Multiple antigen concentrations in delayed-type hypersensitivity (DTH) and response diversity during and after immunization with a peptide-based HIV-1 immunotherapy candidate (Vacc-4x)

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Abstract

Delayed-type hypersensitivity (DTH) testing represents a simple in vivo method for monitoring cellular immune responses. In a phase II clinical trial on HIV-infected patients (n = 38) of the peptide-based HIV-1 immunotherapy candidate Vacc-4x, we monitored DTH responses to three antigen concentrations of Vacc-4x. We have shown that DTH can be used quantitatively to measure immune reactions to Vacc-4x and its individual peptide components. Our data stress the qualities and differences of induration and erythema, both in discriminating individual antigens and in monitoring immunizations over time. The data also indicate that DTH baseline-status might have important impact on immunization kinetics.

Keywords: Delayed-type hypersensitivity; Induration; Erythema; HIV; Immunotherapy

1. Introduction

Effective immunotherapy in human immunodeficiency virus (HIV) type 1 infection aims to attenuate the progression of immunodeficiency [1–3] and might provide therapeutic options even in poor areas. The efficacy of immunotherapies should preferably be monitored in a simple and reliable way. Current knowledge suggests that effective immunotherapies in chronic HIV infection should enhance cellular rather than humoral immune responses [3]. The simplicity of delayed-type hypersensitivity (DTH) testing and its widespread use in basic immunology [4–7] has encouraged the exploratory use of DTH testing in clinical immunotherapy trials for cancer [8–14] and to some extent for HIV [7,15–18]. DTH tests have mainly been used qualitatively based on induration for monitoring cellular immune responses to antigens [7].

The skin infiltrate and erythema from DTH reactions are induced by complex immunological and inflammatory interactions [6,7] but nevertheless represent true in vivo responses to specific antigens. However, the potential clinical predictive values of different DTH antigens and their relation to T-cell responses must be carefully tested in each case, since immunogenicity is not necessarily linked to clinical efficacy. Moreover, DTH might become a simple, clinical tool to examine the longevity of immune responses which can be used to decide whether re-boosting with an immunotherapy is required. The use of DTH in chronic HIV infection shares both these challenges, in that correlates to effective immunity to HIV are still unknown [3] and since HIV-specific immune responses usually diminish over time [19,20].

We have used DTH tests in combination with in vitro immune response assays during a phase II clin-
ical immunotherapy trial with the HIV peptide-based immunotherapy candidate Vacc-4x [21,22]. In order to explore the reliability of the DTH test, three different antigen concentrations of Vacc-4x were used in eight consecutive DTH tests over a period of 52 weeks. Among the 38 chronically HIV-infected patients who completed the study, 31 were later tested for DTH responsiveness approximately 1 year after the end of the study.

We have recently described the potential impact of skin induration at one DTH concentration in relation to the immunogenicity and efficacy of Vacc-4x [21–23]. Here, we present and discuss new data reflecting time dependent relations between the different DTH antigen concentrations and the relevance, not only of skin infiltrate size, but in addition concurrent development of erythema. We also present DTH responses obtained after separate injections of each of the four Vacc-4x peptides which show different DTH response patterns. Potential complications of DTH-testing such as induction of tolerance or allergy to the antigens are also discussed.

