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Peptide immunization in humans: a combined $CD8^+/CD4^+$ T cell-targeted vaccine restimulates the memory CD4 T cell response but fails to induce cytotoxic T lymphocytes (CTL)

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SUMMARY

Immunization with short antigenic peptides represents a potential strategy to induce peptide-specific CTL *in vivo*. In this study, a synthetic vaccine consisting of an HIV-derived, HLA-A2.1-binding CTL epitope and a tetanus toxin-derived T helper epitope was evaluated for its capacity to induce peptide-specific CTL in humans. Thirteen volunteers were immunized and boosted twice with 100 μg of the CTL epitope plus 300 μg of the T helper peptide (p30). Peripheral blood mononuclear cells (PBMC) were regularly analysed for cytotoxic and proliferative responses before, between and after the immunizations, and the serum was tested for anti-peptide antibodies. No unequivocal induction of HIV peptide-specific CTL in any of the volunteers was observed. However, a wide pattern of mild and transient side reactions was observed, ranging from local redness at the injection site to generalized exanthema, myalgias, arthralgias and fever. The side-effects were related to the T helper epitope, as they were similar to the side-effects experienced after tetanus immunization, correlated to the magnitude of the p30-specific *in vitro* proliferative response, and occurred only if p30 was co-injected. No antibodies against the HIV-derived peptides nor against p30 were detectable in the serum after repeated immunizations. The data suggest that the CTL peptide, at the concentration used in this study, failed to induce a cytotoxic immune response *in vivo*, although the T helper peptide seems to be capable of restimulating the specific memory T cells.

Keywords HIV peptide immunization CTL vaccine T helper epitope

INTRODUCTION

Cytotoxic T lymphocytes represent one of the most important and effective mechanisms of the host to control viral infections [1,2]. As in other viral infections, a strong $CD8^+$, MHC class I-restricted CTL response is also detectable in HIV-infected persons [3–7]. The induction of such CTL became the main goal of different anti-viral immunization strategies, since only low benefits are expected from a humoral immunity to protect from HIV infection [8–10]. Humoral responses are generally induced by vaccination with killed virus or intact viral proteins, leading to antigen presentation to MHC class II-restricted T cells and the subsequent secretion of cytokines essential for antibody production [11]. In contrast, the induction of MHC class I-restricted CTL relies on intracellular replication of the pathogen and cytoplasmic processing of its proteins in order to allow efficient antigen presentation on MHC class I. Peptide vaccination strategies can bypass the requirement of intracellular replication of a pathogen, since the short antigenic peptides delivered with the vaccine may directly bind to the class I molecules on antigen-presenting cells (APC). Indeed, several animal studies showed that immunization with short, MHC class I-binding peptides was successfully inducing a CTL response *in vivo* which can protect from persistent infection. Furthermore, it was shown that the simultaneous injection of a MHC class II-restricted T helper epitope and a MHC class I-binding CTL epitope strongly enhanced the generation of peptide-specific CTL, compared with injection of the CTL peptide alone [9,14–20]. The beneficial effect of this co-administration is thought to be due to the induction of $CD4^+$ T cell help supporting the generation of $CD8^+$ CTL. However, the adaptation of effective vaccination protocols from animals to the human system bears manifold problems, as there are open questions of adjuvants, antigen concentrations, number and intervals of booster immunizations, as well as how to prove the effectiveness of the immunization.

Therefore, the aim of this study was to adapt a very simple vaccination protocol, which was used to induce CTL in the murine model, for possible use in humans and to examine its immunogenicity and safety [16,17]. In order to guarantee appropriate help

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for the growth of peptide-specific CTL, we used as adjuvant the 'p30' peptide derived from TT (tetanus toxin/tetanus toxoid; [21]). In the Caucasian population, this epitope should be recognized by most individuals, as it represents an ubiquitous T helper epitope in the TT protein [22,23]. Moreover, the human population is widely vaccinated with TT, and therefore co-immunization with the p30 epitope from this common 'recall' antigen should be suitable to stimulate a part of the TT-specific T helper memory immune response in most vaccinated individuals.

