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Oral Immunization with Recombinant *Mycobacterium bovis* BCG Simian Immunodeficiency Virus *nef* Induces Local and Systemic Cytotoxic T-Lymphocyte Responses in Mice

MICHELINE LAGRANDERIE,[1] ANNE-MARIE BALAZUC,[1] BRIGITTE GICQUEL,[2] AND MARINA GHEORGHIU[1]*

Laboratoire du BCG[1] and Unité Génétique Mycobactérienne,[2] Institut Pasteur, 75724 Paris Cedex 15, France

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Recombinant live *Mycobacterium bovis* BCG vectors (rBCG) induce strong cellular and humoral immune responses against various antigens after either systemic or oral immunization of mice. Cytotoxic T-lymphocyte (CTL) responses may contribute to the control of human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) infections whose portal of entry is the gastrointestinal or genital mucosa. In this study, we immunized BALB/c mice with a recombinant BCG SIV *nef* and observed its behavior in oropharyngeal and target organ lymphoid tissues. The cellular immune responses, particularly the intestinal intraepithelial and systemic CTL responses, were investigated. The results showed that rBCG SIV *nef* translocated the oropharyngeal mucosa and intestinal epithelium. It diffused to and persisted in target lymphoid organs. Specific SIV Nef peptide proliferative responses and cytokine production were observed. Strong systemic and mucosal CTL responses were induced. In particular, we demonstrated direct specific anti-Nef CTL in intestinal intraepithelial CD8β$^+$ T cells. These findings provide evidence that orally administered rBCG SIV *nef* may contribute to local defenses against viral invasion. Therefore, rBCG SIV *nef* could be a candidate vaccine to protect against SIV infection and may be used to develop an oral rBCG HIV *nef* vaccine.

Mucosa-associated lymphoid tissues of the respiratory, gastrointestinal and genital tracts produce local and systemic immune responses after local stimulation with specific antigens (21, 34). These tissues are particularly important for protection against diseases for which entry and pathogenesis involve the mucosal system, such as salmonellosis, tuberculosis (14), and AIDS. Human immunodeficiency virus type 1 (HIV-1) infection of colorectal and vaginal epithelial cells seemed possible in vivo (23, 30, 32) and in vitro in intestinal cell cultures (2, 10, 36). Therefore, the induction of both mucosal and systemic immune responses may contribute to prevent HIV-1 infection and AIDS.

Simian immunodeficiency virus (SIV) infection of rhesus monkeys causes an immunodeficiency syndrome similar to that observed in humans (31) and is a practical model for HIV vaccine research. Ororectal immunization of rhesus monkeys with SIV antigen seems to elicit T- and B-epitope responses at both the mucosal and systemic levels (29). A live, partially attenuated *nef* deletion mutant of SIV induces cellular immune responses (9) and protects against a challenge with a high dose of pathogenic SIV (7). These findings raise safety concerns about the use of attenuated viruses as AIDS vaccine candidates because reversion to virulence is not excluded. In contrast, systemic immunization with recombinant live attenuated BCG vectors induces humoral and cellular responses in mice against a variety of antigens. Cytotoxic T-cell induction against HIV-1 and SIV *gag* or *env* antigens has been described following immunization of mice or rhesus monkeys with recombinant *Mycobacterium bovis* BCG (rBCG) expressing these antigens (1, 45, 47). Moreover, protective immune responses were obtained in small animals immunized with rBCG expressing HIV *env* (22). In this study, an rBCG vector secreting the V3 epitope of HIV-1 induced cellular and humoral immune responses that protect against virus infection in a severe combined immunodeficiency (SCID) mouse model. More recently, we induced specific cytotoxic T-lymphocyte (CTL) responses in mice by subcutaneous immunization with rBCG expressing the SIV Nef antigen (46). We have also shown that BCG priming will not be a limitation to use of BCG recombinants. Immunization of mice with *lacZ* or HIV-1 *nef* rBCG did not suppress cellular immune responses but strongly potentiated anti-β-galactosidase antibody responses (16). rBCG is an efficient oral vaccine delivery system. Indeed, the rBCG expressing *lacZ* gene elicited cellular and humoral immune responses to β-galactosidase in orally immunized guinea pigs and mice, and specific immunoglobulin A antibodies have been found in mouse intestinal secretions (27). The antibody response to the major HIV-encoded proteins in infected individuals has been described (24, 40). However, the poor correlation between the antibody response and disease progression (3, 12) suggests that these antibodies raised against the virus may not be of primary importance in controlling the evolution of the infection.

CTL immune responses are more likely to contribute to mucosal immunity to HIV and SIV by controlling cell-associated infection. Particularly, the intestinal intraepithelial lymphocytes (i-IEL) are considered to be the first-line defense against invasion by members of the intestinal flora or pathogens. The majority of i-IEL are CD8-expressing cells, of which there are two populations, expressing either CD8$^+$ α/β heterodimeric or CD8$^+$ α/α homodimeric coreceptors (21). The CD8$^+$ α/β T lymphocytes are expressing T-cell receptor α/β and depend on major histocompatibility complex class I molecules.

