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Recognition of Prominent Viral Epitopes Induced by Immunization with Human Immunodeficiency Virus Type 1 Regulatory Genes

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Mice immunized with the regulatory genes *nef*, *rev*, and *tat* from human immunodeficiency virus type 1 developed both humoral and cellular immune responses to the gene products Nef, Rev, and Tat. This study demonstrates that it is feasible to induce immune reactions to all of these regulatory gene products. Humoral responses were seen after DNA boosts, while potent T-cell proliferative responses were noted already after a single immunization. A Th1-directed immune response was demonstrated early after immunization. A 3- to 75-fold-stronger T-cell response was seen in animals receiving DNA epidermally compared to that in animals receiving intramuscular injections. Nef, Rev, and Tat putative B- and T-cell epitopes were clearly mapped by using peptides derived from the regulatory proteins and were similar to those which are detected in human immunodeficiency virus infection. Although immunization by the Nef, Rev, and Tat proteins raised high immunoglobulin G titers in serum, the epitope spreading appeared broader after DNA immunization. The combination of all of these regulatory genes together with two genes for structural proteins, the envelope and *gag* genes, demonstrated that a combined approach is feasible in that reactivities to all antigens persisted or were even augmented. No interference between plasmids was noted.

Genes introduced as naked DNA in plasmids have been shown to induce immune reactivities toward a number of infectious agents, such as influenza A virus, hepatitis B virus, bovine herpesvirus, rabies virus, and the tuberculosis bacterium and malaria organism (5, 7, 8, 11, 17, 24, 28, 39, 46, 47). In several studies, even protection from lethal infection could be demonstrated (28, 32, 39, 40, 44, 48, 49). An efficient approach to inducing protective immunity toward human immunodeficiency virus (HIV) type 1 (HIV-1) has yet to be discovered, although T- and B-cell responses to HIV-1 proteins have been induced by using a variety of antigens, immune stimulators, and vaccination routes (25, 27, 30). HIV proteins and glycoproteins have not been able to reproducibly induce protective effects. Lysates of whole infected cells and attenuated virus (HIV-2, simian immunodeficiency virus) have met with better success. These approaches, however, may not be suitable for human use because of their retroviral nature. The aim of using naked DNA would therefore be to obtain antigen presentation, via the endogenous major histocompatibility complex (MHC) class I pathway, similar to that which can be achieved with live vector vaccines, without their potential risks. The HIV-1 regulatory proteins, which appear early in the infected cells, are attractive targets for immunization (13, 23, 29, 31). If HIV-1-infected cells expose epitopes from the regulatory proteins and if an immune response to these epitopes could be induced, termination of early virus replication might be possible. Immune reactivity to the HIV-1 envelope proteins and regulatory proteins Nef, Rev, and Tat has been described previously (15, 16, 33, 41, 42). It remains to be demonstrated that immunity to these regulatory proteins can induce protection. In our study, the capacities of cDNA plasmids containing the regulatory genes *rev*, *tat*, and *nef* of HIV-1 to induce immune responses to the gene products were investigated in four strains of mice. A comparison of the abilities of injected and gold particle-administered plasmid DNAs to elicit immune responses to the HIV-1 regulatory proteins was performed with several mouse strains. The aim was to investigate which B- and T-cell epitopes were immunogenic when plasmid DNA was used for immunization. We also wished to analyze the feasibility of combined immunization with several genes and to identify whether the responses to combination treatment were as good as those seen with the single components.

MATERIALS AND METHODS

Animals. Ten- to 12-week-old DBA/2 ($H\text{-}2^d$) and (C57BL/6 × DBA/2)F_1 ($H\text{-}2^{b/d}$) mice were used to test DNA dose responses, and BALB/c mice ($H\text{-}2^d$) were used to investigate the kinetics of the immune response after a single-dose primary immunization. C57BL/6 ($H\text{-}2^b$) and CBA/J ($H\text{-}2^k$) mice received protein immunizations. Ten- to 12-week-old and 6- to 8-week-old scid mice (C.B-17 scid/scid) were given 2×10^7 human peripheral blood lymphocytes (HLA-A2) intraperitoneally (huPBL-scid mice). The scid mice were kindly provided by C. A. K. Borrebaeck (Bioinvent, Lund, Sweden). Each experimental group contained four to eight animals.

DNA immunogens and immunization. Plasmids contained the HIV-1 *nef* (HXB3), *tat*, *rev*, $p37^{gag}$ (HXB2), and gp160 (Bru) genes under the control of the human cytomegalovirus (CMV) immediate early promoter (3, 27). The *rev* coding sequence is followed by the rat preproinsulin gene poly(A) signal, the *tat*-encoding plasmid contains the human papillomavirus type 16 (HPV-16) late poly(A) signal (nucleotides 7272 to 7453; numbers refer to the genomic HPV-16 molecular clone), and the *nef* gene utilizes the HIV-1 3′ long terminal repeat poly(A) signal. Plasmid pCMV37*gag* has been described before as pC41M (21) and contains the HIV-1 clone pHXB2 $p37^{gag}$-encoding sequence with multiple mutations in the RNA sequence which do not alter the protein sequence and which allow Rev-independent, high-level $p37^{gag}$ production (37). Transcription from this plasmid is driven by the human CMV promoter, and the $p37^{gag}$ coding

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TABLE 1. Antibody responses to HIV-1 regulatory and structural genes after DNA-based or protein immunization

Immunization and mouse strain	Frequency (median OD) of IgM/IgG response to[a]:					Memory B-cell stimulation[b] (frequency)		
	Nef	Rev	Tat	p24	gp160	Nef	Rev	Tat
Plasmid DNA								
BALB/c	15/16 (0.52)	0/6	1/6 (0.54)	ND[c]	ND	15/16	4/6	5/6
DBA/2	6/6 (2.00)	6/6 (1.24)	5/6 (1.20)	ND	ND	6/6	6/6	6/6
(C57BL/6 × DBA/2)F_1	30/43 (0.60)	9/29 (0.56)	22/29 (0.51)	28/29 (0.51)	22/29 (0.50)	27/33	13/19	26/29
huPBL-scid	5/11 (0.39)	10/11 (0.35)	2/11 (0.41)	ND	5/5 (0.42)	5/11	10/11	6/11
Protein								
BALB/c	4/4 (3.00)	4/4 (3.00)	4/4 (3.00)	ND	ND	4/4	4/4	4/4
DBA/2	5/5 (3.00)	5/5 (3.00)	5/5 (3.00)	ND	ND	ND	ND	ND
C57BL/6	5/5 (3.00)	5/5 (3.00)	5/5 (3.00)	ND	ND	ND	ND	ND
CBA	3/3 (3.00)	3/3 (3.00)	3/3 (3.00)	ND	ND	ND	ND	ND
None (control)								
BALB/c	0/4 (0.10)	0/4 (0.04)	0/4 (0.12)	ND	ND	0/4	0/4	1/4
(C57BL/6 × DBA/2)F_1	0/6 (0.08)	0/6 (0.03)	0/6 (0.09)	0/5 (0.02)	0/5 (0.04)	1/6	0/6	1/6
huPBL-scid	0/15 (0.04)	0/15 (0.09)	0/15 (0.09)	0/5 (0.03)	0/5 (0.02)	0/5	0/5	0/5

[a] The mice received ≥2 immunizations. Median OD, median optical density derived from a serum dilution of 1/50.
[b] All tested mice with one or more immunizations.
[c] ND, not done.

sequence is followed by the HPV-16 late poly(A) signal (nucleotides 7272 to 7453). Thus, none of the plasmids were constructed for secretion of the cDNA products. The doses of plasmid DNA varied from 1 to 100 μg. They were given one to three times with 3- to 4-week intervals. The DBA/2 and BALB/c mice were used to test which dose of plasmid is needed to evoke an immune response with the regulatory proteins Rev, Tat, and Nef only. Six mice were used in each group. BALB/c mice were used for comparison of intramuscular (i.m.) doses administered by injection in the right quadriceps muscle and epidermally in the abdominal skin by use of the helium pulse Accell device (courtesy of J. Haynes, Auragen, Madison, Wis.) with 1- to 3-μm-diameter gold particles (Aldrich Chemical Co., Milwaukee, Wis.) coated with cDNA (9, 15, 16). BALB/c mice were used to test the kinetics of the immune response to the regulatory proteins after primary DNA immunization.