The selection of the CTL epitope used in this study was based on earlier investigations on HLA-A2.1-restricted, HIV-derived CTL epitopes [24]. From the entire HIV protein sequence, 73 peptide sequences were selected, all possessing the HLA-A2.1-specific binding motif [24–28]. Twenty of these peptides were synthesized and assayed for binding to HLA-A2.1. Two peptides, p33 (pol, aa 652–600) and p35 (env, aa 199–207), which bind to the HLA-A2.1 molecule and are recognized by CTL from HIV-infected individuals, were finally selected as CTL epitopes in this vaccination trial.

SUBJECTS AND METHODS

Recruitment of volunteers

Healthy donors between 20 and 48 years of age were HLA-typed and tested for the presence of HIV-specific antibodies. In peripheral blood mononuclear cells (PBMC) from 12 HIV^-, HLA-A2.1-positive and one HLA-A2.1-negative individuals, the proliferative and cytotoxic responses against TT, the TT-derived peptide p30 and the HIV-derived p33 and p35 peptides were measured twice before the first immunization. All responded to TT and p30, but none of the volunteers showed a proliferative or cytotoxic response to p33 or p35.

The participants in this trial were informed about the possible risks of the study and signed an informed consent after approval of the immunization protocol by the local ethical committee.

Selection of HIV-derived CTL epitopes and the T helper peptide

The selection of the CTL peptides used in this study is described in detail elsewhere [24]. Briefly, 20 nonameric peptides from different HIV proteins of the HXB2R strain were synthesized and tested for binding to HLA-A2.1 in a cell binding assay with the mutant T2 cell line [25–28]. Four strongly binding peptides were selected and their recognition by PBMC from HIV-infected individuals was analysed. Peptide p35 from the HIV env protein (aa 199–207, TLTSCNTSV; mol. wt 925 D) was recognized in six out of 14 HIV-infected, asymptomatic patients, and was previously described in a longer form to be involved in the natural immune response to HIV [29]. Peptide p33 (pol, 652–660, ALQDSGLEV; mol wt 931 D) was recognized by PBMC from three out of the 14 HIV^+ patients [24].

The T helper epitope p30 from TT, which corresponds to the TT sequence 947–967 (FNNFTVSFWLRVPKVSASHLE; mol. wt 2479 D) was described in earlier reports to be recognized in most Caucasians [21–23]. All of the volunteers responded to this epitope at least at two time points with a stimulation index (SI) of >4 and were thus considered to be p30 responders.

Preparation of different vaccine formulations

All peptides used were synthesized and purified according to good laboratory practice. Amino acid analysis and high performance liquid chromatography (HPLC) confirmed their appropriate sequence. One part of the peptides was lyophilized after purification and solubilized in water just before injection, whilst the second part was adsorbed to aluminium hydroxide. The different preparations of the vaccine contained (i) 100 μg p33 (= 107 nmol) + 300 μg p30 (= 121 nmol), both in water; (ii) 100 μg p33 + 300 μg p30, both separately adsorbed to alum; (iii) 100 μg p35 (= 106 nmol) + 300 μg p30 in water; and (iv) 100 μg p35 + 300 μg p30, both in alum. The final volume of these preparations was 0·5 ml. The preparations contained up to 280 μg/ml trifluoracetic acid (TFA), which is in the range of TFA serum concentrations after halothane narcosis and thus should not induce adverse reactions [30]. Furthermore, all different vaccine preparations were tested for toxicity in two guinea pigs receiving two human doses and in five mice injected with one human dose. The animals did not develop side reactions upon i.p. injection of the vaccine.

