins associated with the IKKα/IKKβ/NEMO complex for IRF-3 regulation, because the NAK/TBK1 and IKK-*i*/IKKε are IKK-like molecules. We used two well-characterized stimuli to trigger IRF-3 signaling pathways, engagement of TLR3 with polyIC and infection with NDV, which indeed activated ISRE-dependent transcription in a manner that depends on NAK/TBK1 and IRF-3 (Fig. 1, *A* and *C*). As a result, we found that A20 inhibited TLR3 stimulation- or NDV infection-induced ISRE-dependent transcription in a dose-dependent manner (Fig. 1, *B* and *D*). In contrast, expression of A20 did not affect IFN-α-induced activation (Fig. 1*B*), indicating that A20 specifically regulates IRF-3 signaling. We next asked whether A20 modulates the activity of the IFN-β promoter, which harbors ISRE recognized by IRF-3. Transfection studies revealed that A20 potently inhibited TLR3- or NDV-induced IFN-β promoter activation (Fig. 1, *E* and *F*). A20 also inhibited NAK/TBK1-induced, but not the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 2, *A* and *B*). In addition, A20 inhibited the polyIC- or NDV-induced dimerization of endogenous IRF-3 (Fig. 2, *C* and *D*). These results strongly suggest that A20 acts on a common mediator(s) that regulates IRF-3 activation induced by TLR3 engagement or NDV infection.

A20 interacts with NAK/TBK1 and IKK-i/IKKε

Because NAK/TBK1 is a common regulator of TLR- or virus-induced IRF-3 activation, we next examined interaction of A20 with NAK/TBK1 and found that A20 was coimmunoprecipitated with NAK/TBK1 (Fig. 3*A*). In addition, A20 was associated with another IRF-3 kinase, IKK-*i*/IKKε (Fig. 3*B*). Interestingly, when A20 was cotransfected with NAK/TBK1 or IKK-*i*/IKKε, A20 was found to migrate more slowly in immunoblot studies (Fig. 3, *A* and *B*), as had been shown for TRIF, which associates with NAK/TBK1 (12). A20 has a deubiquitinase domain at the N terminus and repeated zinc finger domains at the C terminus (Fig. 3*C*). Previous studies demonstrated that TNFR-associated factor (TRAF)2 and TRAF6 interact with the N terminus of A20, whereas A20-binding inhibitor of NF-κB interacts with the C terminus of A20 (13). NEMO was reported to interact with both the N and C termini of A20 (24). To determine which functional domain(s) of A20 is required for the interaction with these kinases, we generated deletion constructs capable of expressing the N terminus (aa 1–378) or C terminus (aa 379–790) of A20 (Fig. 3*C*). Immunoblot analyses detected these mutants at expected positions, although the level of expression of the N terminus of A20 was higher than the others (Fig. 3*D*). Immunoblotting studies coupled with immunoprecipitation revealed that NAK/TBK1 and IKK-*i*/IKKε interacted with the C-terminal zinc finger and N-terminal deubiquitinase domains of A20 (Fig. 3, *E* and *F*).

Because A20 interacts with NAK/TBK1, which phosphorylates IRF-3, we examined whether A20 inhibits NAK/TBK1-induced IRF-3 phosphorylation. Immunoblotting with phosphospecific anti-IRF-3 Ab following native PAGE demonstrated that NAK/TBK1-induced phosphorylation of IRF-3 at Ser^{386}, one of the important residues for the dimerization of IRF-3 (5), was impaired in cells expressing the full length or C terminus of A20 (Fig. 3*G*, *upper panels*). In contrast, NAK/TBK1-induced phosphorylation of IRF-3 was not inhibited in cells expressing the N terminus of A20. As reported previously (7), slowly migrating, multiply phosphorylated species of IRF-3 were detected by SDS-PAGE followed by immunoblotting with phosphospecific or conventional anti-IRF-3 Abs when IRF-3 was cotransfected with NAK/TBK1, and these modifications were weakened by coexpression of the full length or C terminus of A20 (Fig. 3*G*, *lower panels*). We verified in transfection studies that the biochemical activity of each A20 domain correlated with the functional requirement for inhibition of TLR3- or NDV-induced ISRE-dependent transcription (Fig. 3, *H* and *I*). The C-terminal zinc finger domain of A20 suppressed ISRE-dependent transcription induced by TLR3 stimulation or