We investigated the behavior of rBCG SIV *nef*-expressing bacilli in the orogastrointestinal tract. We observed its viability in the intestinal lumen, where it encounters unfavorable con-

* Corresponding author. Mailing address: Laboratoire du BCG, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: (33).01 45 68 82 35.

ditions for its survival. Its translocation into inductive lymphoreticular tissues along the orointestinal epithelia and its persistence in target organs were investigated.

The second goal was to study the specific cellular immune responses induced against the Nef antigen by T cells isolated from different oropharyngeal and intestinal lymphoid organs. We then analyzed the cytotoxic activity induced in the i-IEL by oral immunization with rBCG SIV *nef*.

After oral ingestion, rBCG was translocated into the oropharyngeal epithelia and intestinal mucosa. The rBCG bacilli were then disseminated in Peyer's patches (PP) and systemic target organs, where they persisted. Large numbers of rBCG survived in the intestinal lumen. They induced specific cellular responses such as proliferative and cytokine production in oropharyngeal and systemic lymphocytes. A specific anti-Nef CTL in i-IEL CD8β^+ T-cell subset was detected. An important local and systemic cytotoxicity was demonstrated in mice orally immunized with rBCG expressing SIV *nef*. These findings provide evidence that oral immunization with recombinant BCG SIV *nef* may contribute to regional host defense against intestinal viral invasion.

MATERIALS AND METHODS

Animals. Specific-pathogen-free BALB/c mice (6 weeks old) from Iffa Credo (Saint-Germain sur l'Arbresle, France) were used in this study.

Microorganisms. The *M. bovis* BCG Pasteur strain 1173P2 was used as the host for the construction of the recombinant BCG SIV *nef* strain as previously described (46). Briefly, the $SIV_{mac\ 251}$ *nef* gene was cloned in *M. bovis* BCG under the control of P_{AN}, a promoter from *Mycobacterium paratuberculosis*. Nef was expressed as a fused polypeptide with ORF2, an open reading frame adjacent to P_{AN}. The rBCG SIV *nef* strain and the wild-type strain 1173P2 were grown as dispersed bacilli in Beck-Proskauer medium (15). The resulting vaccine suspensions were stored at −70°C until use, and the number of CFU per milliliter was determined by plating suitable saline dilutions on Middlebrook 7H10 agar with or without kanamycin (10 μg/ml).

Immunization. For oral immunization, mice were deprived of water overnight. Next morning, they received 100 μl of BCG containing 10^{10} CFU per ml for five consecutive days (total dose, 5×10^9 CFU). The thirsty animals easily swallowed the 100 μl of BCG suspension which was dropped into their mouth with a yellow tip of an Eppendorf pipette. To avoid large differences in the BCG dosage among animals, after each BCG ingestion, 100 μl of 3% sodium bicarbonate was given to the mice.

rBCG SIV-nef growth in target organs. The growth of rBCG was monitored by counting the CFU in the oropharyngeal lymphoid tissues, called by others (35) salivary-associated lymphoid T tissue. These organs are located in the oral region, just under the mandible: the submandibular glands (SMG) are on the median line, the periglandular lymph nodes (PGLN) are on each side of it, and the cervical lymph nodes (CLN) are lateral to them. At various intervals after oral immunization, these individual tissues and PP were carefully isolated, transferred to separate petri dishes, and dissociated into single cells. The mesenteric lymph nodes (MLN), spleens, lungs, and livers of mice were homogenized (Stomacher, Lab-Blender 80; Bioblock). Suitable dilutions were plated on Middlebrook 7H11 medium supplemented with kanamycin (10 μg/ml). To recover rBCG from the intestinal lumen, feces were decontaminated with a solution of 0.2% malachite green and 3% sodium hydroxide and then neutralized with 2% chlorhydric acid solution. The samples were centrifuged at 14,470 × *g* and the pellet was diluted in saline and plated on culture medium as described above. The number of CFU was counted after 18 days of incubation at 37°C.

Proliferation assay. At various time intervals after oral immunization, the spleen, MLN, CLN, and PGLN nodes were removed and single-cell suspensions were prepared in RPMI 1640 medium containing 5% fetal calf serum (FCS), 1% glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were cultivated at 8×10^5 cells in 0.2 ml per well in 96-well flat-bottom culture plates (Nunc) in the presence of SIV Nef 146-160 peptide (1 μg/ml). This peptide was used previously (46) at different concentrations and at 1 μg/ml induced the highest proliferation of lymph node cells from mice subcutaneously immunized with rBCG SIV *nef*. The cells were incubated for 4 days at 37°C under 7% CO_2, 0.4 mCi of [^{3}H]thymidine (Amersham, Little Chalfont, United Kingdom) was added to each well, and the cells were grown overnight. The cells were harvested on fiberglass filters and washed with an Automash 2000 (Dynatech, Bioblock, Illkirch, France), and the incorporated radioactivity was measured in a liquid scintillation counter (Beckman). Results are given as geometrical means of triplicate determinations, and the stimulation index (SI) calculated as (cpm with antigen stimulation)/(cpm without antigen stimulation).