Immunization protocol and reporting of side-effects

The 13 volunteers were separated into four groups: six volunteers (group 1) received p33/p30 in water; two volunteers (group 2) p33/p30 in alum; three volunteers (group 3) p35/p30 in water; and two volunteers (group 4) p35/p30 in alum. Immunization started with an injection of the immunogen subcutaneously at the inner side of the upper arm. At days 21 and 56, the volunteers were boosted twice. Blood samples were always taken just before immunizations and PBMC were isolated for *in vitro* analysis. To monitor the effects of the third immunization (on day 56), blood was taken additionally on days 80 and 140. One volunteer (no. 2) belonging to group 3 did not follow this schedule: after a first immunization with p35/p30 in water, he developed side-effects and received at day 21 only p35 alone. Two months after this immunization, he was boosted again with p35/p30 in water. Finally, a fourth injection of p35/p30 was given after an additional 3 weeks.

After each injection, volunteers monitored their side-effects and were asked to measure temperature and present themselves when skin symptoms appeared. In two volunteers with exanthema, pictures were taken and blood was collected 6 h and 24 h after immunization for measurement of cytokines (IL-3, IL-4, IL-5 and tumour necrosis factor-alpha (TNF-α), detection limit 100 pg/ml, except IL-4 50 pg/ml), for C-reactive protein (CRP) and differential blood counts and FACS analysis of PBMC.

In vitro *analysis of PBMC during the study*

Freshly isolated PBMC were used to analyse the proliferative response against TT, p30, p33 and p35. The HLA-A2.1-restricted flu peptide, derived from the influenza virus matrix protein (aa 57–68, CKKALGFVFTLDK), was included in all proliferation and cytotoxicity assays as a control peptide [31,32]. The complete culture medium (CM) consisted of RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 25 μg/ml transferrin (no. 663.710; Biotest, Dreieich, Germany), 100 U/ml penicillin, 10 μg/ml streptomycin and 10% pooled heat-inactivated human AB serum (the same batch was used throughout the study). The medium for culture of the T2 cell line was RPMI 1640 supplemented with 20% fetal calf serum (FCS), 5 mM HEPES buffer and 2 mM L-glutamine.

To measure specific proliferation, 1×10^5 cells/well were

cultured in a 96-well plate in 200 μl CM. To perform dilution curves, TT and p30 were used at concentrations of 20 ng/ml to 6 μg/ml. The flu peptide and the HIV peptides were tested in the range 0·7–250 μg/ml. After 7 days, ^{3}H-thymidine was added for 16 h, the cultures were harvested and ct/min were measured. SI was calculated as (ct/min in cultures with antigen)/(ct/min in cultures without antigen). In unstimulated control cultures, the background varied from 120 to 6700 ct/min.

To enhance the sensitivity of the cytotoxicity assays, we expanded the culture period *in vitro* to 28 days and used two different culture conditions: (i) in the micro-assay, 1×10^6 PBMC were stimulated with soluble peptides at a concentration of 100 μg/ml in 200 μl/well CM in a 96-well round-bottomed plate (no. 3077; Falcon, Becton Beckinson, Rutherford, NY). After 3, 6 and 9 days, half of the medium was changed with fresh CM supplemented with 40 U/ml IL-2 (EuroCetus, Amsterdam, The Netherlands). After 12 days, the cells were used as CTL lines in a cytotoxicity assay [24]; (ii) in the macro-assay, 4×10^6 PBMC per well of a 24-well culture plate (no. 3047; Falcon) were incubated with the flu peptide, p33 or p35 or without antigen. IL-2 was added as in the micro-assay and cytotoxicity was measured on day 12. One part of these cells were restimulated after 12 days and on day 20 with autologous, antigen-pulsed and irradiated PBMC. After an additional 8 days, the cultures were tested again in a cytotoxicity assay.

Cytotoxicity assay

The mutant T2 cell line was used as target cell in all cytotoxicity assays. This cell line exclusively expresses HLA-A2.1 and thus no allospecific killing had to be expected [27]. The target cells were pulsed with the different peptides at a concentration of 200 μg/ml for 3 h and ^{51}Cr (no. NEZ030, NEN; Du-Pont, Regensdorf, Switzerland) was added for the last 90 min. Cells were washed three times with CM before use and resuspended at 5×10^4 cells/ml in CM. Targets and effector cells were incubated at different ratios (1 : 2, 1 : 5, 1 : 15) in a total volume of 200 μl CM for 4 h. Supernatant (100 μl/well) was harvested and released ^{51}Cr was measured in a γ-counter. Average spontaneous release values ranged from 10% to 20% of the totally incorporated ^{51}Cr. Percentage of specific lysis was calculated as 100 $\times$ (experimental–spontaneous release)/(total ^{51}Cr incorporated–spontaneous release).

