Delayed-Type Hypersensitivity Skin Testing using Third Variable Loop Peptides Identifies T Lymphocyte Epitopes in Human Immunodeficiency Virus–Infected Persons

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Delayed-type hypersensitivity (DTH) skin testing has been used for nearly 100 years as a diagnostic tool for numerous infectious diseases, and more recently, as an important criterion in the evaluation of suspected immunodeficiencies [1]. Microbial recall antigen responsiveness by DTH testing has been shown to be an independent predictor of human immunodeficiency virus type 1 (HIV-1) disease progression [2–4]. The prognostic value of DTH responses to specific HIV proteins is currently under investigation. Preliminary reports suggest that acquisition of DTH reactivity to an HIV-1 whole inactivated vaccine may confer a more favorable prognosis [5]. In order to define and exploit potentially protective cellular immune responses in a diverse human population, it is necessary to identify the individual epitopes that contribute to the whole protein response.

We have previously identified T lymphocyte epitopes in the HIV-1 gp120 protein from HIV-1–infected volunteers participating in a phase II gp160 vaccine therapy trial using HIV-1 envelope–specific CD4 T lymphocyte lines [6]. In a subsequent study [7], fine epitope mapping of the third variable (V3) loop sequence was performed using CD4 cell lines and a series of truncated V3 loop peptides in 4 persons. However, these in vitro techniques for CD4 epitope mapping are labor-intensive and have uncertain in vivo sensitivity and specificity. Using 2 of these HIV-infected volunteers, selected because of their nonoverlapping V3 loop CD4 cell epitopes and being available at a proximal research site, the CD4 cells of volunteer Henry M. Jackson Foundation for the Advancement of Military Medicine (cooperative agreement no. DAMD17-93-V-3004).

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Materials and Methods

Subjects. Two HIV-1–infected volunteers, 1 man and 1 woman, recent recipients of recombinant (r) gp160 (NL4-3 sequence; MicroGeneSys, Meriden, CT) as participants in a vaccine therapy trial, were enrolled in an approved clinical protocol for this study. Both volunteers had CD4 cell counts of 500–700/mm³, were not anergic to routine microbial antigens, and did not have any acute illness at the time of the study. They were selected based on having nonoverlapping V3 loop CD4 cell epitopes and being available at a proximal research site. The CD4 cells of volunteer 1 recognized an epitope on the carboxy half of the V3 loop (AFVTIGKIG); volunteer 2 recognized an epitope on the amino half of the V3 loop (RKSIRIQGRG).

Peptides. The sequences of synthetic peptides used in the skin test assay represent the amino half (LAI-A: TRPNNNTKRKSIRIQGRG), the carboxy half (LAI-B: GPGRAFVTIGKIGNM-RQAH), the middle portion (LAI-P18: RIQRGPGRFVTIGK)
[8], and the complete linear sequence (LAI-D: TRPNNTRKSI-1QRGPGRAFVTTGKIGNMRQAH) of the V3 loop. The underlined sections indicate the presence of the volunteers’ CD4 cell complete epitope sequence in the peptides. These V3 loop peptides (HIV-1 LAI sequence, matching the rgp160 vaccine) were manufactured under good laboratory practice standards using standard FMOC technology and then purified at a facility under good manufacturing practices using reverse-phase-high-pressure liquid chromatography. Analytical methods necessary to support the identity and purity of the peptides were used as part of the Investigational New Drug Application 6306. The V3 loop peptides were reconstituted with sterile 0.9% saline prior to intradermal injection.

**Skin test application and analysis.** Volunteer 1 was injected intradermally with 0.1 mL of a 5-µg/mL concentration of each V3 loop peptide, a 1:10 dilution of tetanus toxoid, a 1:10 dilution of candida antigen, and 0.9% saline. Measurements performed at 48 h were shown prior to skin biopsy. Volunteer 2 received similar treatment, except that 0.1 mL of a 50-µg/mL concentration of V3 loop peptides was required to elicit a response. Punch biopsies (3 mm) were taken from three sites (as described above), placed in a formaldehyde fixative, and processed according to standard techniques. Paraffin-embedded sections were stained with hematoxylin-eosin or OPD4.