Cytokine ELISPOT assay. An adaptation of the enzyme-linked immunospot (ELISPOT) assay (6, 41) was used to enumerate gamma interferon (IFN-γ)- or tumor necrosis factor alpha (TNF-α)-specific spot-forming cells (SFC). To detect IFN-γ-producing cells, 96-well nitrocellulose plates (Millititer, HA; Millipore, Molsheim, France) were coated with anti IFN-γ monoclonal antibody (MAb) (R4-6A2; Pharmingen) at 4 μg/ml in bicarbonate buffer (100 μl/well). Control wells were coated with bicarbonate buffer alone. All wells were then blocked for 60 min at 37°C with RPMI 1640 containing 10% FCS. T cells were stimulated for 24 h with 1 μg of SIV Nef 146-160 peptide per ml or with 10 μg of mycobacterial purified protein derivative (PPD; Ministry of Agriculture, Weybridge, United Kingdom) per ml. Cells were then added to wells at various concentrations ranging from 2.5×10^5 to 1×10^6 cells/100 μl/well. They were incubated for 20 h at 37°C under 7% CO_2 in the presence of the same antigens. The plates were then thoroughly washed once with H_2O and then five times with phosphate-buffered saline (PBS) containing 0.05% Tween (PBS-Tween) and were incubated overnight at 4°C with 100 μl of biotinylated anti-IFN-γ MAb (4 μg/ml) (XMG 1-2; Pharmingen) per well. The plates were washed again with PBS-Tween at least five times, avidin-phosphatase alkaline (Sigma) diluted 1:1,000 was added, and the mixture was incubated overnight at 4°C. After the plates were washed with PBS-Tween, spots (representing IFN-γ-secreting cells) were developed with the substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate–Nitro Blue Tetrazolium [Sigma]). The number of spots was determined with the aid of a dissecting microscope (GSZ; Bioblock Scientific, Illkirch, France).

A similar assay was used to determine the number of TNF-α-specific SFC. Appropriate numbers of T cells were stimulated in vitro with the same SIV Nef or PPD antigens as described above and then incubated in 96-well nitrocellulose plates coated with 4 μg of anti-TNF-α MAb (MP6-XT22; Pharmingen) per ml. To determine the number of TNF-α-secreting cells, 100 μl of biotinylated-polyclonal anti-TNF-α antibody (Pharmingen) was added (4 μg/ml). After appropriate incubation and washing, the spots were developed and the TNF-α SFC were enumerated as described above.

Isolation of i-IEL. i-IEL were prepared as described elsewhere (19). Briefly, the small intestine was cleaned of the mesentery and intestinal contents were flushed out with PBS, and PP were removed. Then, the small intestine was cut into 2-cm-long pieces opened longitudinally. The mucosa were dissociated by mechanical disruption under magnetic stirring for 15 min at room temperature in medium 199 (Gibco BRL, Life Technologies, Paisley, United Kingdom) containing 10% FCS (Seromed; Biochrom KG, Berlin, Germany) and 1 mM dithioerythritol (Sigma). Tissue debris and cell aggregates were removed by passage through a glass wool column (1.6 g packed in a 20-ml-capacity syringe) in medium 199 containing 10% FCS. The lymphocytes were obtained by centrifugation on a Ficoll layer ($d = 1.077$) (Nycoprep; Nycomed, Oslo, Norway). The cells were then suspended in complete medium consisting of RPMI 1640 (Seromed) containing 10% FCS, 100 U of penicillin per ml and 100 μg of streptomycin per ml (Seromed), 1% L-glutamine (Seromed), and 5×10^{-5} M 2-mercaptoethanol (Gibco BRL).

Purification of i-IEL T-cell subsets. The total population of i-IEL obtained from the small intestine of 12 mice was suspended in PBS containing 5% FCS. Aliquots of 3×10^7 cells were each incubated with 2 μg of rat anti-mouse CD8β MAb (53-5.8; Pharmingen, San Diego, Calif.) for 30 min at 4°C and then for 15 min with goat anti-rat IgG microbeads (Miltenyi Biotec, Bergish, Gladbach, Germany) at 4°C. The cells were applied to a column prewashed with PBS–5% FCS on a small magnetically activated cell sorter (mini-MACS system; Miltenyi). Nonadherent CD8β^- cells were collected and reapplied to the column three times. The separation column was then removed from the mini-MACS, and CD8β^+ cells were eluted by washing with PBS–5% FCS. The i-IEL CD8β^+ and CD8β^- cells were then suspended in complete medium for cytotoxicity assays.

Local cytotoxicity assay. Fresh i-IEL CD8β^+ and CD8β^- T-cell subsets were assayed for cytolytic activity against ^{51}Cr-labeled P815 mastocytoma target cells. Thus, both CD8β^+ and CD8β^- i-IEL T-cell subsets obtained from naive and immunized mice were incubated for 4 h with P815 target cells at 37°C under 7% CO_2. Radioactivity released into supernatants was counted, and the percentage of specific lysis was calculated as 100 × [(cpm released from target cells in the presence of effector cells − cpm released from target cells alone)/(cpm released from target cells lysed by 10% Triton X-100 − cpm released from target cells alone)]. P815 target cells were preincubated for 24 h before the assay with an SIV Nef 46-60 peptide (10 μg/ml). This peptide is a CTL epitope in mice immunized subcutaneously with rBCG SIV-Nef (46).