RESULTS

Induction of peptide-specific CTL

The cytotoxic response to the control flu peptide and either p33 or p35 before, during and after the immunizations is summarized in Table 1. Eleven out of the 12 HLA-A2.1-positive volunteers showed a moderate to substantial flu peptide-specific cytotoxic response at least at two time points during the study. This indicates that the assays used were suitable to detect CTL directed against one certain epitope and at frequencies encountered in a non-persisting infection like influenza. Together with a recent report by Vitiello *et al.* [18] and the fact that the volunteers were boosted twice, this suggests that the assays used here should have been sensitive enough to monitor a successful CTL induction.

Nevertheless, no consistent cytotoxic immune response to p33 or p35 peptide was detectable after the immunizations, even when the cells were repeatedly restimulated *in vitro.* The occasionally observed cytotoxicity to the HIV-derived peptide (i.e. volunteer 10 or 12) is probably related to the variability of the test system. Therefore, no or only low and thus not detectable levels of peptide-specific CTL were induced *in vivo* by the vaccine regimen used in this study.

Alteration of the proliferative response to the vaccine compounds

To monitor possible alterations of the proliferative response against the peptides in the vaccine, PBMC from volunteers were stimulated with different concentrations of TT, TT-derived p30, the flu peptide and the HIV peptides (Table 2). The specific proliferative responses are given in SIs, which seem to us to account better for the variable background proliferation. In volunteer 2, the p30-specific proliferative response was not detected on day -30 and day 0, but became detectable after p30 injection. In contrast, donor 1 showed a weak response against p30 before entering the study and seemingly lost this response upon p30 immunization, as he was negative in all stimulation assays after day 0. The proliferative response against TT remained unaltered in all volunteers, except one, who reached maximal stimulation values at 10 times lower concentrations than before entering the study. The proliferative response against the HLA class I-restricted flu peptide and HIV-derived p33 and p35 was low (SI < 3) at the beginning of the study and did not increase after repeated injections.

Side-effects upon injection of p33/30 and p35/p30 vaccine

To monitor the safety of the vaccine formulations used, all volunteers were carefully observed for occurring side-effects. A summary of the adverse reactions observed is given in Table 3. They ranged from local reactions at the injection site (redness, pain and itching) to systemic effects such as fever, exanthema and flu-like symptoms. Local reactions were observed in seven out of 13 vaccinated persons, whereas systemic side-effects occurred in eight individuals. Only three out of the 13 volunteers did not show any adverse reaction. It is noteworthy, that the severity of the symptoms decreased with increasing number of booster immunizations. In general, the adverse reactions appeared within hours (4–8 h) after peptide injections and were mild and transient, as they lasted for maximally 24 h. In two persons (volunteers 7 and 12) a rapid development of local redness and systemic exanthema was seen within 2 h. Blood samples, taken 6 h and 24 h after injection, did not show significant changes of CRP or of activated T cells, as no CD25$^+$ or HLA-DR-positive T cells were detectable in their PBMC. Cytokines (IL-3, IL-4, IL-5, TNF-α) were detectable before and after immunization, but no post-immunization increases were noted (data not shown). To rule out that antibodies against p30 and subsequent immune complex formation accounted for the observed side-effects, the serum was analysed for the presence of anti-p30 and anti-HIV peptide antibodies. Using a polyvalent anti-human immunoglobulin antibody in a standard ELISA and a control anti-p30-specific MoAb, no p30-specific immunoglobulin was detectable in the serum taken before, during and after the study, indicating that p30 mainly acts as a T cell epitope, but is not able to stimulate B cell responses. Also the shorter, HIV-derived peptides p33/p35 did not elicit detectable antibody production (data not shown).