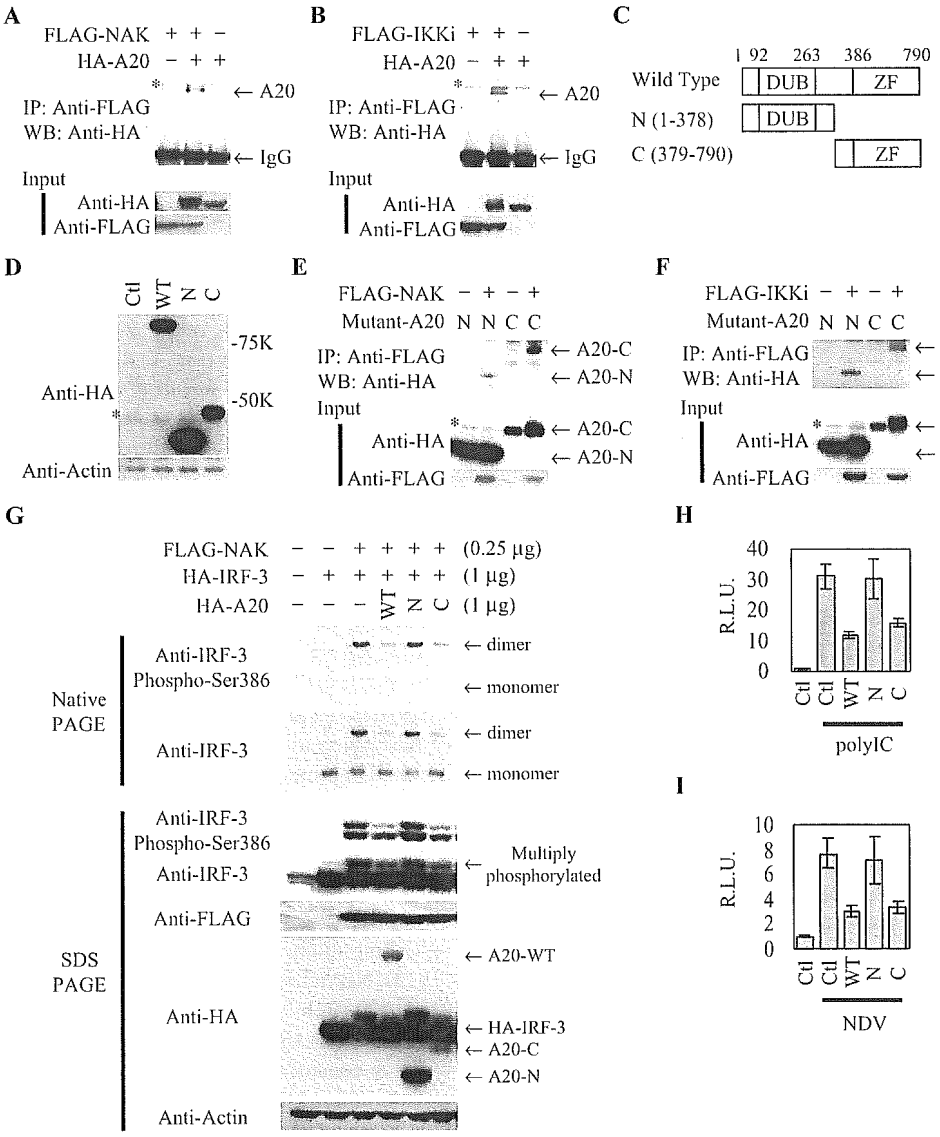

FIGURE 3. A20 interacts with NAK/TBK1 and IKK-*i*/IKKε. *A* and *B*, 293 cells were transfected with 5 μg of the indicated expression plasmids for 24 h. Total amount of plasmid (10 μg) was kept constant by addition of pcDNA3. The cell lysates were subjected to immunoprecipitation with anti-FLAG Ab followed by Western blotting with anti-HA and anti-FLAG Abs. IP, Immunoprecipitation; WB, Western blotting. The asterisks indicate nonspecific bands. *C*, Schematic representation of wild-type (WT) and mutant A20 proteins (N and C). The mutant N encodes the N-terminal 378 aa (1–378) and contains the deubiquitinase domain (DBU). The mutant C encodes the C-terminal 412 aa (378–790) and has seven zinc finger motifs (ZF). *D*, 293 cells were transfected with the indicated expression constructs. Cells extracts (10 μg) were subjected to Western blotting analysis with anti-HA and anti-actin Abs. Ctl, Control vector transfection. The asterisk indicates nonspecific bands. *E* and *F*, 293 cells were transfected with 5 μg of the indicated plasmids for 24 h. Total amount of plasmid (10 μg) was kept constant by addition of pcDNA3. The cell lysates were subjected to immunoprecipitation with anti-FLAG Ab followed by Western blotting with anti-HA and anti-FLAG Abs. The asterisks indicate nonspecific bands. *G*, 293 cells were transfected with indicated expression plasmids. Total amount of plasmid (2.25 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, whole-cell extracts were prepared and subjected to native PAGE or SDS-PAGE. Each membrane was sequentially probed with the indicated Abs. *H* and *I*, Reporter assays. 293/TLR3 cells (*H*) and 293 cells (*I*) were transfected with 1 μg of the indicated expression plasmids along with pEF1-*lacZ* and pISRE-luc, and then treated with polyIC for 3 h (*H*) or infected with NDV for 12 h (*I*), respectively. Ctl, Control vector transfection.