2. Materials and methods

2.1. Study protocol, patients and immunizations

The Vacc-4x study protocol has been described in detail elsewhere [21,22]. In brief, Vacc-4x consists of four synthetic peptides (Vac-10, -11, -12 and -13), each corresponding to conserved domains on the HIV-1 p24 capsid protein representing the native Gag regions with residues 23–293, 288–308 and 359–378, respectively [15]. The peptides were produced according to good manufacturing practice (GMP) with a purity >97% [15]. They were stored lyophilised and reconstituted in sterile water immediately prior to injection. Stability testing of the peptides have shown that they are stable for more than 18 months. Forty HIV-infected non-AIDS patients with asymptomatic chronic HIV infection who had been on stable and effective combination antiretroviral therapy (CART) for more than 6 months were enrolled, 38 completed the study, having CD4 T-cell counts >200 (445–723)/μl and HIV RNA <50 copies/ml. A total of 23 (61%) patients were HLA-A2 positive. The patients were randomised into two dosage groups of Vacc-4x: low dose (LD, 0.4 mg in 0.1 ml; n = 18) and high dose (HD, 1.2 mg in 0.1 ml; n = 20). Granulocyte-macrophage colony stimulating factor (GM-CSF, 30 μg) was used as a local intradermal adjuvant 15 min prior to Vacc-4x injection at the same site. Ten immunizations were given over an initial period of 26 weeks together with CART (single immunizations at weeks 1, 2, 3, 4, 6, 12, 13, 21, 22 and 26). An observation period of 26 weeks without immunizations then followed and which included two periods with CART treatment interruptions lasting 4 weeks (weeks 26–30) and 14 weeks (weeks 38–52), respectively [22]. About 1 year after the end of the study, i.e. at week 109 (108–114) (median, interquartile range), 31 of the 38 patients were again challenged with the three DTH tests in order to investigate longevity of the immune responses generated.

2.2. DTH skin tests

DTH tests (0.1 ml) were always performed at three concentrations (triple DTH) of the Vacc-4x peptide mix (0.2, 1.0, and 4.0 mg/ml; low-DTH, medium-DTH, and high-DTH) on eight occasions (weeks 1, 3, 6, 12, 21, 26, 38, and 52). In addition, on weeks 18 and 24 DTH tests were performed using each of the four peptides Vac-10 to Vac-13 at 1.0 mg/ml (single peptide DTH), respectively. However, due to a technical problem too few vials were available of Vac-10, therefore only 25 and 20 patients were tested using this peptide, at weeks 18 and 24, respectively. All DTH tests were injected on the left and the right forearms without adjuvant. Induration and erythema areas were recorded by the same study nurse after 48 h, and calculated as circular areas based on the average of vertical and horizontal diameters.

Both DTH induration and erythema was considered positive when the area was >10 mm². This limit value was determined from placebo-testing all patients at baseline using water alone [21].

2.3. Statistical analysis

Qualitative response distributions were calculated using the Fisher exact test. Otherwise non-parametrical tests were used for all analyses, including Mann–Whitney U-test, Wilcoxon matched pairs test, and Spearman R for comparison between patient groups, for paired comparisons, and correlations, respectively. All tests were two-tailed, and p-values were considered statistically significant when <0.05. The analyses were performed using Statistica software (Statsoft Inc., Tulsa, USA).

3. Results

3.1. Triple DTH at baseline

At baseline (week 1) positive DTH induration areas >10 mm² were observed in nine patients (24%). However, only one patient was baseline-positive for all three DTH antigen concentrations. Three patients responded to two concentrations whereas five responded to only one (either high-DTH or medium-DTH). In contrast, positive DTH erythemas were not observed at baseline in any patients (p ≤ 0.05, Fisher exact test).

3.2. Differences in DTH responses between the Vacc-4x dosage study groups

The low-DTH, medium-DTH, and high-DTH induration and erythema areas throughout the study are presented in
We have previously described that significantly larger DTH induration was induced in Vacc-4x HD compared with LD patients using the highest DTH concentration at weeks 3, 6, 26, 38, and 52[21,22]. Because different Vacc-4x dosages might induce diverse cellular immune responses both in a quantitative and qualitative manner, the HD and LD study-arm patients were compared in induration and erythema at all three concentrations and all time points. DTH indurations in at least one of the three DTH antigen concentrations were higher for HD patients ($p<0.05$, Mann–Whitney U-test) at all time points from weeks 3 to 109 except in week 12, whereas this was only sporadically true for DTH erythema (Fig. 1, asterisks). Similarly, qualitative comparisons of positive versus negative DTH between the HD and LD dosage arms showed that the number of induration-positive patients again differentiated the HD from the LD group ($p<0.05$, Fischer exact test), whereas this was rarely the case for erythema.