Systemic cytotoxicity assay. At various times after oral immunization with wild-type or rBCG SIV *nef*, single suspensions of MLN cells and splenocytes were prepared in complete medium supplemented with 10 U of interleukin-2 IL2 (Genzyme, Cambridge, Mass.) per ml. Cells were cultivated in 24-well flat-bottom plates (Nunc, Rostkilde, Denmark) at 4×10^6 cells/ml in the presence of SIV Nef 46-60 peptide. The effector cells were incubated for 5 days at 37°C under 7% CO_2 and were tested for cytotoxic activity on P815 target cells as described above.

To characterize cytotoxic effectors, the cells were cultivated for 5 days in the presence of the synthetic SIV Nef peptide 46-60; effector cells were then incubated for 1 h at 37°C under 7% CO_2 in the presence or absence of GK1-5 anti-CD4 or H35-17-2 anti-CD8 MAb (20 μg/ml). These antibodies have been described previously (8, 38). Effector cells were then washed, serially diluted, and added to target cells and the cytotoxicity was assayed.

TABLE 1. Recovery of rBCG SIV *nef* CFU in gastrointestinal lymphoid tissues and in target organs after oral immunization[a]

Day	Mean rBCG CFU ± SD in[b]:								
	Feces (1 g)	PP	SMG	PGLN	CLN	MLN	Liver	Spleen	Lungs
1	ND[c]	12 ± 3	20 ± 3	0	0	0	0	0	0
3	$1.1 \times 10^6 \pm 0.2$	82 ± 9	30 ± 12	57 ± 5	33 ± 2	0	0	0	0
5	$2.6 \times 10^6 \pm 0.2$	220 ± 28	133 ± 35	70 ± 22	14 ± 6	12 ± 2	0	0	0
8	$5.1 \times 10^4 \pm 0.005$	25 ± 2	80 ± 5	57 ± 5	17 ± 5	0	0	0	0
10	0	13 ± 2	95 ± 15	70 ± 4	32 ± 8	0	62 ± 8	0	0
15	ND	0	140 ± 43	247 ± 45	580 ± 62	18 ± 2	187 ± 9	137 ± 46	50 ± 40
30	ND	0	103 ± 12	917 ± 62	850 ± 41	117 ± 6	12 ± 2	583 ± 154	25 ± 20
60	ND	0	32 ± 4	410 ± 8	0	52 ± 2	0	883 ± 47	0

[a] BALB/c mice were immunized with 10^9 CFU of rBCG SIV *nef* given orally on five consecutive days (total dose, 5×10^9 rBCG CFU).
[b] Results are given as the mean number of rBCG CFU per gram of feces and per mouse organ ± standard deviation ($n = 3$).
[c] ND, not done.

To determine the MHC class I restriction, EL4 cells obtained from a lymphoma of C57BL/6 $H2^b$ mice were also used as targets for cytotoxic effectors.

Statistical method. Significant differences in CTL responses were determined by Student's *t* test.

RESULTS

Recovery of rBCG in gastrointestinal lymphoid tissues and target organs. The wild-type and rBCG SIV *nef* were administered orally on five consecutive days at a dose of 10^9 CFU per day. The rBCG was recovered 1 day after ingestion in PP and 3 days after ingestion in the SMG, PGLN, and CLN (Table 1). Between 10 and 15 days after immunization, rBCG could be recovered from the major target organs, earlier in the liver (day 10) than in the MLN, spleen, and lungs (day 15), but not in the axillary lymph nodes. rBCG persisted for 2 months in the SMG, PGLN, MLN, and spleen. Few CFU were found in oropharyngeal and intestinal mucosa: less than 50 to 900 CFU per organ. This is in accordance with previous findings (28). The largest number of viable rBCG was found in feces (more than 10^6 CFU per g). There were no significant differences between wild-type BCG and rBCG recovery (17). Intragastric administration was also performed, but BCG could not be recovered from SMG, PGLN, or CLN (data not shown).

Induced proliferative responses. After oral immunization with wild-type BCG or rBCG expressing SIV *nef*, in vitro proliferative responses to SIV Nef 146-160 peptide of splenocytes and PGLN, CLN, and MLN cells were analyzed. Specific proliferative responses were induced 1 month after oral immunization with the rBCG strain, peaked at 2 months, and decreased after 3 months, remaining positive in all tissues studied and slightly higher in splenocytes (SI > 4) as shown in Fig. 1. The higher proliferation obtained with oropharyngeal lymphoid tissues (SI = 7 to 15) correlated with the late post immunization response.

Induced cytokine production. At 4 weeks after oral immunization with wild-type BCG or rBCG SIV nef, local (i-IEL and PP) and systemic (spleen and lymph node) cells were recovered and analyzed for in vitro cytokine production. A proportion of T cells from local or systemic sites, stimulated with the SIV Nef 146-160 peptide, produced IFN-γ and TNF-α (Table 2). Oropharyngeal PGLN, PP, and splenic cells contained the largest number of SFC for both cytokines. Very few cells from mice immunized with wild-type BCG stimulated in vitro with the SIV Nef peptide produced IFN-γ or TNF-α. The wild-type BCG and rBCG immunizations led to similar numbers of IFN-γ- and TNF-α-positive cells when stimulated with mycobacterial antigen PPD.