NDV infection as efficiently as the full-length A20, whereas the N terminus of A20 failed to do so.

siRNA to A20 enhances the signal-induced IRF-3-dependent transcription

To verify the role of endogenous A20, we generated a siRNA expression construct capable of knocking down the A20 expression. Expression of siRNA specific to A20 effectively reduced the level of transfected A20, whereas it did not affect that of IRF-3 (Fig. 4*A*). Expression of this siRNA specifically enhanced ISRE-dependent transcription induced by TLR3 stimulation or NDV infection, whereas it barely caused additional ISRE-dependent transcription in unstimulated 293 and 293/TLR3 cells or in 293/TLR3 cells stimulated with IFN-α (Fig. 4, *B* and *E*). Similar results were obtained with HeLa cells (Fig. 4*C*) and immunologically relevant THP-1 cells (*D*). Consistent with the ability of A20 to suppress NAK/TBK1-induced ISRE-dependent transcription (Fig. 2*A*), expression of siRNA to A20 enhanced this transcriptional activation (Fig. 4*F*). In contrast, it did not alter the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 4*G*). These results indicate that endogenous A20 negatively regulates IRF-3 signaling following initial stimulation of the system.

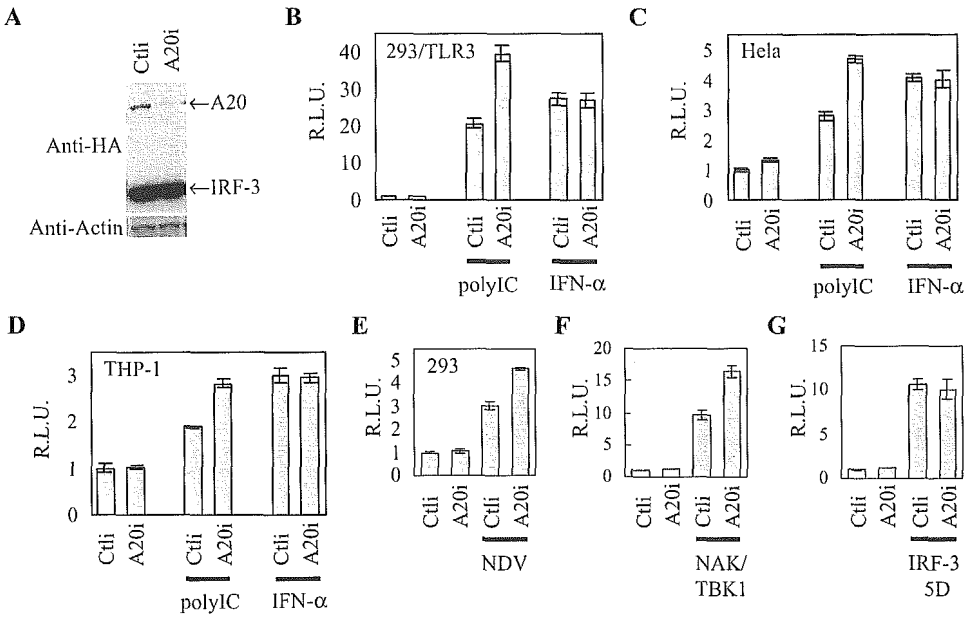

FIGURE 4. Suppression of A20 expression by RNA interference enhances the signal-induced IRF-3-dependent transcription. *A*, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with 100 ng of pcDNA3-HA-A20 and 100 ng of pEF-HA-IRF3. Cells extracts (10 μg) were subjected to Western blotting analysis with anti-HA and anti-actin Abs. Ctli, Control siRNA; A20i, siRNA specific to A20. *B* and *C*, 293/TLR3 cells (*B*) or HeLa cells (*C*) were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were stimulated with polyIC or IFN-α for 12 h. *D*, THP-1 cells were transfected with 1 μg of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were stimulated with polyIC or IFN-α for 8 h. *E*, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-*lacZ* and pISRE-luc. Thirty-six hours after transfection, the cells were infected with NDV for 12 h. *F* and *G*, 293 cells were transfected with 50 ng of pcDNA3-FLAG-NAK (*F*) or 15 ng of pEF-p50-IRF-3 5D (*G*) along with indicated siRNA expression plasmid, pEF1-*lacZ* and pISRE-luc for 36 h. The harvested cells were subjected to luciferase reporter assay.