Six mice each were used to study the responses toward Rev and Tat, and 27 mice were used to study in detail the response toward Nef.

The F_1 mice were given plasmids by the same routes as the BALB/c mice, with doses ranging from 2 to 100 μg. These mice were also given combinations of genes. The same amounts of each plasmid were used (2, 5, or 10 μg of plasmid per animal). Forty-three F_1 mice were given plasmids; of these, 14 mice received only the *nef* plasmid, 22 mice received the *nef*, *rev*, *tat*, gp160, and $p37^{gag}$ plasmid combination, and 7 mice received *nef*, *rev*, $p37^{gag}$, and gp160. Structural cDNA genes were always given together with the *rev* plasmid.

The huPBL-scid mice were given plasmids by intraperitoneal and intradermal (i.d.) injection in doses ranging from 1 to 100 μg. Six huPBL-scid mice received the *nef*, *rev*, or *tat* plasmids, and 10 received *rev* and gp160 plasmids combined. Control animals, given empty plasmid vector and/or phosphate-buffered saline (PBS) (pH 7.4), were included in all groups (see Tables 1 and 2).

Protein immunization. Eight- to 12-week-old BALB/c, DBA/2, CBA, C57BL/6, and F_1 hybrid mice were immunized subcutaneously with recombinant protein Nef (20), Rev, or Tat in doses ranging from 5 to 50 μg of protein in Freund or Ribi adjuvant (Sigma, St. Louis, Mo.). The mice were given booster immunizations 3 weeks after the primary immunization with the same antigen dose as in the primary immunization.

Immunoassays. Serological responses were measured by enzyme-linked immunosorbent assay (ELISA). The antigens used were affinity-purified recombinant proteins Tat (kindly provided by J. Karn, Medical Research Council, United Kingdom) and Nef (kindly provided by B. Kohleisen and V. Erfle, GSF, Germany). Recombinant Rev (Swedish Institute for Infectious Disease Control, Stockholm, Sweden) expressed in a baculovirus system was also used. The synthetic peptides used were 20-mers with a 5-amino-acid (5-aa) overlap covering the complete HIV-1 LAI strain regulatory proteins Nef, Rev, and Tat. Amino acids were according to the Los Alamos Data Base on Retroviruses (26). The peptides were synthesized by the 9-fluorenylmethoxycarbonyl method (34).

The concentrations used for coating were 1 μg/ml for recombinant proteins and 10 μg/ml for synthetic peptides. Antigens were diluted in 0.1 M carbonate buffer (pH 9.6), and 100 μl/well was added to 96-well plates (Nunc, Aahus, Denmark). The plates were sealed and incubated for 18 to 20 h at room temperature and stored at 4°C. Prior to use, the plates were blocked with 5% dried milk in PBS. Sera were diluted in PBS (pH 7.4) with 1% bovine serum albumin (BSA), 2% goat serum, 2.5% dried milk, and 0.05% Tween 20. A 100-μl-sample of serum per well was incubated for 60 min at 37°C before a washing and addition of horseradish peroxidase-labelled goat anti-mouse immunoglobulin M (IgM) (Southern Biotechnologies, Birmingham, Ala.) or IgG (Bio-Rad, Richmond, Calif.) or goat anti-human IgG or IgM. The 60-min incubation was repeated, the wells were washed, and *o*-phenylene diamine dissolved in 0.1 M citric acid (pH 5.5) and activated with H_2O_2 was added as a substrate. Color development was terminated with 100 μl of 2.5 M H_2SO_4 per well. The absorbance was measured at 490 nm. Absorbance values higher than twice the preimmunization serum absorbance were considered positive.

B-cell stimulation in vitro. Spleen cells (100,000/well in 200 μl of RPMI 1640 supplemented with 5% inactivated fetal calf serum) were cultured at 37°C in 96-well flat-bottomed cell culture plates (Nunclon; Nunc) with stimulating antigens (1 μg/well) for 72 h. After a washing, antigen-specific immunoglobulins were measured by ELISA as described above.

T-cell stimulation. Spleen cells (200,000/well) were cultured for 5 to 6 days in RPMI 1640 supplemented with 4 mM L-glutamine, 50 IU of penicillin per ml, 50 μg of streptomycin per ml, and 10% fetal calf serum in the presence of 0.1 to 1 μg of antigen. Cell rinsings from the peritoneal cavity of the repopulated huPBL-scid mice were used. A 50-μl volume of ^{3}H-labelled thymidine was added per well (1 μCi), and the cells were incubated for 16 h before they were harvested and thymidine incorporation was measured in a β-counter. The stimulation index (SI) was calculated by dividing the mean counts per minute in triplicate wells of the antigen-stimulated wells by the mean counts per minute of medium control wells. A >5-fold SI with whole proteins and a >3-fold SI with synthetic peptides was considered positive. Cells from mice immunized with, for instance, *rev* DNA were used as controls for testing T-cell reactivity to Nef or Tat.

DTH. Delayed-type hypersensitivity (DTH) was measured by swelling 24 to 72 h after injection of 10 μg of antigen in the skin of the footpad. The DTH reaction was measured only for the DBA/2 and BALB/c mice. A difference of more than 0.1 mm in footpad swelling between the antigen- and control protein-injected mice was considered a positive DTH reaction.

IL-2 secretion. Interleukin-2 (IL-2) secretion in supernatants of in vitro-stimulated spleen cells was measured after 48 to 60 h. Human IL-2- and murine IL-2- or IL-4-dependent CTLL-2 bioassays were used (4). A 50-μl sample of the supernatants from in vitro-stimulated spleen cells was added in triplicate to 5,000 CTLL-2 cells in 50 μl/well in round-bottomed Nunc 96-well plates. The CTLL-2 cell line was a kind gift from A.-M. Geretti, Erasmus University, Rotterdam, The Netherlands. When secretion of IL-2 was measured, 5 μg of monoclonal anti-mouse IL-4 antibodies or anti-mouse IL-2 (a kind gift from G. Shearer, National Institutes of Health, or from Collaborative Biomedical Products, Bedford, Md.) per ml was added to the bioassay supernatants. The cells were incubated for 18 h at 37°C in 5% CO_2 before [$^3H^+$]thymidine (1 μCi) was added. The plates were kept at 37°C for 4 h before the cells were harvested and [^{3}H]thymidine incorporation was measured as described above. IL-2 production (in units per milliliter) was quantitated by using standard curves generated by culturing CTLL-2 cells with known concentrations of recombinant IL-2 (Sigma).