### 3.3. Developments of induration and erythema over time

Induration and erythema areas were positively correlated with a non-parametrical test in all three DTH antigen concentrations and at all time points after baseline (Spearman $R = 0.34–0.78$, $p < 0.04$). Nevertheless they developed differently: after the initial immunization period beyond week 6 the DTH induration areas stabilized throughout the study with no significant differences between the various time points (Fig. 1). In contrast, the erythema components increased further between weeks 26 and 52 in all three DTH antigen concentrations (Fig. 1, $p < 0.05$), i.e. in the period with two consecutive treatment interruptions of CART.

The erythema areas were generally larger than the induration areas after baseline, particularly for high-DTH and medium-DTH ($p < 0.01$) but less frequently for low-DTH. However, it should be noted that the DTH antigen dose-relations to erythema and induration developed differently: from week 3 and beyond, the proportions between the three different DTH erythemas were almost similar to those between the DTH antigen dosages (low-DTH:medium-DTH:high-DTH = 1:5:20) (Table 1) except a disproportional increase from weeks 38 and beyond. Conversely, for induration areas the corresponding dose–response relationship was lower, 1:1.7:2.7 (average) and stable even after week 26 (Table 1).

### 3.4. Comparison of DTH-baseline positive and negative patients

The Vacc-4x peptides are modified compared to the native p24-sequences [15]. The fact that nine patients recognised Vacc–4x peptides by a positive DTH even before immunizations started, suggested that these patients had pre-existing memory T-cells which could potentially induce different response patterns to Vacc-4x compared with the baseline DTH-negative ones. When the DTH baseline-positive and baseline-negative patients were compared within the HD and LD Vacc-4x dosage arms, differences in the development of DTH responses were observed, despite the small number of baseline-positive patients in each subgroup: within the LD group, baseline-positive patients ($n = 4$) experienced a successive increase in DTH induration areas that became significantly larger than in the DTH baseline-negative patients ($n = 14$), particularly from week 21 and beyond, whereas this was not the case for erythemas (Fig. 2, left panels). In contrast, within the HD group the baseline-positive patients ($n = 5$) developed larger initial DTH induration areas than the baseline-negatives only at week 3, while the baseline-negative patients ($n = 15$) rather tended to develop larger erythemas at the end of study (Fig. 2, right panels).

### 3.5. DTH after challenge with Vacc-4x single peptides

To evaluate the contributions of each of the four Vacc-4x peptides (Vac-10 to Vac-13) and to evaluate the effects of the injections at weeks 21–22, Vacc-4x single peptide DTH were performed at weeks 18 and 24 (Fig. 3). These tests were made using 1.0 mg/ml of each peptide, a dose corresponding to Vacc-4x high-DTH. No differences were observed in either DTH induration or erythema for any of the peptides as determined with paired tests comparing week 18 with week
Table 1

Median DTH area and corresponding ratios

(a) DTH areas (mm²)

<table>
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<td></td>
<td>4.0²</td>
<td>1.0²</td>
<td>0.2²</td>
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<td>3</td>
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<td>50</td>
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(b) Ratioes (compared to the low-DTH's)

<table>
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<td>1.0</td>
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*a Concentration (mg/ml).
*b Concentration ratio.

24. However, the DTH responses divided the peptides into three distinct groups (Fig. 3): (i) Vac-13 were at these two time points less immunogenic than the other peptides by inducing smaller induration and erythema areas (*p* < 0.01; indicated by large circle around origo in Fig. 3 representing a majority of anergic patients with overlapping values). Moreover, fewer patients responded to Vac-13 (*p* < 0.05, Fisher exact test); (ii) Vac-11 and Vac-12 had similar median induration areas (28 mm²) which also correlated with the erythema component at both time points (0.56 < *R* < 0.72, all *p* < 0.001), in contrast to both Vac-13 and Vac-10. Moreover, the Vac-11 and Vac-12 erythema areas were moderate and comparable to corresponding induration areas (36 mm² and 44 mm², respectively); and (iii) Vac-10 induced considerably larger erythema than the other three peptides (*p* < 0.05), but similar induration areas to Vac-11 and Vac-12. Thus, Vac-10 induced higher numerical differences between corresponding erythema and induration areas (median, 352 mm²) compared to Vac-11 and Vac-12 (both median 0 mm², *p* < 0.001).