The observed side reactions are most probably due to the TT-derived p30 T helper epitope, since: (i) the HLA-A2.1-

negative volunteer (no. 13), who probably does not bind p35, also experienced flu-like symptoms; (ii) one volunteer (no. 2) did develop systemic side reactions (fever, flu-like symptoms) after the first and third injection consisting of p35/p30, but not after the second injection with p35 alone; (iii) the severity of the side-effects correlated to the p30-specific, proliferative *in vitro* response (Table 4); and (iv) briefly before starting the study, four volunteers with low TT titre (volunteers 6, 9, 10 and 11) were vaccinated with the whole TT protein to boost the p30-mediated helper effect. Two of them (nos 9 and 11) had similar symptoms to the vaccination with TT as to the p30/p33 vaccine (myalgia, arthralgia, fever), whilst two others (nos 6 and 10) did not develop side reaction to TT nor to the peptide vaccine.

DISCUSSION

In this study we used a mixture of MHC class I and class II binding

Table 1. HIV peptide and flu-specific CTL responses in peripheral blood mononuclear cells (PBMC) from vaccinated volunteers

a.

Volunteer				Specific killing of HIV peptide-pulsed targets (effector to target ratio = 15 : 1) on day					
No.	Sex	Vaccine		−30	0	21	56	80	140
1	M	p30/p35	W	0	7	0	0	0	0
2	M	p30/035	W	0	6	0	0	0	0
3	M	p30/p35	A	0	0	0	0	0	0
4	F	p30/p35	A	0	0	0	0	8	0
5	M	p30/p33	W	0	0	5	8‡	0	0
6	F	p30/p33	W	0	0	0	0	0	0
7	F	p30/p33	W	0	0	0	0	0	0
8	F	p30/p33	W	0	0	0	0	0	9
9	M	p30/p33	W	0	0	0	0	0	7
10	M	p30/p33	W	9	5	0	0	0	21
11	F	p30/p33	A	0	0	0	0	0	0
12	M	p30/p33	A	0	0	11‡	10‡	0	0
13	(A2.1–)	p30/p35	W	0	0	0	0	ND	ND

b.

	Specific killing of flu peptide-pulsed targets (effector to target ratio = 15 : 1) on day					
No.	−30	0	21	56	80	140
1	31‡	21‡	14‡	62‡	22‡	20‡
2	0	0	24†	0	0	35†
3	0	0	35†	30‡	19‡	58†
4	0	0	38*	0	0	27†
5	8	0	13*	25†	11*	0
6	0	0	7‡	0	9†	0
7	ND	15	11‡	5‡	0	0
8	28	5‡	52‡	20‡	13‡	18‡
9	0	22*	0	10†	0	0
10	0	0	0	0	5*	0
11	0	19*	0	13†	14‡	11†
12	0	13*	0	10†	10†	9*
13	0	0	0	0	ND	ND

Cytotoxic response in vaccinated volunteers to the HIV-derived peptides (a) and the flu peptide (b). Percentage of specific killing is shown after subtracting the killing of control (unlabelled) target cells. In cases where micro-assay was negative, values from (*) macro-assay without or †with two restimulations are shown; ‡beside micro-assays (from which data are shown), also in macro-assays specific killing could be measured. Vaccines were either applied in water solution (W) or adsorbed to aluminium hydroxide (A).

Table 2. Proliferative responses to TT and p30 over time

Volunteer no.	Vaccine		Proliferative response to TT on day						Proliferative response to p30 on day					
			−30	0	21	56	80	140	−30	0	21	56	80	140
1	p30/p35	W	120	34	87	147	203	6	7	3	1	1	1	1
2	p30/p35	W	97	217	612	210	843	39	1	1	6	13	95	6
3	p30/p35	A	39	47	4	48	35	18	3	1	5	2	8	1
4	p30/p35	A	3	540	50	149	20	10	3	2	32	33	9	2
5	p30/p33	W	38	52	13	20	50	50	6	6	2	1	4	3
6	p30/p33	W	114	139	9	10	18	18	10	2	1	1	6	2
7	p30/p33	W	62	210	5	4	28	292	12	32	2	4	19	79
8	p30/p33	W	44	88	75	71	157	1	21	5	1	5	7	4
9	p30/p33	W	38	70	60	4	7	88	1	1	11	1	1	16
10	p30/p33	W	23	272	170	12	15	50	1	1	2	1	2	6
11	p30/p33	A	30	293	651	9	9	96	7	19	23	1	1	6
12	p30/p33	A	407	471	259	10	49	100	70	32	92	5	13	38