Discussion

Previous studies identified critical mediators in TLR signaling pathways that play important roles in innate immune responses. In addition, recent reports showing that mice deficient in suppressor of cytokine signaling-1, IL-1R-associated kinase M, or ST2, a negative regulator of NF-κB signaling, are hypersensitive to endotoxin shock, strongly suggest that negative feedback regulation of TLR signaling is important to protect the host from excessive immune response (25–28). Because IRF-3 is known to mediate endotoxin shock or virus-induced cell death, the IRF-3 activity should also be strictly controlled (29, 30). Several negative regulators of TLR-triggered NF-κB signaling were identified, but those of IRF-3 signaling have not been described except for IRF-2, which competes with IRF-3 for the recruitment of CREB binding protein (31). In this study, we have demonstrated for the first time a negative regulation of IRF-3 signaling triggered by TLR3 engagement or virus infection at the level of IRF-3 kinases. Because A20 was identified as a polyIC- or LPS-inducible protein (13–14), A20 might participate in the negative feedback regulation of IRF-3 signaling. Thus, our present study provides a molecular basis for a role of A20 in evasion of fatal excessive immune response in hosts suffering from virus infection. Pitha and colleagues (32) reported that pretreatment of cells with LPS impaired virus-induced phosphorylation and subsequent nuclear translocation of IRF-3. Because A20 is induced by LPS stimulation, our results that A20 interferes with the NAK/TBK1 and IKK-*i*/IKKϵ-mediated IRF-3 signaling could partly explain the impaired IRF-3 activation after LPS treatment. Studies using A20-deficient mice will clarify the roles of A20 for the negative regulation of antiviral innate immune responses in vivo.

We showed potent suppression of the IFN-β promoter by A20. This may partly be due to A20 inhibition of NF-κB activation (data not shown). Because A20 was reported to inhibit NF-κB activation through TRAF6 or receptor interacting protein, which transduce signals from TLR3-TRIF and interact with NEMO, it is reasonable to assume that A20 interferes with the TRAF6, receptor interacting protein, and NEMO signaling axis triggered by TLR3 stimulation or virus infection (12, 13, 24, 33, 34).

We have demonstrated the important role of the C-terminal zinc finger domain of A20 for inhibition of IRF-3 activation induced by TLR3 stimulation or NDV infection (Fig. 3, *H* and *I*). The C terminus of A20 interacts with NAK/TBK1 and inhibits NAK/TBK1-induced phosphorylation and subsequent dimerization of IRF-3 (Fig. 3*G*). In contrast, the N terminus of A20 does not inhibit NAK/TBK1-induced IRF-3 activation, although it also interacts with NAK/TBK1. These results indicate that A20 inhibits NAK/TBK1 from phosphorylating IRF-3, and that this inhibition cannot simply be explained by the binding of A20 or A20 mutant to NAK/TBK1. Beyaert and colleagues (33, 35) reported that the C terminus of A20 was also required for the inhibition of NF-κB activation, and that the zinc finger motifs of A20 compensate the function each other. However, the functional consequences of the A20 C terminus remain to be clarified, and further study will be required to define the molecular mechanism responsible for the inhibition of IRF-3 activation by A20.

Recently, several proteins that contain A20-like functional domain were reported. Cezanne and Trabid share deubiquitinase and zinc finger domains with A20, and Cezanne inhibits TNF-α-induced NF-κB activation (36). ZNF216 is another A20-like protein that has zinc finger domain and inhibits TNF-α-, IL-1β-, and TLR4-induced NF-κB activation via interaction with NEMO (37). It would be interesting to examine whether these A20-like molecules also regulate IRF-3 activation.

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Sequence Note

Phylogenetic Heterogeneity of new HTLV Type 1 Isolates from Southern India in Subgroup A

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ABSTRACT

Seven isolates of human T cell leukemia virus type 1 (HTLV-1) were taken in southern India and phylogenetically analyzed to gain new insights into the origin and dissemination of HTLV-1 in the subcontinent. The new Indian HTLV-1s were found to be members of subgroup A (Transcontinental subgroup) of the Cosmopolitan group. They formed three different clusters (South African/Caribbean, Middle Eastern, and East Asian clusters). These results demonstrate that Indian HTLV-1s are genetically heterogeneous and include the most divergent strain of subgroup A. On the basis of these results, we speculate that subgroup A HTLV-1s may have been present for thousands of years in India.