Intestinal CD8β$^+$ i-IEL cytotoxicity. Fresh i-IEL showed a strong nonspecific cytotoxicity when stimulated with anti-CD3

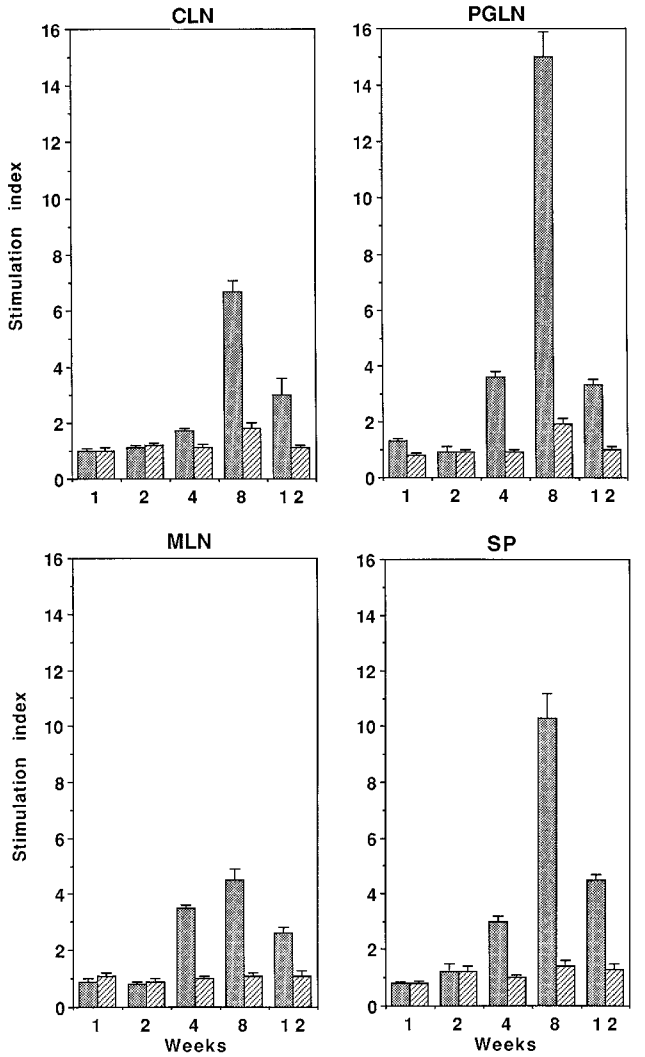

FIG. 1. Proliferative responses induced in BALB/c mice at various times after oral immunization with rBCG expressing SIV *nef*. BALB/c mice (five per group) were orally immunized with rBCG SIV *nef* (▨) or wild-type BCG (▨) (5×10^9 CFU). Cells from PGLN, CLN, MLN, and spleen (SP) were harvested at various times and stimulated in vitro for 5 days with medium or with SIV Nef 146-160 peptide. The results are expressed as the SI (counts per minute with antigen/counts per minute without antigen). Counts per minute without antigen never exceeded 10^3.

TABLE 2. Enumeration of IFN-γ and TNF-α cytokine-producing cells obtained from BALB/c mice orally immunized with rBCG SIV *nef* or wild-type BCG[a]

Origin of cells	Strain used for immunization	No. of IFN-γ SFC[b] after stimulation in vitro with:		No. of TNF-α SFC[b] after stimulation in vitro with:	
		SIV-Nef peptide (1 μg/ml)	PPD (10 μg/ml)	SIV-Nef peptide (1 μg/ml)	PPD (10 μg/ml)
PP	rBCG SIV *nef*	70 ± 10	90 ± 12	105 ± 20	100 ± 15
	Wild-type BCG	6 ± 2	100 ± 30	8 ± 2	115 ± 30
i-IEL	rBCG SIV *nef*	30 ± 5	35 ± 6	30 ± 10	35 ± 5
	Wild-type BCG	3 ± 1	30 ± 5	2 ± 0	30 ± 4
PGLN	rBCG SIV *nef*	100 ± 15	115 ± 20	250 ± 35	280 ± 30
	Wild-type BCG	6 ± 2	120 ± 30	6 ± 0	250 ± 35
MLN	rBCG SIV *nef*	30 ± 5	35 ± 2	90 ± 25	100 ± 18
	Wild-type BCG	10 ± 4	40 ± 10	12 ± 2	98 ± 10
SP	rBCG SIV *nef*	100 ± 18	120 ± 18	350 ± 60	480 ± 35
	Wild-type BCG	10 ± 4	150 ± 25	40 ± 8	350 ± 55

[a] BALB/c mice (five per group) were orally immunized with rBCG SIV *nef* or wild-type BCG (5×10^9 CFU). Four weeks later, cells from PP, i-IEL, PGLN, MLN, and spleen (SP) were harvested and stimulated with SIV-Nef 146-160 peptide or with PPD. Cytokine-producing cells were then counted.
[b] Number of SFC/10^6 cells.