Individual stimulation indices of all 12 HLA-A2.1-positive volunteers on days −30 to 140 are shown. Although dilution curves of the different antigens were performed, always the values from stimulation with 6 μg/ml are included. Vaccines were either applied in water solution (W) or adsorbed to aluminium hydroxide (A).

peptides to induce $CD8^+$, MHC class I-restricted CTL in human volunteers. The results obtained are dichotomous, as we obtained strong evidence that the T helper peptide stimulated the immune system, but no evidence for the induction of cytotoxic $CD8^+$ T cells.

The failure to induce specific CTL in the volunteers may have different reasons: (i) the concentration of the MHC class I binding peptide was chosen to be equimolar with the T helper peptide, and thus might have been too low. In particular, peptide degradation, i.e. by serum proteases, may be more relevant for the short, HLA-A2.1 CTL peptides, which exactly fit into HLA-A2.1, than for the longer T helper peptide, as the removal of one or two amino acids from the nine amino acid-long CTL peptide will already abrogate its ability to bind to HLA-A2.1 [33]. In contrast, the removal of some amino acids from the longer p30 T helper peptide does not interfere with MHC binding [34]; (ii) we had hoped that the p30 peptide may stimulate the immune system and thus serve as adjuvant. The strength and pattern of the side-effects suggest that TT-specific memory T cells were indeed stimulated. However, it is not clear whether the amounts and composition of cytokines produced are appropriate to support the induction of a $CD8^+$ CTL response; (iii) it may be necessary that the T helper and

Table 3. Side reactions to repeated peptide immunizations

Volunteer no.	Vaccine		Local reaction			Systemic effects (transient <24 h)		
			Pain	Redness	Itching	Fever max. 38·3°C	Exanthema urticaria	Arthralgies/myalgies, flu-like symptoms
1	p30/p35	W						2
2	p30/p35	W	1,2			1		1,2
3	p30/p35	A						1
4	p30/p35	A		1,2				
5	p30/p33	W		3				
6	p30/p33	W						
7	p30/p33	W		1,2	1		1,2,3	
8	p30/p33	W						
9	p30/p33	W	1	1,3		1,3*		
10	p30/p33	W						
11	p30/p33	A		2	2			1
12	p30/p33	A		1,2,3	2			1
13	A2.1 neg.	W						1

Numbers (1–3) indicate reactions after first, second and third immunization. Volunteer 2 received in the first booster injection p35 alone and thus number indicates reactions after third (the second complete) injection. *After first injection, also swelling of the local lymph node was observed.

Table 4. Correlation of p30-specific proliferation to side-effects

Volunteer no.		Last TT vaccination	TT-specific titre	Mean stimulation index to		Side effects	
				p30-peptide	TT	local	systemic
12	A	1989	5·2	48	249	+++	+++
4	A	1982	1·7	27	38	(+)	- - -
7	W	1990	3·9	26	102	+++	+++
2	W	1993	11·2	14	146	++	+++
11	W	1994	42	12	183	++	+++
8	W	1993	11·2	7	86	- - -	- - -
9	A	1994	4·1	6	55	++	+++
6	W	1994	63	5	56	- - -	- - -
5	W	1987	2·2	5	43	- - -	- - -
10	W	1994	5·1	4	103	- - -	- - -
3	A	1992	11·7	3	357	- - -	(+)
1	W	1991	6·6	2	121	- - -	(+)

The proliferative responses against p30 and TT are given as the mean of stimulation indices obtained in six different experiments performed between days −30 and 140. Volunteers were ranked in this table according to the magnitude of the p30-specific response.