RESULTS

B-cell responses. Humoral immune responses developed to all genes given as single plasmids. A total of 45 to 100% of the

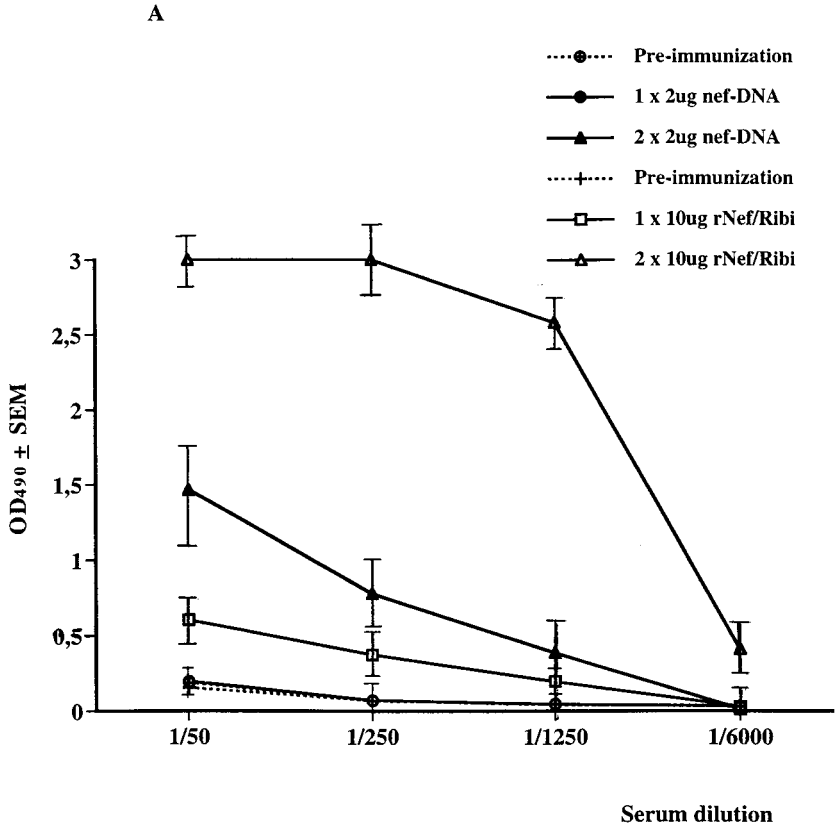

FIG. 1. (A) Comparison of serum IgG ELISA reactivity to recombinant Nef protein in one BALB/c mouse immunized i.d. with the gene gun/Accell after one and two doses of 2 μg of plasmid containing the *nef* gene (HXB3) and reactivity in a BALB/c mouse subcutaneously given 10 μg of recombinant Nef protein twice in Ribi adjuvant by injection. OD_{490}, optical density at 490 nm; SEM, standard error of the mean. (B to D) Percent HIV-1 regulatory Nef, Rev, and Tat peptide serum IgG responders and in vitro B-cell IgG responder spleen cells from three cDNA-immunized immunocompetent mouse strains: BALB/c (10 to 16 mice), DBA/2 (6 mice), and F_1 (16 to 33 mice). The serum IgG (upper graphs) and in vitro IgG synthesis (lower graphs) epitope patterns are shown.

immunized mice responded serologically to Nef, but fewer responded to Rev and Tat (Table 1). They were seen with various frequencies, depending primarily on the strain of mice and the immunizing dose (Table 1 and Fig. 1). The responses characteristic for each strain are presented in Table 1 and Fig. 1 and 2.

(i) BALB/c strain. BALB/c mice developed a high frequency of HIV Nef-specific humoral responses after the second *nef* DNA immunization (Fig. 1A). The frequencies of BALB/c responders to Rev and Tat were lower when measured serologically but increased when measured as specific IgG synthesis in vitro.

(ii) DBA/2 strain. DBA/2 mice also readily developed antibodies toward the gene products of *nef*, *rev*, and *tat* (Fig. 1B to D). One microgram of *nef* DNA was sufficient to induce specific IgG. Anti-Rev responses developed more slowly than those to Nef and Tat. Four of six *rev* DNA-immunized DBA/2 mice had low but detectable IgG titers in serum 2 weeks after the second immunization. After the third immunization, all mice became seropositive. The serum IgG titers of all the antigens ranged between 70 and 1,250, while the IgM values were between 50 and 500.

(iii) (C57BL/6 × DBA/2)F_1 strain. The (C57BL/6 × DBA/2)F_1 mice received DNA as single plasmids or in combination. Seventeen to 96% responded serologically. The least frequent response again was the response to Rev. Mice that received combinations of plasmids responded to the same regulatory HIV-1 antigens as mice that received a single plasmid.

Fourteen of the 43 F_1 mice were given the *nef* DNA as a single plasmid. Of these, seven received the plasmid by i.m. injection and seven received it by the epidermal route by use of a gene gun and DNA-coated gold particles. All gene gun-immunized animals and three of the i.m. DNA-immunized mice responded by developing serum IgG toward Nef.

We also introduced immunizations with genes for the virion structural components gp160 and *gag* protein p24 in the F_1 hybrid mice. Twenty-nine F_1 animals received combinations of both regulatory and structural plasmids which resulted in 96.5% IgG responders to p24 and 75.8% responders to envelope gp160. Both intramuscular and epidermal immunizations induced p24 antigen-specific IgG.

The most frequent response toward the regulatory proteins in animals that received these combinations was elicited toward the Tat protein (79% serum IgG responders). The poorest response was seen toward Rev, to which only a few responses could be detected.

(iv) huPBL-scid mice. Eighteen to 100% of the huPBL-scid mice responded by developing IgM but not IgG antibodies toward the gene products. The best response was seen among the five mice receiving three doses of Rev and gp160 (10 μg of each plasmid).

(v) Protein immunization. Protein immunizations were performed as controls (Table 1 and Fig. 1A). All mice immunized with recombinant protein in adjuvant responded serologically to all recombinant proteins. Recombinant proteins used for