MAb, consistent with previous reports (18, 20). To measure the antigen-specific cytotoxicity, we purified the CD8β$^+$ i-IEL T-cell subset by a magnetically activated cell-sorting procedure. The CD8β$^+$ i-IELs, recovered from BALB/c mice 2 weeks after oral immunization with rBCG expressing SIV *nef*, expressed specific cytolysis of P815 target cells coated with SIV-Nef 46-60 peptide (45 ± 3% at a ratio of 1:100), as illustrated in Fig. 2A. CD8β$^+$ i-IELs from naive or wild-type BCG-immunized mice were not cytolytic (≤10%) (Fig. 2A). This demonstrated the specificity of the cytotoxic response of this purified T-cell subset induced by oral administration of the rBCG SIV *nef* ($P < 0.0001$). Depletion of the CD8β$^+$ i-IEL population abrogated the CTL activity. Indeed, CD8β$^-$ i-IELs isolated from either naive or immunized mice showed no antigen-specific cytotoxicity against the same P815 target cells (≤10%) (Fig. 2B).

Splenic and MLN cell cytotoxicity. To analyze SIV Nef-specific CTL at the systemic level, splenic and MLN cells from orally immunized mice were stimulated in vitro for 5 days with SIV Nef 46-60 peptide and assayed for cytotoxicity against P815 target cells coated with the same SIV Nef peptide. A strong cytotoxic activity was observed either with MLN cells (61 ± 3%) or with splenocytes (64 ± 2%) (Fig. 3) and was abrogated by the addition of an anti-CD8 MAb. In contrast, the cytotoxic activity of both MLN cells and splenocytes was not affected by the addition of the anti-CD4 MAb (Fig. 3). Thus, at the systemic level, orally administered rBCG SIV *nef* induced cytotoxic responses. This cytotoxicity was specifically directed against SIV-Nef antigen ($P < 0.0001$).

A memory-specific CTL response lasting 3 months was obtained in splenocytes (about 60% at ratio of 1:100), and a response lasting 2 months was obtained at a lower level in MLN cells (Fig. 4).

No cytotoxicity was found when $H2^d$ effector cells from BALB/c mice were tested against $H2^b$ target cells obtained from a lymphoma of C57BL/6 mice (data not shown). The cytotoxicity of CD8β$^+$ i-IELs and splenic or MLN CD8$^+$ T cells demonstrated that local and systemic cytotoxicities were MHC class I restricted.

DISCUSSION

CTL may control the spread of viral infections by lysing infected host cells (42, 44). In particular, in HIV or SIV infections, local cytotoxicity of intestinal IELs seems to be a major defense against HIV enteric invasion (39). In animal experiments, parenteral immunization with various HIV or SIV BCG recombinants induced CD8 MHC class I-restricted CTL (25, 47). In contrast, immune responses after oral immunization with HIV or SIV BCG recombinants has not previously been studied. Oral immunization with rBCG expressing SIV *nef* deserved investigation in view of the known advantages of BCG. In particular, this bacillus has already been orally administered as vaccine to humans and confers a degree of protection against tuberculosis. Orally administered *lacZ* rBCG induces bivalent immune responses against both mycobacterial antigens and the antigens carried in animals. It also persists for a long time in the host, thereby providing lasting immunogenic stimulation and adjuvant behavior. The lack of recovery of BCG from oropharyngeal tissues after intragastric administration correlates with its recovery from these organs earlier than from other sites after oral ingestion and strongly suggests that BCG translocates across the oropharyngeal mucosa. Moreover, the incidence of cervical adenitis observed in children after oral BCG administration may be explained by a similar route of translocation (43). The large quantity of viable BCG in feces shows that it can traverse the entire length of gastrointestinal tract, resisting factors that were assumed to be mycobactericidal, such as gastric acidity and enzymatic lysis. The late strong proliferation and cytokine production obtained with T cells from the oropharyngeal organs suggest that BCG can induce immune responses in any segment of the gut by invading and multiplying. It would be interesting to determine (by preventing BCG contact with the oral mucosa) the part played by the oral mucosa compared to the intestinal mucosa in induced immune responses.

Mucosal CTL responses have shown (20) that fresh i-IEL lysed target cells only after T-cell receptor ligation with MAbs specifically raised against CD3 complexes. This cytolysis was not specific, since i-IEL from naive mice are cytotoxic when