HUMAN T CELL LEUKEMIA VIRUS type 1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–3] HTLV-1 has unique geographic and ethnologic distribution patterns. It is endemic mainly in Melanesia, the Caribbean basin, sub-Saharan Africa, and southwestern Japan and is highly prevalent among certain ethnic populations including Jews in Iran, the Ainu in Japan, Aboriginals in Australia, and Native Americans. These sporadic infections are thought to be vestiges of various migrations of HTLV-1 carriers from endemic areas, although how this distribution was established is not clear.

Seroepidemiological surveys have shown sporadic HTLV-1 infections in India. In a previous phylogenetic study,[4] Indian HTLV-1s were found to belong to the phylogenetic group that includes the Middle Eastern HTLV-1 strains, suggesting a possible link between these two areas. However, from that work it was unclear whether Indian HTLV-1 strains are indeed the closest to Middle Eastern strains, because clear phylogenetic separation of HTLV-1 strains could not be accomplished on the basis of the protein-encoding regions (*gag*, *pol*, *env*, and *pX* genes) analyzed in the previous work. In contrast to analyses based on these regions, analysis of the long terminal repeats (LTR) region permits finer separation of taxa within the genotype that includes Indian and Middle Eastern HTLV-1s. Thus, to gain new insights into the origin and dissemination of HTLV-1 in India, we phylogenetically analyzed seven new HTLV-1s from southern India on the basis of the LTR region.

The prevalence of HTLV-1 in southern India was examined by serological assays. Sera were collected in three districts of southern India (Kerala, Andhra Pradesh, and Tamil Nadu), and were tested by a particle agglutination (PA) test (Serodia HTLV-1; Fujirebio, Tokyo, Japan). Reactive sera in the PA test were further confirmed by an immunofluorescence assay as described previously.[2] Three serum samples were collected from Dravidian speakers living in Andhra Pradesh in southern India (Table 1) and phylogenetically analyzed. In addition, we analyzed HTLV-1s of four reported seropositives (two from the

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TABLE 1. SEVEN HTLV-1s ISOLATED FROM SOUTHERN INDIA IN THIS STUDY

HTLV-1 isolate	*Place*	*Age (years)*	*Sex*	*Disease*	*Subject*			*Notes*
					PA[a]	*Western blot*	*IFA*	
AP15	Andhra Pradesh	60s	M	Nervous disease	NI	NI	NI	Inhabits inland near the Tamil Nadu district
IND001	Andhra Pradesh	NI	F	NI	+	NI	NI	
IND002	Andhra Pradesh	11	NI	NI	NI	NI	NI	Blood sample on filter paper
IN2	Kerala	47	F	NI	NI	NI	NI	Wife of the subject from whom IN3 was isolated
IN3	Kerala	52	M	ATL	+(16)	+	+	A migrant worker in the Middle East
Ma1	Tamil Nadu	20	F	ARC	+(8192)	Not done	+	Also positive for HIV-1
TNA	Tamil Nadu	43	F	NI	+(512)	NI	NI	Also positive for HIV-1

Abbreviations: ARC, AIDS-related complex; ATL, adult T cell leukemia; IFA, immunofluorescence assay; NI, no information available; PA, particle agglutination test.
[a]Figures in parentheses represent the titer of HTLV-1 antibodies.

Tamil Nadu district[5] and the other two from the Kerala district[6]) (Table 1). The two seropositives from Kerala were also Dravidian speakers. Two of the three Andhra Pradesh subjects (from whom IND001 and IND002 were isolated) said that they had had blood transfusions. It is unknown whether the other five subjects had received blood transfusions. Of the two Tamil Nadu subjects, the one from whom TNA was isolated was a commercial sexual worker and the one from whom Mal was isolated had sexual contacts with prostitutes.

For all but one of the subjects, chromosomal DNA (containing HTLV-1 proviral DNA) was extracted from either cultured or uncultured peripheral blood mononuclear cells (PBMCs) by a standard procedure with proteinase K. For the subject from whom IND002 was isolated, chromosomal DNA was extracted from whole blood that was blotted onto filter paper.[4] Randomly selected regions (approximately 1 cm^2) of the filter paper were cut into more than 100 pieces with sterile scissors. The pieces were put into 3 ml of 0.85% NaCl solution and shaken at room temperature for 1 hr. Supernatants were centrifuged for 1 min to precipitate free PBMCs, which were utilized for the subsequent extraction of DNA.