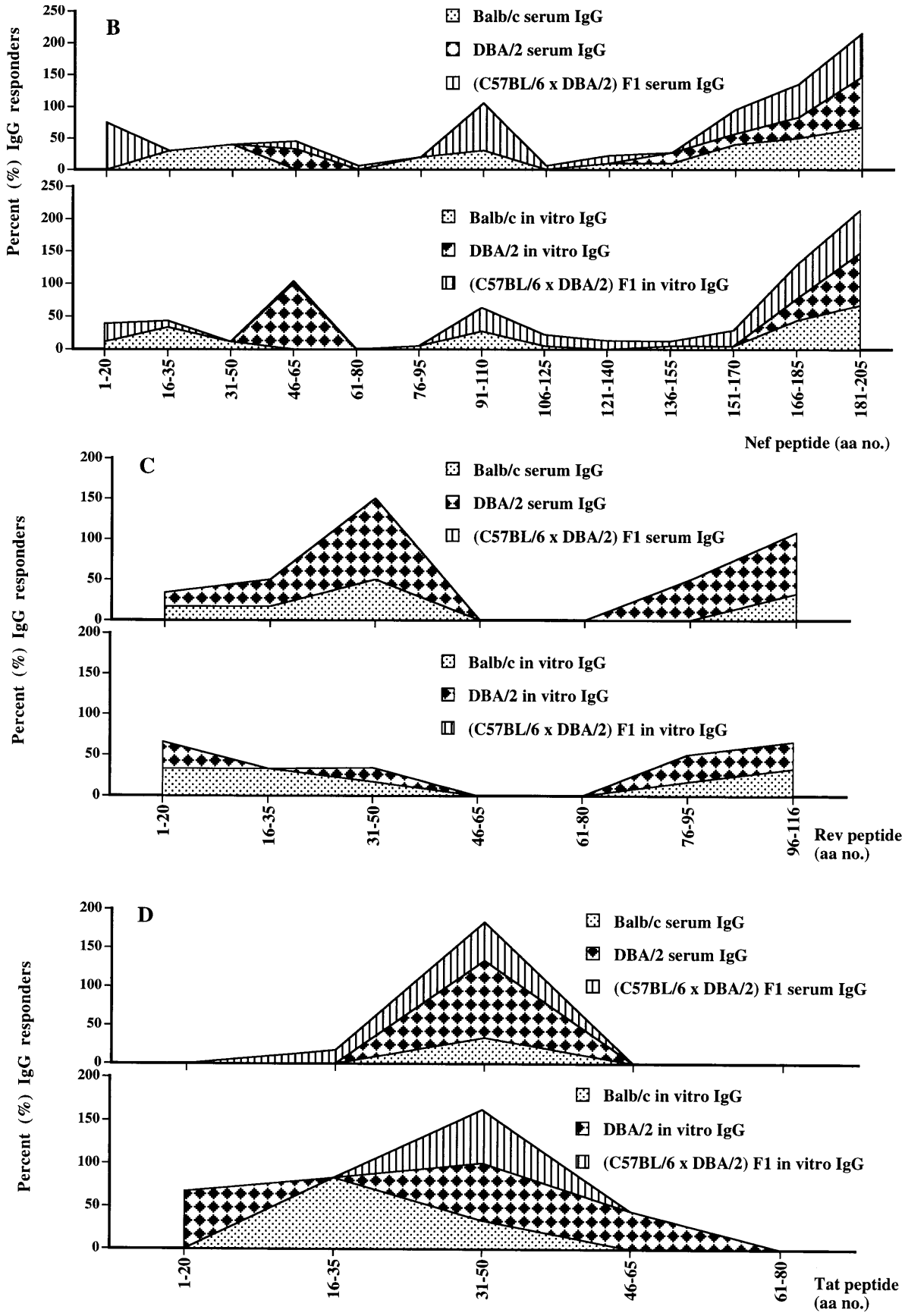

FIG. 1—*Continued.*

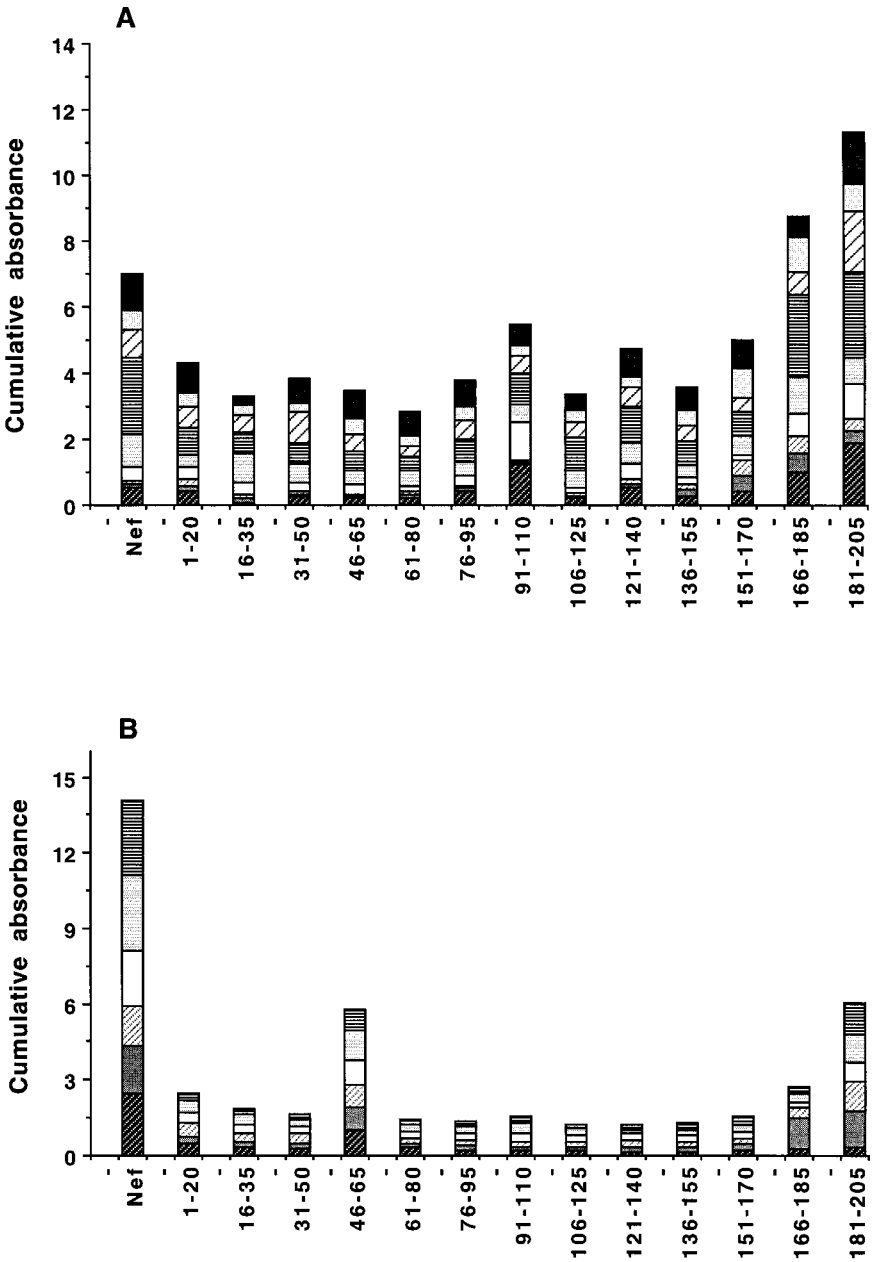

FIG. 2. B-cell epitope mapping with synthetic peptides. Serum IgG epitope mapping by ELISA reactivity to synthetic peptides representing Nef for *nef* DNA-immunized BALB/c (A), DBA/2 (B), and (C57BL/6 × DBA/2)F_1 (C) mice is shown. B-cell epitopes were also mapped by using sera from four mouse strains (three mice per strain) immunized with recombinant Nef protein (two 50-μg doses of Nef in Freund adjuvant given subcutaneously) (D). Reactivity to the whole protein (Nef) and Nef peptides is also shown. CAG, control antigen; Abs, absorbance.

immunization induced high IgG titers to the respective proteins, with IgG titers of >10,000 after three injections.

(vi) In vitro IgG synthesis. Spleen cells were restimulated in vitro with the respective antigens, in order to evaluate whether immunoglobulin memory had developed already after the first immunization. These studies showed a positive reaction in almost all cases (Table 1). Using this assay of antibody synthesis in vitro, all seven F_1 animals in a gene gun-immunized group and six of seven F_1 animals in a group injected i.m. were shown to respond by developing IgG toward the Nef protein and to synthetic Nef peptides. Using in vitro B-cell stimulation, more frequent secretion of antibody even to Rev protein or Rev peptides was seen.