3.6. Follow-up measurements 1 year after the study

About 1 year after the end of the study, i.e. at week 109 (108–114)(median, interquartile range), 31 of the 38 patients were again challenged with Vacc-4x high-DTH, medium-DTH, and low-DTH. Positive DTH were still present in 87% (*n* = 27) of those tested, which was almost identical to what we observed after the immunization period [21]. Neither the induration nor the erythema areas were different compared to week 52 with the exception of high-DTH erythema (*p* = 0.04) (Fig. 1). The patients in the HD arm still had larger induration and erythema areas for the high- and medium-DTH (*p* < 0.05) (Fig. 1). Moreover, no difference (Mann–Whitney U-test, *p* > 0.1) were found between the 12 patients who had resumed CART and those who were still off therapy (*n* = 19).

4. Discussion

The great potential of DTH for monitoring immune responses to antigens lies both in its simplicity for large-scale applications and that DTH reflects de facto in vivo responses. However, the relevance to factors such as clinical efficacy or to dosage guidance must be cautiously documented. The challenges concerning DTH is first to ensure intradermal delivery, because deeper deposition of antigen could easily cause greater antigen “loss”, particularly with highly diffusible peptides (as in Vacc-4x), and presence of fewer Langerhans cells. We tried to minimized such errors by having the same study nurse to perform all immunizations, DTH-injections and read-outs throughout the study. We estimated that less than 3% errors were made for the more than
1000 DTHs injected, based on the assumption that single episodes of anergic responses alone antigen concentration in a series of positive DTHs might have been false negative; this could either be caused by inadequate subdermal application or by faulty dilutions of Vacc-4x by the hospital pharmacy (data not shown). Another potential problem with repetitive DTH is the immunization effect of the DTH per se [7], and even the possibility of inducing tolerance [24] or allergy by injecting peptides without adjuvant. To our understanding induction of tolerance was not evident in this study since immune responses increased or stabilized in the majority of the patients. Allergy was not evident either and necrosis was not observed in any of the patients. However, we did observe three cases (once in each of weeks 26, 38 and 52) where the high-DTH injection caused transient, but generalized erythemas and vaso-vagal reactions lasting less than 1 h [22].

DTH in clinical practice is usually interpreted qualitatively [13–16], but determination of cut-off levels may be difficult, particularly in immunocompromised patients [7]. We defined cut-off at 10 mm$^2$ ($\Omega \approx 3.5$ mm), according to the maximal infiltrates seen with placebo DTH (water) [21]. This cut-off is comparable to that suggested for other antigens in HIV [16,17]. However, we here discuss several quantitative aspects of the DTH parameters. This appears relevant because we recently found significant relations between the magnitude of Vacc-4x DTH induration areas and efficacy in terms of improved viral control [22]. Moreover, Vacc-4x DTHs were related to Vacc-4x-specific T-cell proliferation in vitro [21], in keeping with three ACTG-studies reported by Katzenstein et al. [16]. It should also be pointed out that Vacc-4x induced even HIV p24 protein responses, suggesting a cross-reactivity between the Vacc-4x peptides and HIV p24 protein responses [21]. This possibility was further supported by a parallel decrease in Vacc-4x peptide and p24 responsiveness in blood during onset of HIV viraemia, in contrast to non-HIV antigens [23].