**CD4 cell line proliferation assay.** A gp120-specific CD4 T lymphocyte line from volunteer 2 was developed as previously described [6]. A total of 3 × 10^8 T cells was incubated in a 96-well flat bottom plate with 1 × 10^5 irradiated autologous peripheral blood mononuclear cells (PBMC) and each peptide at 5 µg/mL, rgp120, or medium alone. After 2 days of incubation, the cells were pulsed with 1.67 µCi/well[^3H]thymidine for 18 h, harvested, and counted.

**PBMC assay.** A total of 1 × 10^6 PBMC was incubated in a 96-well round bottom plate with each peptide at 50, 5, or 0.5 µg/mL (figure 1B, only a 5-µg/mL concentration was used), rgp160, or medium alone. The assay was performed using triplicate wells for each peptide or protein. After 7 days of incubation, the cells were pulsed with 1.67 µCi/well[^3H]thymidine for 18 h, harvested, and counted.

**Results**

Two HIV-infected persons, who had distinct, nonoverlapping CD4 cell epitopes in the V3 region, were selected for this proof of concept study. Four peptides were selected for testing: LAI-A, the amino half of the V3 sequence, containing the in vitro epitope for volunteer 2; LAI-B, the carboxy half of the V3 sequence, containing the in vitro epitope for volunteer 1; LAI-P18 [8], the crown of the V3 sequence, containing neither complete epitope; and LAI-D, the complete linear V3 sequence.

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**Figure 1.** Proliferative responses induced by third variable (V3) loop peptides before and at time of delayed-type hypersensitivity skin test protocol. A, gp120-specific CD4 T lymphocyte line from volunteer 2 (6 months prior to skin testing) proliferated in response to peptides LAI-A and LAI-D and recombinant (r) gp120 protein, as predicted by past in vitro epitope mapping [7]. B, Peripheral blood mononuclear cells (PBMC) from volunteer 1 (3 months prior to skin testing) proliferated in response to peptides LAI-B and LAI-D and rgp160 protein, as predicted by past in vitro epitope mapping [7], thus confirming biologic activity of peptides. C, PBMC from volunteer 2 (day of skin testing) did not adequately define T lymphocyte epitope despite proliferation to entire rgp160 protein and recall antigens. D, PBMC from volunteer 1 (day of skin testing) did not adequately define T lymphocyte epitope despite proliferation to entire rgp160 protein. Data are expressed as lymphocyte stimulation index (LSI) (specific proliferation/background proliferation). Horizontal bars represent LSI ≥ 3 (standard antigen specificity in such assays). C and D, Nos. in parentheses represent antigen concentration in µg/mL.
containing the in vitro epitopes of both volunteers. The study incorporated internal positive and negative peptide controls by injecting both volunteers with each of the four peptides; each volunteer was predicted to respond to two of the V3 loop peptides. Two standard microbial recall antigens, tetanus toxoid and candida, and 0.9% saline were used as additional positive and negative controls, respectively.

The V3 loop peptides and controls were injected intradermally in a fashion identical to clinical tuberculin and anergy testing. The results (figures 2, 3) were reported 48 h after injection, the typical interval for maximal human DTH reactions. The clinical course of the peptide-induced induration was comparable to that seen with the standard recall antigens. The photographs (figure 2A, B) of the skin test readings demonstrate the simplicity of this technique and document that the V3 loop peptide responses are qualitatively and quantitatively similar to the standard tetanus toxoid response.

Both volunteers responded to the DTH skin test as predicted by previous in vitro techniques (figures 1A, B, 3); volunteer 1 responded to V3 loop peptides LAI-B and LAI-D and volunteer 2 responded to LAI-A and LAI-D. Neither volunteer responded to saline and LAI-P18, which lacked the predicted epitopes. Both subjects had V3 loop peptide responses of the same magnitude and character as their own tetanus toxoid responses, although volunteer 1 had more vigorous responses in general (figure 3). The threshold dose for response differed between the 2 subjects: Volunteer 1 responded to 0.5-μg doses of the peptides, while volunteer 2 required 5-μg doses for measurable reactivity. Each of the positive peptide responses was of a magnitude (10–20 mm of induration) that was easily measured and distinct from the negative responses. Volunteer 2 also had moderate cutaneous immediate hypersensitivity responses to both the experimental and control antigens that resolved in <1 h.