(vii) B-cell epitope mapping. To analyze the epitope reactivities, sera were tested for binding to synthetic 20-mer peptides representing the three regulatory proteins. Comparisons of the epitopes detected by direct serology and by in vitro B-cell antigen activation are shown in Fig. 1B to D. Nef peptide epitopes detected in the three immunocompetent mouse strains tested are shown in Fig. 2A to C. Immunization with recombinant regulatory protein Nef, Rev, or Tat showed linear B-cell epitopes which appeared more restricted than with gene

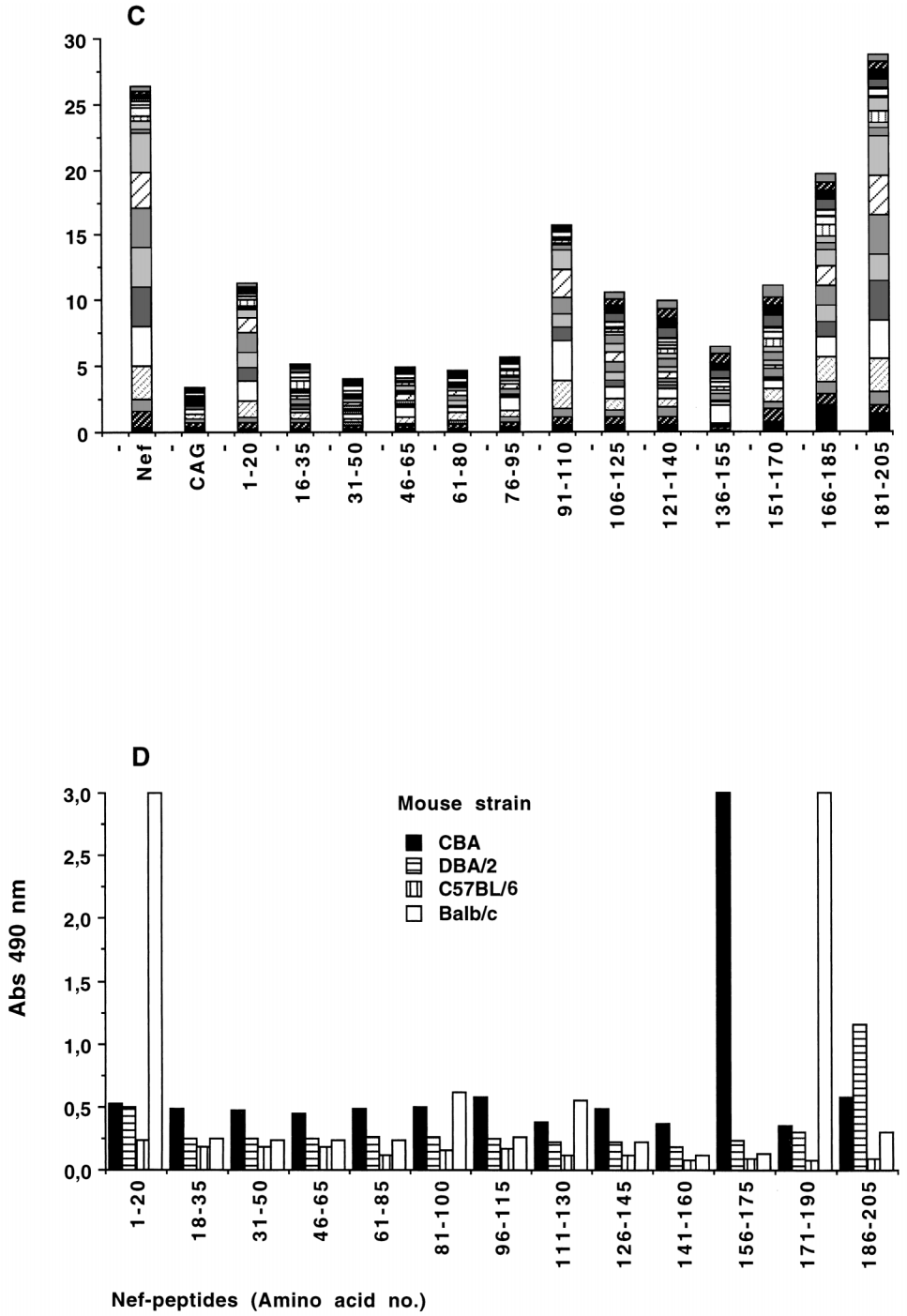

FIG. 2—*Continued.*

vaccination. An example of the pattern of reaction toward the linear Nef peptides is shown in Fig. 2D.

In the DBA/2 mouse strain, the IgG epitope reactivities to Nef, Rev, and Tat could be seen only after the second DNA injection as IgG secretion after in vitro culture of spleen cells (data not shown). After the third immunization, the IgG could be detected directly in serum by ELISA. Following protein immunization with easily measurable titers of antibody to the whole Rev protein, linear Rev B-cell epitopes in DBA/2 mice could not be mapped with our synthetic peptides. Linear Tat peptide epitopes could still be mapped in the N-terminal aa 1 to 20, and linear Nef B-cell epitopes could still be mapped in the Nef C-terminal aa 181 to 205.

The F_1 mice had the broadest epitope reaction pattern. In the Nef peptide epitope mapping, frequent and strong reactivities to aa 1 to 20, 91 to 110, and 151 to 205 were seen (Fig. 1). Mice that received combinations of genes responded to the same regulatory HIV-1 epitopes as mice that received the respective single genes. The epitopes analyzed after in vitro B-cell IgG synthesis revealed a pattern similar to that of serum

TABLE 2. Cellular immune responses to HIV-1 regulatory and structural proteins after DNA-based or protein immunization

Immunization and mouse strain	Frequency (median SI) of T-cell response to:					DTH[a] (frequency)		
	Nef	Rev	Tat	p24	gp160	Nef	Rev	Tat
Plasmid DNA								
BALB/c	11/16 (49)	4/6 (7)	6/6 (6)	ND[b]	ND	4/6	1/6	6/6
DBA/2	5/6 (18)	3/6 (6)	6/6 (22)	ND	ND	4/6	3/6	0/6
(C57BL/6 × DBA/2)F_1	37/43 (14)	2/29 (9)	22/29 (25)	17/29 (15)	18/29 (13)	ND	ND	ND
huPBL-scid	1/11 (5)	2/11 (3)	0/11 (1)	ND	2/5 (3)	ND	ND	ND
Protein								
BALB/c	3/3 (14)	ND	ND	ND	ND	ND	ND	ND
None (control)								
BALB/c	0/4 (2)	0/4 (0)	0/4 (1)	ND	ND	0/4	0/4	0/4
(C57BL/6 × DBA/2)F_1	0/4 (1)	0/4 (1)	0/4 (1)	0/4 (1)	0/4 (1)	ND	ND	ND
huPBL-scid	0/5 (1)	0/5 (1)	0/5 (1)	0/5 (1)	0/5 (1)	ND	ND	ND

[a] A difference of more than 0.10 mm in footpad swelling between antigen- and control protein-injected mice was considered as a positive reaction.
[b] ND, not done.

IgG antibody. Only Tat peptides gave broader reactivities after in vitro activation (Fig. 1D). When induction of epitopes in DNA- or protein-immunized animals was analyzed, we found that DNA induced a broader epitope pattern than proteins (Fig. 2).

The human B-cell epitope mapping performed via the huPBL-scid responses revealed N- and C-terminal epitopes in the Rev protein representing peptides aa 1 to 20 and 76 to 115 (not shown). Tat peptide reactivity was seen for aa 61 to 80. Due to low numbers of cells harvested from the *nef* DNA-immunized scid mice, B-cell epitope mapping by in vitro B-cell antibody synthesis could not be performed with all antigens.