The extracted DNA was subjected to nested polymerase chain reaction (PCR) to amplify a part of the LTR region that corresponds to nucleotide positions 99 to 685 of ATK, a prototypic Japanese HTLV-1 strain, with special care to avoid cross-contamination of the amplified products as described previously.[7] Throughout this study, all negative controls gave negative signals. Nucleotide sequences of a part of the amplified LTR fragments that correspond to positions 122 to 628 of ATK were determined in both directions, using an automated DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were aligned with the computer software CLUSTAL W with minor manual modifications. Phylogenetic trees were constructed by the neighbor-joining (NJ) method. For construction of NJ trees, bootstrapping was done to generate 1000 resamplings of the original sequence alignments, and pairwise genetic distances were estimated on each resampling by the Kimura two-parameter method. Phylogenetic trees were then constructed with CLUSTAL W and the trees were visualized by TREEVIEW. Detailed procedures and references for construction of the trees are described elsewhere.[7] The GenBank accession numbers for the new strains are AY607576 to AY607582.

We amplified a part of the LTR region of seven new HTLV-1s from India (Table 1). HTLV-1 isolates are phylogenetically separated into three major genotypes. Most HTLV-1 isolates from the world form a large group called the Cosmopolitan group or HTLV-1a. HTLV-1 isolates from Central Africa and those from Melanesia have diverged from those of the Cosmopolitan group, and are thus collectively called the Central African (HTLV-1b and -1d) and Melanesian (HTLV-1c) groups, respectively. The LTR sequences of the newly isolated HTLV-1s exhibited higher genetic similarities to HTLV-1 strains of the Cosmopolitan group (97.1%) than to those of the Central African (94.4%) and Melanesian groups (91.3%). The phylogenetic analysis also placed the newly isolated HTLV-1s into the Cosmopolitan group (Fig. 1). This group has been divided into five subgroups (A to E) on the basis of their LTR sequences. Each of the five subgroups closely correlates with the geographic origins of its HTLV-1 isolates. According to our data (Fig. 1), all of the new HTLV-1 strains belong to subgroup A (Transcontinental subgroup). This was confirmed by their restriction fragment length polymorphism (RFLP) profiles, which are consistent with those of subgroup A in our classification. Namely, the analyzed LTR fragments of the new HTLV-1s have *Nde*I and *Sac*I sites, but not *Dra*I and *Mae*III sites, which is consistent with the RFLP patterns of subgroup A strains. All of these data demonstrate that the prevalent genotype of HTLV-1 in southern India is subgroup A of the Cosmopolitan group.

Because all of the new Indian isolates belong to subgroup A (Fig. 1), their nucleotide alignments were compared with those of subgroup A strains. The new strains formed three different clusters (Fig. 2). One cluster contained two of the new strains (IND001 and IND002) together with South African and Caribbean HTLV-1s (Caribbean/South African/Indian cluster). Another cluster contained two other Indian HTLV-1s (AP15 and TNA) together with mostly Middle Eastern HTLV-1s (Middle Eastern cluster). The third cluster contained the other three Indian isolates (IN2, IN3, and Ma1) together with Japanese HTLV-1 strains. We tentatively refer to this cluster as the East Asian cluster. These clusters had the following characteristic nucleotide substitutions: HTLV-1s of the Caribbean/South African/Indian cluster have a G-to-A substitution at nucleotide position 246 in ATK, a C-to-T substitution at position 386, and an A-to-G substitution at position 575. Two of the Indian isolates (AP5 and TNA), like strains of the Middle Eastern cluster, have G rather than A at 240. It is noteworthy that within subgroup A, AP15 and TNA were phylogenetically located at the nodes of the Middle Eastern cluster, while Ma1 was branched off at the node of the East Asian cluster (Fig. 2). Collectively, these results indicate that subgroup A HTLV-1s circulating in India are polyphyletic.

The finding that HTLV-1s in southern India formed three different clusters indicates that the HTLV-1s of southern India are highly heterogeneous. This heterogeneity is in sharp contrast to the homogeneity of subgroup A HTLV-1s from other geographic areas. For example, HTLV-1s of South America and the Middle East form respective clusters (the Latin American and Middle Eastern clusters) within subgroup A. In addition, as shown in Fig. 1, India has the most divergent strain of subgroup A (SG).[8,9] The present analysis further showed that within subgroup A, some of the new Indian HTLV-1s are phylogenetically located at or branched off the node of the Middle Eastern and East Asian clusters. As a result, subgroup A HTLV-1s in southern India are clearly distinguishable from those of other geographic areas in that southern India has both the most divergent HTLV-1 and highly heterogeneous HTLV-1s.

These findings have an interesting implication with respect to the origin of HTLV-1 in India. Earlier studies hypothesized that HTLV-1 was introduced into India by recently migrated peoples such as African slaves several hundreds years ago, or by Jewish populations that migrated from the Middle East about 1000 to 1300 years ago.[4] However, our results indicate that Indian HTLV-1 is much more heterogeneous and includes more divergent strains than subgroup A HTLV-1 of other geographic origins such as Africa and the Middle East. This implies that HTLV-1 has been present in India for much longer than 1300 years.