CTL peptide are intimately presented by the same APC, because this co-localization of CTL and T helper cell would ensure a high local concentration of the cytokines necessary to stimulate specific $CD8^+$ T cells. To achieve that, it could be advantageous to chemically link the T helper epitope and the CTL peptide [16,18,35]. Such linkage would also contribute to the stability of the two peptides, but may have the drawback that it could lead to the generation of antibodies and the formation of new CTL epitopes. Furthermore, attachment of a lipophilic tail to the conjugate may improve the uptake and appropriate processing. On the other hand, the use of lipophilic compounds may complicate the manufacturing and purification of the vaccine [18,20]. Indeed, a most recent study in mice indicates that both the linkage of the two T cell epitopes and the modification with a lipo-tail (lipopeptides) strongly enhanced the immunogenicity of a peptide-based vaccine. This vaccine formulation was also used in humans, and led to the induction of MHC class I-restricted, peptide-specific CTL [18].

Another reason for the failure to measure specific CTL could be that the *in vitro* assays used were not sensitive enough to detect very low CTL precursor frequencies. As seen with the flu peptide, the precursor frequencies against one common epitope are low and vary widely between individuals and over time in the same individual [31,36]. As Vitiello *et al.* have shown, successful CTL induction can lead to strong cytotoxic responses [18]. Thus, although we can not rule out that a moderate HIV peptide-specific cytotoxicity was generated, we consider our results negative. Nevertheless, a more sensitive screening assay, such as the determination of precursor frequencies by limiting dilutions analysis, might have revealed an increase of HIV peptide-specific CTL upon peptide immunization [37,38].

In mice, low antigen concentrations given subcutaneously may preferentially lead to immunity, while higher concentrations given intravenously could induce tolerance [39]. Since the repeated subcutaneous injection of our vaccine did not change the p30-specific proliferative response, it seems that no p30-specific tolerance was induced.

The observed side-effects were closely related to the peptide immunization. They were observed repeatedly in the same individual and were mild and transient. In particular, they included local and some systemic effects like arthralgias and myalgias, which might have been missed in an animal model. Aluminium-adsorbed peptides did not induce more severe side-effects than alum-free vaccines. However, due to the low number of volunteers receiving the alum vaccine and the failure to detect CTL in any volunteers, it is not possible to comment on the effects of the aluminium hydroxide in the preparation. The strength of side-effects correlated to the p30-specific *in vitro* proliferative response and, interestingly, effects were similar to the side-effects observed after TT immunization, namely fever, arthralgia and myalgia. Since they did not correlate to the immunoglobulin titre specific for TT, and since no p30-specific antibodies were detected in the serum of our 13 volunteers, the clinical symptoms may be due to T cell-derived cytokines rather than to a B cell-mediated mechanism. However, the interaction of p30 with specific memory cells did not result in a boosting of the p30- (or TT)-specific T cell response as revealed by the *in vitro* assays. Thus, no evidence for tolerance induction was found, although the severity of the observed side reactions became weaker with increasing numbers of injections, or even disappeared on the follow-up injection.

It is noteworthy that in the study using the lipopeptides, only local side-effects were reported, whereas in our study and in a recent trial with cat Fel-d1 peptides also systemic side-effects were observed [18,40]. The goal of the Fel-d1 study was the induction of tolerance (or anergy) in cat-allergic individuals. Two 26 amino acid-long peptides derived from Fel-d1, the major cat allergen, were injected subcutaneously in doses ranging from 7·5 to 750 μg/ml [41]. Interestingly, these MHC class II binding peptides induced allergy-related reactions such as conjunctivitis and chest tightness. In contrast, the side-effects in our study with the TT-derived p30 peptide were similar to those after TT immunization. Thus, the side-effects observed after immunization with a peptide derived from a ‘re-call’ antigen might be similar to the reactions after the administration of the original protein. In addition, this study suggests that some side-effects,

attributed to humoral mechanisms, may actually be due to T cell-mediated reactions.

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