T-cell responses. (i) Cell proliferation in vitro with recombinant proteins. The BALB/c, DBA/2, and F_1 mice showed antigen-specific T-cell proliferation in vitro in response to the Nef, Rev, or Tat recombinant proteins with high frequencies (Table 2). The antigen-specific T-cell responses were more frequent with the 1-μg DNA dose than with 100 μg of DNA. Among BALB/c mice, the strongest T-cell proliferative responses with high SIs were seen 6 weeks after a primary single-gene immunization. The frequency of responses to the recombinant Rev protein was lower than the frequencies of responses to Nef or Tat proteins.

(ii) DTH. The tested mouse strains also developed DTH reactions to these gene products (Table 2). In the BALB/c mice, the median DTH reactions ranged from 0.15 to 0.18 mm for Nef, 0.20 mm for Rev, and 0.31 to 0.36 mm for Tat. In the DBA/2 mice, the median DTH reactions were 0.20 mm (range, 0.15 to 0.25 mm) for Nef, 0.22 mm (range, 0.19 to 0.23 mm) for Rev, and not detectable for Tat. Lymphocytes from the huPBL-scid mice seldom showed induction of specific T-cell reactivity.

(iii) T-cell epitope mapping. All strains had prominent T-cell reactivities, as shown by proliferative responses to proteins as well as to peptides (Tables 2 and 3). Figure 3 shows how the three immunocompetent mouse strains responded to Nef peptides after DNA immunization. The T-cell responses to all regulatory proteins and peptides are shown in Table 3. T-cell proliferation in response to Rev was measurable only when synthetic peptides were used as stimulating antigens.

(iv) BALB/c strain. BALB/c responses to Nef peptides were seen with aa 16 to 50 and 151 to 205 (Fig. 3A). i.m. injection and gene gun-delivered DNA both gave prominent T-cell reactivity to Nef and caused IL-2 secretion (Fig. 4). The gene gun delivery recruited a different and broader epitope response (Fig. 4B).

The kinetics of the T-cell proliferative responses were monitored after one *nef* DNA immunization given epidermally by gene gun or by i.m. injection (Fig. 4). After two immunizations with the respective delivery systems, all animals in the epidermal/gene gun-immunized group (7 of 7) responded and 5 of 7

TABLE 3. T-cell proliferation epitopes of Nef, Tat, and Rev mapped in vitro[a]

Protein and aa	Response (%)[b]		
	BALB/c	DBA/2	(C57BL/6 × DBA/2)F_1
Nef			
1–20	+ (6)	++ (33)	++ (48)
16–35	+ (12)		+ (20)
31–50	+ (12)		+ (11)
46–65	+ (11)	+ (17)	
61–80			++ (27)
76–95			+ (18)
91–110			+ (11)*
106–125			+ (11)
121–140			+ (14)
136–155	+ (6)*		+ (18)*
151–170	+ (12)		+ (14)
166–185	++ (38)*		+++ (59)*
181–205	++ (25)*	+ (17)	+++ (50)*
Rev			
1–20	—*		—*
16–35		+ (17)*	—*
31–50	+ (17)		
61–80			
76–95			+ (6)*
96–116	+++ (50)*		
Tat			
1–20		+ (17)	
16–35	+ (17)	++ (33)	
31–50	++ (33)	++ (33)	++ (38)*
46–65		+++ (50)	++ (38)
61–80		+ (17)	
67–86		++ (33)	

[a] The following numbers of mice were tested with the indicated proteins: BALB/c, 16 for Nef, 6 for Rev, and 6 for Tat; DBA/2, 6 each for Nef, Rev, and Tat; and (C57BL/6 × DBA/2)F_1, 43 for Nef, 29 for Rev, and 29 for Tat.
[b] +, <20% responders; ++, 20 to 50% responders; +++, >50% responders; —, >3 SI; *, IL-2 stimulation = >3 SI (IL-2 secretion range, 0.05 to 0.8 U/ml).

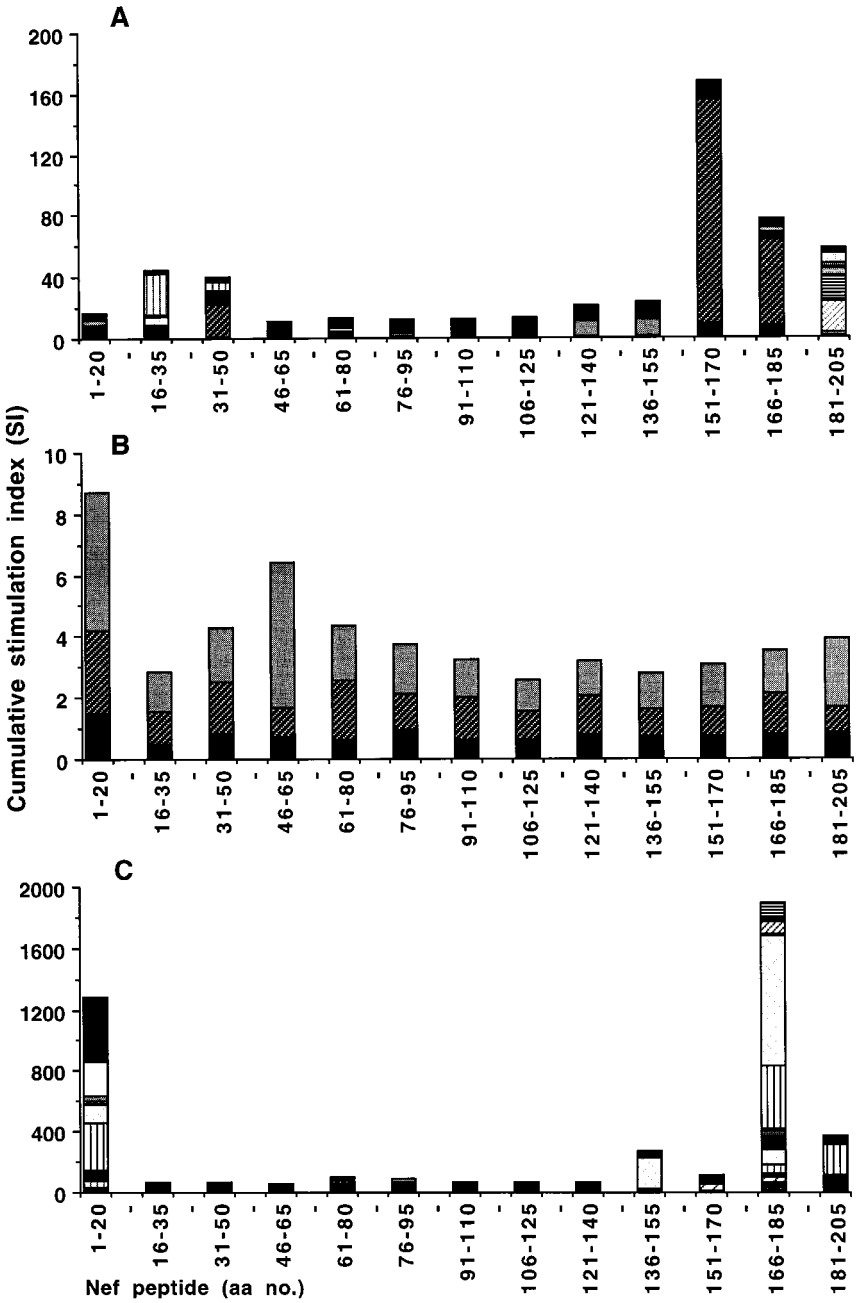

FIG. 3. T-cell proliferation epitope mapping in vitro with synthetic peptides representing the Nef protein. Cumulative Nef peptide SIs are shown for *nef* DNA-immunized BALB/c (A), DBA/2 (B), and F_1 (C) mice.

in the i.m.-immunized group responded. Protein immunization gave a weaker cumulative T-cell response to the Nef protein and its peptides (Fig. 5). Several controls were performed to exclude cross-stimulation. Spleen cells of mice immunized by *rev* DNA were not stimulated in vitro by Nef or Tat antigen. Spleen cells from *nef* or *tat* DNA-immunized mice did not proliferate in vitro when Rev antigen was used as a stimulus (not shown).