If this is correct, how did HTLV-1 get to India? HTLV-1 isolates analyzed in the present study were isolated from the Dravidians, a population that migrated to India several thousand years ago. The high heterogeneity and divergence of HTLV-1 in southern India may be explained by the genetic vari-

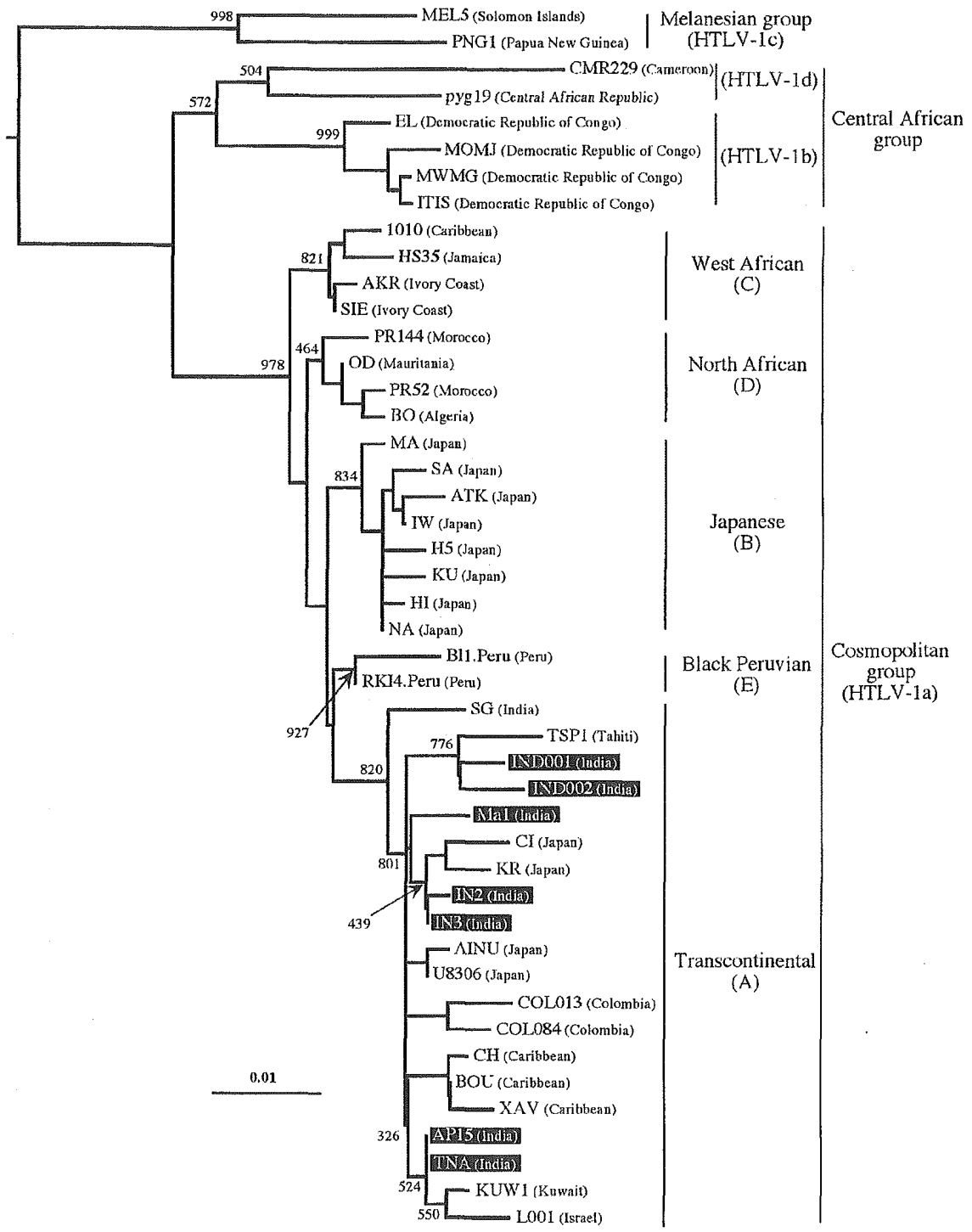

FIG. 1. Phylogenetic tree of HTLV-1 isolates based on a part of the LTR region (nucleotide positions 122–628 in ATK), showing the evolutionary relationships between the new isolates from India and isolates previously reported. Newly isolated HTLV-1s from India are highlighted. The tree was constructed by using the neighbor-joining (NJ) method. The scale at the bottom of the tree indicates the number of nucleotide substitutions per site. The horizontal branch lengths are proportional to the genetic distance. Numbers at nodes are bootstrap values. The tree was rooted with a prototype isolate of HTLV-2, MoT. The other DNA sequences used for construction of the phylogenetic tree have been described previously.[7]