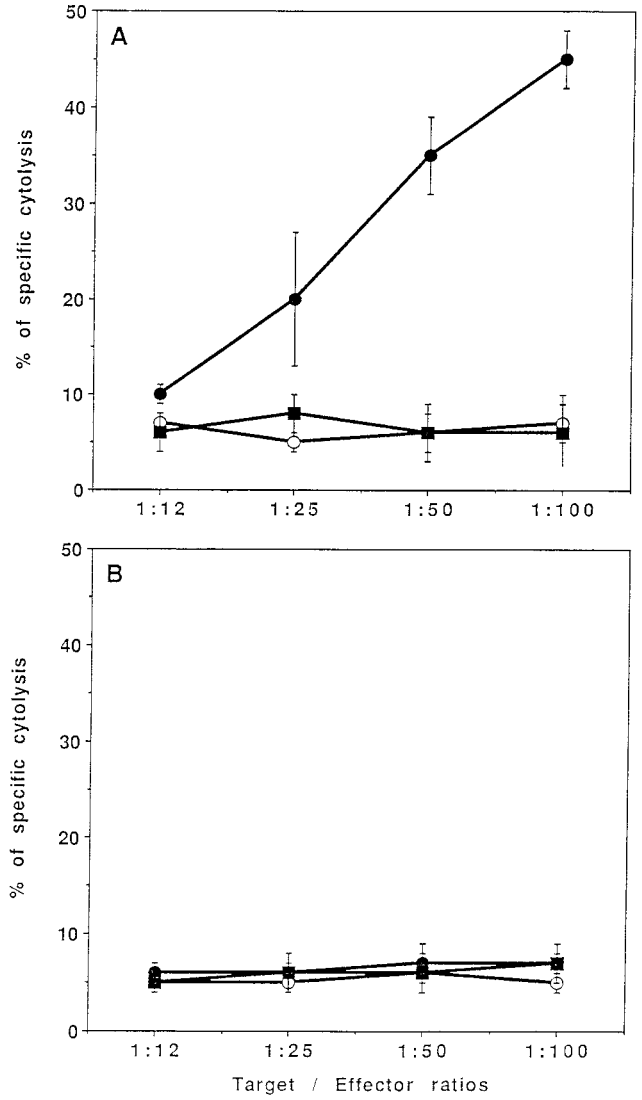

FIG. 2. Intestinal CD8β$^+$ IEL cytotoxicity induced by oral immunization with rBCG expressing SIV *nef*. BALB/c mice were orally immunized with rBCG SIV *nef* (●) or wild-type BCG (○) (5×10^9 CFU). Naive mice (■) were used as controls. After 14 days, fresh CD8β$^+$ and CD8β$^-$ i-IEL subsets (12 mice per group) were purified by magnetically activated cell sorter treatment. The specific cytotoxic activity of CD8β$^+$ (A) or CD8β$^-$ (B) subsets was measured after a 4-h incubation with P815 coated with the SIV Nef 46-60 peptide as target cells. Significant lysis was found only with CD8β$^+$ i-IEL from mice immunized with rBCG SIV *nef*.

stimulated with anti-CD3 MAb. In contrast, the SIV-Nef specific cytotoxicity we obtained with CD8β$^+$ i-IEL shows that these cytotoxic cells are induced by local antigenic stimulation. Indeed, CD8β$^+$ cytotoxic i-IEL have been characterized elsewhere (20) as the progeny of the thymodependent lymphoblasts elicited in PP by antigenic stimulation. Thus, oral immunization with recombinant BCG expressing SIV *nef* led to an antigen-specific CTL response in the thymus-dependent CD8β$^+$ population. The thymus-independent CD8β$^-$ T-cell subset from bone marrow failed to lyse target cells specifically. These i-IELs CTL responses are in agreement with the results of other studies, which have also shown similar specific CTL responses in i-IEL (37) or PP cells (33) after oral immunization with parasites (5) or viruses.

The induction of systemic CTL, T-cell proliferation, and IFN-γ or TNF-α production in splenic or oropharyngeal lymph node cells after oral immunization suggests the recirculation of the specifically activated T lymphocytes of both helper CD4$^+$ Th1-type and cytotoxic CD8$^+$ cells.

The induction of a CTL response against proteins produced early after and at the site of entry of the infecting SIV might allow the elimination of infected host cells before they release infectious viral particles. Therefore, vaccines that stimulate a strong CD8$^+$ Nef-specific CTL could protect against SIV in-