(v) IL-2 secretion. Cell supernatants taken after in vitro stimulation with antigen and/or synthetic peptides were used to measure IL-2 production. Both injection- and gold particle-administered DNA resulted in antigen-specific IL-2 secretion. This stimulation occurred with the same synthetic peptides that stimulated cell proliferation (Table 3 and Fig. 4). Twelve weeks after a single immunization, IL-2 secretion could still be detected, even though no or only low T-cell proliferation was measurable. Control mouse spleen cells were not stimulated to produce IL-2 by recombinant antigens or peptides.

DISCUSSION

Our primary aim was to induce and analyze the immune responses toward the HIV-1 regulatory proteins. Previous investigators have concentrated their efforts mainly on HIV-1 envelope DNA-induced reactivities (16, 27, 41). Plasmid DNA carrying any of the three HIV-1 regulatory genes *nef*, *rev*, and

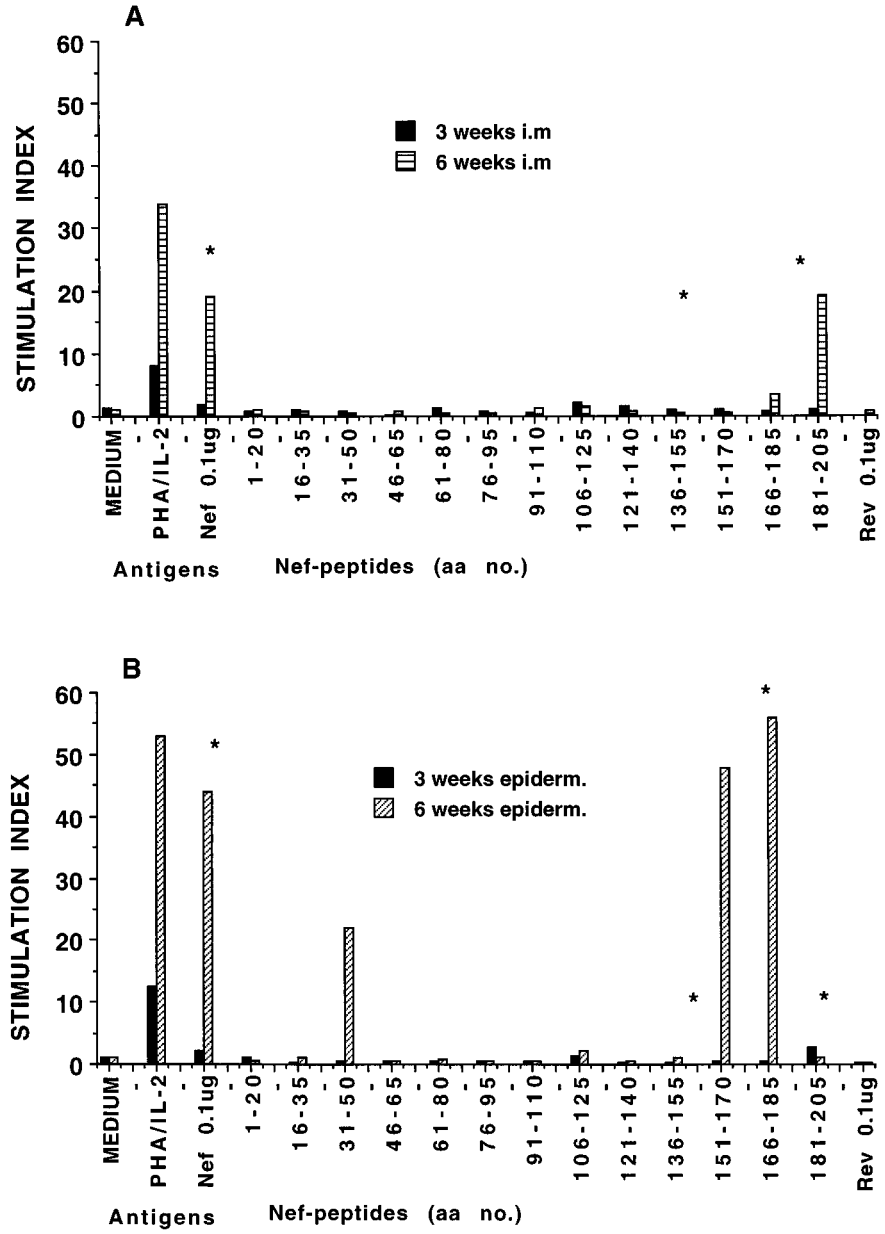

FIG. 4. T-cell proliferation in vitro in response to HIV-1 recombinant Nef protein and synthetic peptides in BALB/c mice given one dose of *nef* DNA by injection i.m. (A) and in BALB/c mice given one dose of *nef* DNA epidermally (B) with the gene gun with *nef* DNA-coated gold particles. *, epitopes for which significant IL-2 synthesis was measured. PHA, phytohemagglutinin.

tat induced murine humoral and cellular immune responses in immunocompetent mice. Strong but variable responses were detected, and it was possible for us to both identify reactivities to the proteins in question and map the B- and T-cell epitopes.

To obtain a detectable humoral IgG response, at least one booster immunization was required. This is similar to what has been shown previously by others (14, 42). Both BALB/c and DBA/2 mice were used to determine the DNA dose required to elicit a humoral immune response. A relatively good response was seen even with as low a dose as 1 to 2 μg of DNA, given twice.

The primary humoral immune response could be identified as an in vitro response to specific antigens. In this system, B cells secrete presynthesized immunoglobulins which can be quantified by ELISA or by enumeration of single antibody-producing cells (6). It was demonstrated that a memory to produce antibodies of the IgM class was induced already after the primary immunization in both immunocompetent mice and scid mice repopulated with human peripheral lymphocytes. B cells synthesizing IgG were easily identified in the immunocompetent mouse strains, even 12 weeks postimmunization.