The significance of DTH erythema is less clear than the significance of induration. It should be noted that although erythema was correlated with induration in non-parametric analysis throughout the study, the impact of different dose–response kinetics is intentionally reduced in rank correlation statistics. In fact, parametric correlations between erythema and induration were only scarcely found, and the few significant correlation coefficients were considerably lower than the non-parametric counterpart Spearman’s $R$. The magnitude of DTH erythema and induration responses...
behaved differently in three aspects: first, only induration correlated with the differences in immunogenicity between the two Vacc-4x dosage study arms. This is consistent with the general notion recognizing DTH induration as the best correlate for T-cell responses [6,7]. Second, the DTH antigen doses gave almost a linear increase in erythema areas (Table 1) whereas induration increased relatively less in response to the higher DTH antigen concentrations. Third, DTH induration areas stabilized early in the study, whereas erythema increased in the last 26 weeks, i.e. in periods off CART and with detectable HIV RNA levels. A discordance between erythema and induration might have clinical implications that should encourage monitoring of both response types in future trials.

Positive Vacc-4x DTH induration at baseline identify those patients with chronic HIV who had circulating memory T-cells specific to epitopes on these HIV-like peptides. Such patients might theoretically benefit from both a different injection schedule and/or antigen dosage than patients with no baseline responses. Although the number of baseline-positive patients in this study (n=9) were too few to statistically document every aspect of baseline DTH, our data suggest that baseline-positive patients might benefit from Vacc-4x LD or fewer HD injections. However, the numerous baseline-negative patients, on the other hand, benefited more from HD than LD. To our knowledge, these data may be the first to indicate that antigen-based immunotherapies should potentially be individualized according to baseline cellular immunity, which can most conveniently be addressed by DTH. However, the low number of baseline-positive patients in this study necessitate that the data should be reproduced in larger studies.

We used two original approaches to study DTH responses in detail. First, DTH was performed with simultaneous application of multiple DTHs. The 20-fold difference in DTH antigen concentration gave almost similar qualitative test response frequencies such as in week 26 with response rates between 71 and 89% (differences not significant, Fischer exact test). However, the lower concentration (low-DTH) gave induration areas that approached placebo level with little erythema. Qualitative DTH measurements using this DTH concentration alone would therefore characterize some of the DTH-positive patients as DTH-negative. High-DTH on the other hand triggered to some extend disproportional erythema, and particularly so in the second half of the study and in baseline-negative patients. It should be noted that only one out of nine patients were baseline-positive to all the three DTH antigen concentrations whereas five patients responded to only one concentration. Thus, acknowledgement of specific T-cell memory would have been lost in some patients by using only one DTH antigen concentration. These observations suggest a complex relationship between re-activation of T-cell memory and DTH antigen concentrations. As a second approach, we undertook DTH testing of
the single Vacc-4x peptide antigens. We found that these peptides induced different induration and erythema profiles, at least after 4–5 months. In fact, most of the DTH erythema, at least between weeks 18 and 24, could be attributed to one of the four Vacc-4x peptides (Vac-10). The reasons for these discrepancies cannot be determined from our present data, but will require further studies that compare factors such as single-peptide estimates of baseline activities, response kinetics with cytokine profiles, and HLA makeup. The frequency of peptide-specific memory T-cells to each of the peptides may be just as variable as observed for Vacc-4x. Thus, even single peptide antigen DTH should perhaps be tested at baseline. Our data indicate that patients recognise the Vacc-4x antigens one and a half years after their last immunization, even though most of them (19 of the 31 tested) still were off CART. Significant differences were again seen between the two injection-groups (LD and HD): Our data thereby indicate that the DTH-test, even over a substantial period of time, can discriminate between adequate and insufficient immunization, and for example point out when boosting injections might be indicated.

5. Conclusions

Performing DTH-tests has several advantages in monitoring of vaccines or immunotherapies; one needs no blood or serum, no expensive equipment or specialised personnel. We have shown that DTH can be used quantitatively to measure immune reactions to an immunotherapy candidate and its individual components. The data stress the qualities and differences of induration and erythema, both in discriminating individual antigens and in immune response monitoring over time. Our data also indicate that baseline DTH may have impact on immunization kinetics. This work should encourage more immunotherapy trials to include DTH as an in vivo response parameter, and that baseline and follow-up DTH should be closely monitored in each case.

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References