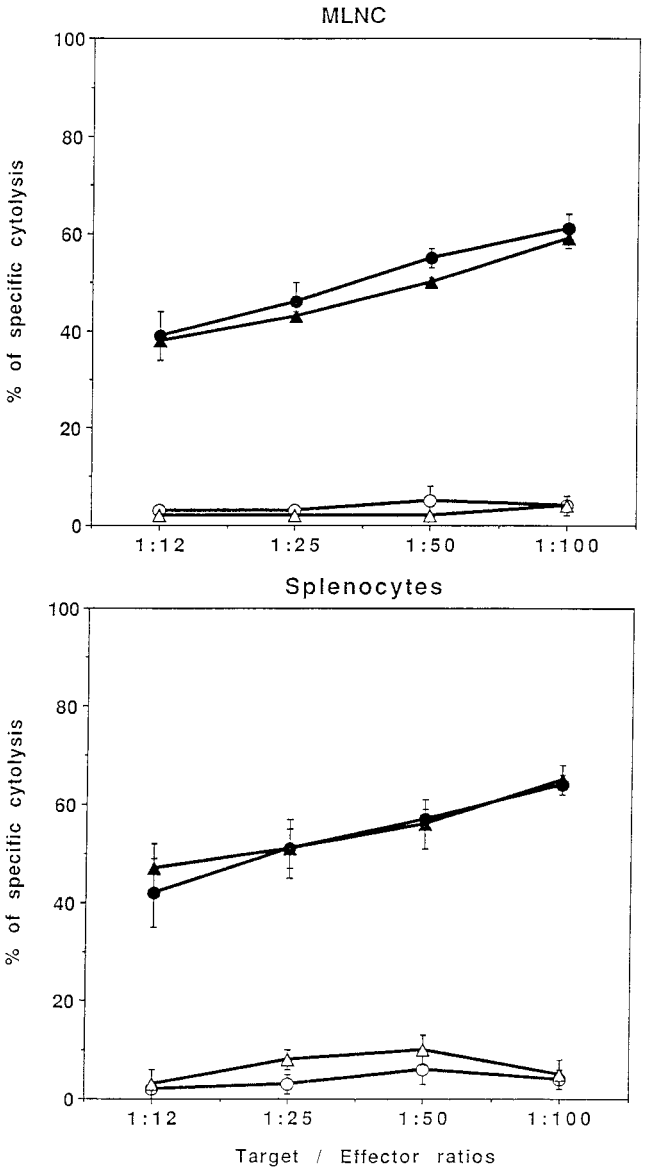

FIG. 3. Splenic and MLN cell cytotoxicity induced by oral immunization with rBCG expressing SIV *nef*. To monitor the SIV Nef-specific cytotoxicity of splenocytes and MLN cells, BALB/c mice were orally immunized with rBCG SIV *nef* (●) or wild-type BCG (○) (5×10^9 CFU). After 30 days, splenocytes and MLN cells were cultured for 5 days with the SIV-Nef 46-60 peptide (1 μg/ml). The cytotoxic activity of these cells was then tested against P815 cells coated with the same peptide. The phenotype of cytotoxic cells was determined by adding medium alone (●) and either anti-CD8 (△) or anti-CD4 MAb (▲) to the effector cells. No SIV-Nef-specific cytolysis was found in wild-type-BCG-immunized mice. Both splenocytes and MLN cells lysed target cells. No lysis was observed in the presence of anti-CD8 MAb.

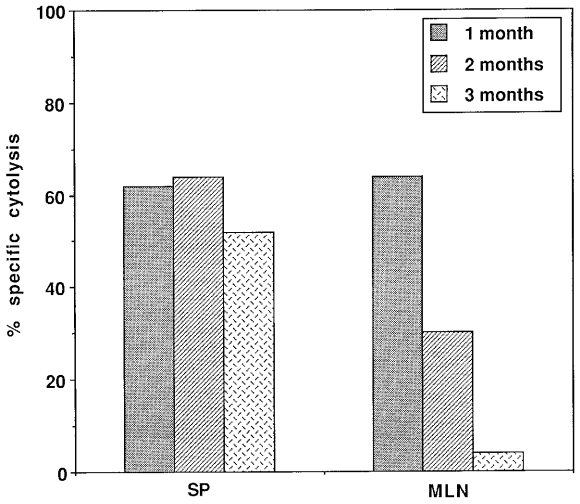

FIG. 4. Memory systemic cytotoxicity induced by oral immunization with rBCG expressing SIV *nef*. To analyze memory SIV Nef-specific cytotoxicity, BALB/c mice were orally immunized with rBCG SIV *nef*, and splenocytes and MLN cells were cultured for 5 days with the SIV Nef 46-60 peptide (1 μg/ml). At various times after immunization, the cytotoxicity of these cells was tested against P815 cells coated with the same peptide. Splenocytes presented a long-lasting CTL response.

fection by early suppression of SIV replication (13). The *nef* gene is of prime interest: SIV mutants deleted for the *nef* gene induce protection against a virulent challenge, clearly demonstrating the importance in vivo of the *nef* gene (7).

For viruses like SIV or HIV-1, which initially contact the epithelium, the presence of virus-specific CTL cells at this site could result in local reduction or containment of the infectious agent before its dissemination. The specific and substantial target cell cytolysis produced by $CD8\beta^+$ IELs suggests that IELs may protect intestinal epithelial integrity by killing SIV-infected cells at the site of entry (26).

The ability of immune IELs to lyse target cells and to produce IFN-γ and TNF-α specific for the SIV Nef peptide may also have important implications for the defense of the epithelium against SIV infection and dissemination. Production of IFN-γ favored the differentiation of CTL, probably by inducing the release of interleukin-12. TNF-α could augment lymphocyte migration and, by macrophage activation, facilitate antigen presentation. The effects of proinflammatory cytokines on SIV or HIV binding to potential target cells is not known, but the initial pathogen-host interactions seem susceptible to the activity of cytokines representative of inflammatory conditions (4, 11).

In this study, we demonstrated that oral immunization with rBCG vaccine expressing SIV *nef* elicited strong mucosal and systemic cellular and, particularly, CTL immune responses. Although the precise nature of the protective responses against HIV or SIV infections has not been elucidated, many reports suggest the importance of the CTL response, particularly against proteins produced early during the viral cycle. These results suggest that orally administered rBCG SIV *nef* could be a candidate vaccine to protect macaques against SIV infection. This could lead to the development of an rBCG HIV *nef* vaccine to be used orally in humans.

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