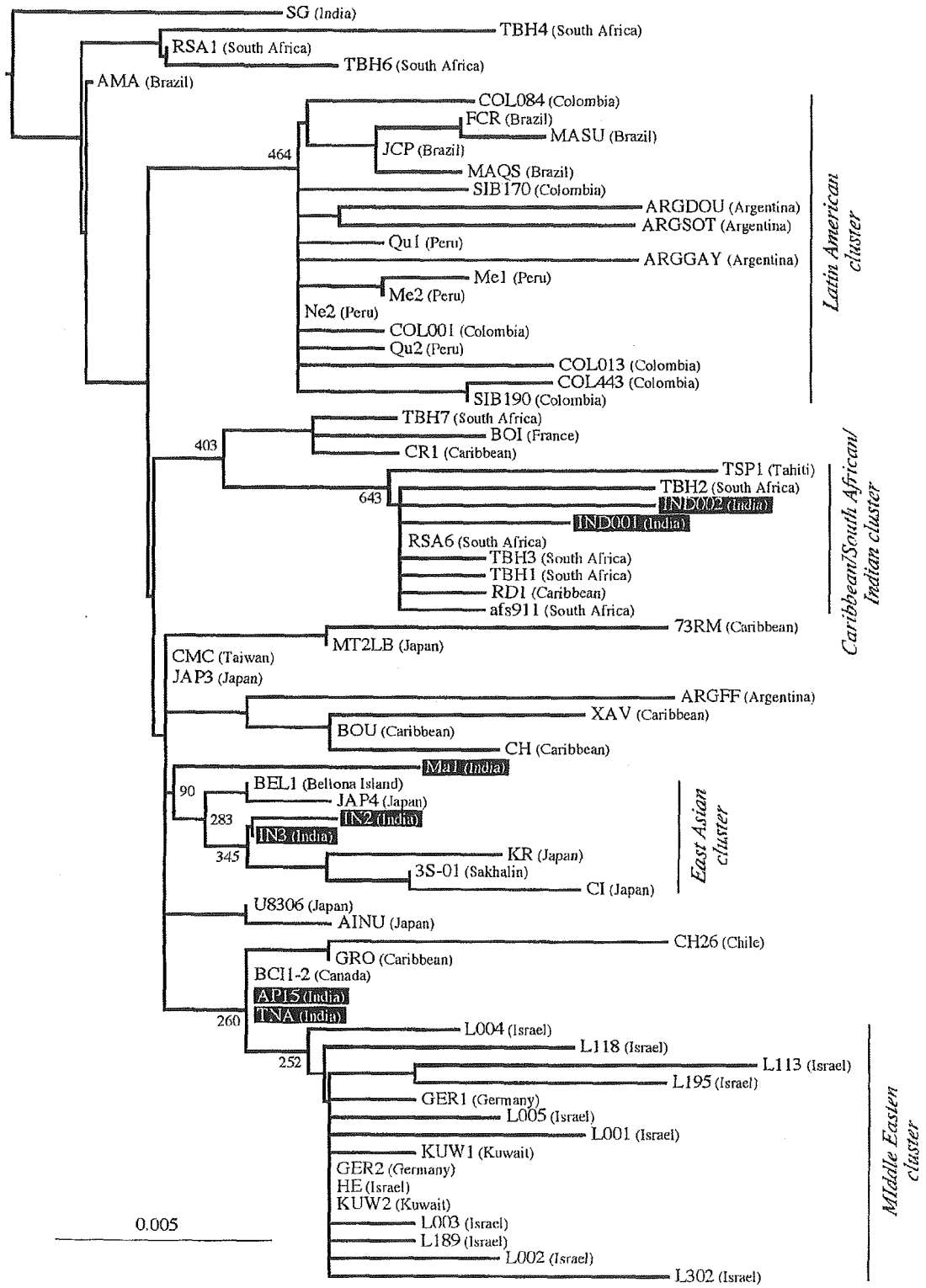

FIG. 2. Phylogenetic tree of HTLV-1 isolates that belong to subgroup A. The new isolates from India are boxed in black. The tree was constructed on the basis of a part of the LTR region (nucleotide positions 122–628 in ATK) by using the NJ method. The tree was rooted with strains of the Central African and Melanesian groups. For details, see the legend to Fig. 1.