When memory B-cell responses in mice were studied after epidermal influenza virus DNA immunizations, anti-influenza virus B-cell responses were seen only in cells collected from the spleen, even though several other B-cell homing organs were investigated (19). Only after challenge with appropriate influenza virus antigen were B cells detectable at the local site of challenge, as measured by the ELISPOT assay. Memory B cells

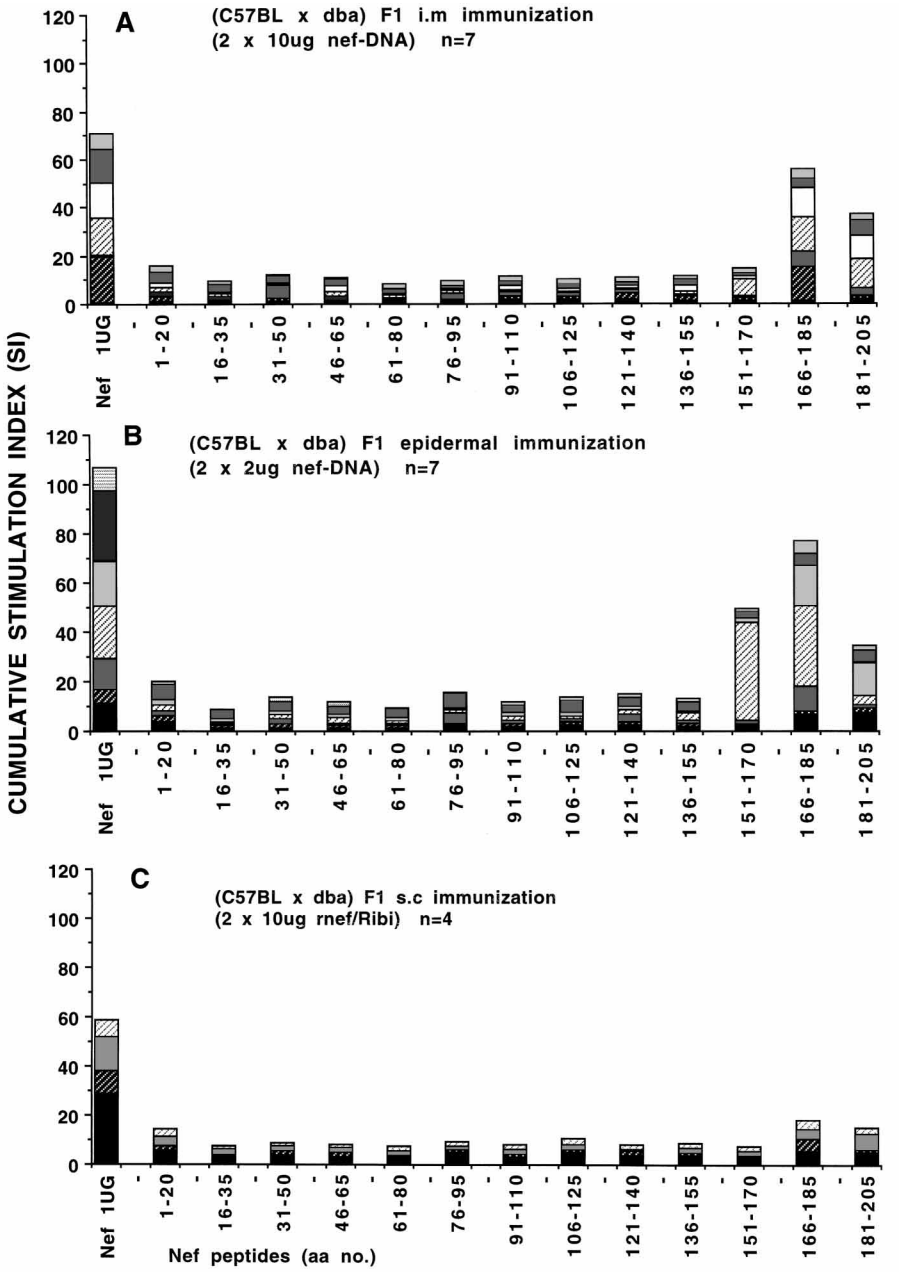

FIG. 5. T-cell epitopes mapped for (C57BL/6 × DBA/2)F_1 mice receiving two immunizations of HIV-1 *nef* plasmids by i.m. injection (A) or epidermally with the gene gun (B) or receiving two immunizations of recombinant Nef protein-Ribi adjuvant injection subcutaneously (C). Reactivity to the whole protein (Nef) and to the Nef peptides is shown. The doses used were 10 (A and C) and 2 (B) μg; $n = 7$ (A and B) or 4 (C).

have been estimated to have a half-life of >7 weeks (35), and the half-life of antigen-specific B cells in transfer experiments has been estimated to be around 2 to 3 weeks (14). In our DNA immunizations, it thus appears likely that memory cells for the humoral arm of the immune system can be induced by a single injection of the relevant gene.

By using synthetic peptides representing specific regions of these proteins, it was possible to map the humoral and T-cell responses. The DNA immunization induced IgG reactive with several characteristic sequences which were the same for the Nef protein in four mouse strains. Also after *rev* and *tat* DNA immunization, antibody sites similar to those found after protein immunization were detectable. This would indicate that the proteins expressed by the DNA plasmids in vivo and the recombinant proteins given as soluble molecules are presented similarly to B cells. When the Nef B-cell epitopes of the immunocompetent mice were compared, the clearest similarities were seen in the C terminus. This region seems to be the main common immunogenic region of this protein (10, 29, 36, 38).

Cellular responses were induced already following the primary DNA immunization with regulatory genes. T-cell proliferation in response to Nef epitopes was found in the N-terminal, middle, and C-terminal regions of the protein. Again, the strongest responses occurred to epitopes located C terminally. Even protein immunization induced responses in this region, although of a smaller magnitude. Similar epitopes have been

reported by Michel et al. (22), who found that immunization by recombinant vaccinia virus containing the *nef* gene induced T-cell responses, mainly with peptides representing Nef aa 179 to 199.

Our *tat* DNA elicited T-cell proliferative responses. These epitopes are similar to the epitopes in HIV-1-infected individuals which are described as T-helper epitopes (2). The induction of IL-2 secretion in the supernatants from the stimulated cells in vitro and the footpad swelling in vivo suggest a Th1-like response.

A comparison of two methods of administering the plasmids favors gold particle delivery of DNA for all three regulatory proteins since the in vitro T-cell immune responses were stronger. The immune response elicited after a single dose of DNA lasted more than 3 months without booster immunization, as shown by T-cell stimulation and IL-2 secretion. By this route of DNA delivery, the protein expression and T-cell presentation probably occur in the Langerhans cells in the skin or in closely located draining lymph nodes. Since spleen or lymph node cells were collected for the T-cell assays, stimulation of circulating T cells must have occurred. The Th1 activation, which was 3- to 10-fold stronger 3 months after gold bead delivery of DNA than after i.m. injection, may be explained by several factors. i.m.-injected plasmids may be more locally restrained than DNA delivered into the skin by gene gun. It has also been shown that plasmids introduced into undamaged muscles are less efficiently taken up by muscle fibers than in regenerating muscles (7, 45). Another reason for the differences may be the less efficient presentation by MHC class I molecules on myocytes than on dermal cells, such as Langerhans cells (1, 18). This may have relevance only for intracellularly located proteins, such as regulatory proteins, and secreted gene products may behave differently.

For HIV genes, both lower efficacy and equal or higher efficiency in inducing a protective immunity with polynucleotide vaccines given i.m. versus epidermally have been described (12, 41–43). The reasons for these variations may be variations in the quality of the plasmids, uptake rate in cells, capacity of the cells to express the gene (overexpression or lack of expression), and presentation on MHC molecules. Further studies of initial events in plasmid uptake and activation in different types of cells are needed.

Due to the polymorphism of the human population, it is likely that most effective vaccines must contain many proteins or glycoproteins or several DNA genes in order to induce an effective barrier to challenge. Our studies have shown that several of the more elusive proteins which are scarce in natural HIV infection still have properties that allow the induction of potent immune responses of both the B-cell and the Th1 cell pathways.

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