The histology at the sites of peptide-induced induration was characterized by superficial and deep perivascular and periadnexal inflammation (figure 2C). The lymphocytic infiltrate was found to be primarily CD3+ (data not shown), with a preponderance of CD4 cells (figure 2D). This pattern of lymphocytic infiltration, with the absence of a granulocytic component, is consistent with previous descriptions of DTH reactions [9].

HIV-1 envelope-specific CD4 T lymphocyte lines from both volunteers proliferated in response to the appropriate V3 loop peptides when evaluated ~6 months prior to skin testing; results from volunteer 2 are shown as an example (figure 1A). Because the CD4 T lymphocyte lines were generated in vitro by repetitive antigen stimulation and interleukin-2 expansion, it can only be concluded that there were precursors present in the hosts capable of responding to those particular epitopes. It should not be assumed that these responding cells are expanded in vivo, since there have been reports of HIV-1-specific CD4 T lymphocyte lines generated from uninfected low-risk subjects [10].

When PBMC proliferation was assayed as a surrogate for in vivo expanded CD4 cell populations, there was a loss of sensitivity. Volunteer 2 never demonstrated a PBMC proliferative response to any of the V3 loop peptides. PBMC from volunteer 2 immediately prior to skin test application failed to proliferate in response to the V3 peptides despite a strong proliferative response to the rgp160 protein (figure 1C). Volunteer 1 had variable PBMC proliferative results. PBMC from volunteer 1 obtained ~3 months prior to skin test proliferation in response to the V3 loop peptides as predicted (figure 1B), but PBMC obtained immediately prior to skin test application failed to provide fully informative results, despite peptide dose titration and an adequate response to the rgp160 protein (figure 1D). These data suggest that PBMC proliferative responses may not always be the most sensitive surrogate for determining expanded in vivo CD4 cell populations.

Discussion

Using small peptides in a DTH assay, we demonstrated that cellular epitopes could be accurately identified in humans. Despite studies in mice [11, 12] and guinea pigs [13, 14] that have demonstrated the feasibility of using small peptides to generate DTH-like reactions, there has been a paucity of work in humans. Although one previous study [15] reported a human responding in a DTH-like skin test assay to two allergenic rye grass peptides that matched the in vitro PBMC proliferative response, the substantial clinical utility of this technique has not been realized. We demonstrate in this study that peptide-based DTH skin testing, typical in clinical course as well as histology, can be applied to HIV-infected persons in a systematic way to clearly demarcate epitopes that are in close proximity. Having demonstrated the clinical potential of peptide-based epitope mapping, we plan further studies in a larger cohort of HIV-infected persons, using peptides containing most of the described epitopes of the HIV-1 gp120 protein, to seek critical information on the cellular immunoregulation of HIV-1.

There are potential limitations to this technique. Both volunteers tested in this study had preserved standard recall DTH reactivity and were therefore not anergic. This technique would be unlikely to provide information in immunodeficient persons who were anergic to common recall antigens. In addition, should a large number of peptides be required to adequately cover a protein sequence, it may be necessary to initially pool groups of peptides and subsequently separate the reactive pools in a second application, making this technique more time-consuming.

Despite these potential limitations, the DTH peptide skin testing technique offers numerous advantages over currently available in vitro techniques to define the regions of cellular immunoreactivity: It is potentially more specific than CD4 T lymphocyte lines and therefore more informative of the in vivo state, since it measures expanded lymphocyte populations and not in vitro expanded precursors; it is potentially more sensitive, as suggested by our comparison to contemporaneous PBMC proliferation, since the entire lymphocyte population of the host is capable of responding and not just a small venous
aliquot; it is a simple, well-established, clinical technique that requires no laboratory training and could therefore be utilized in remote regions of the world where laboratory facilities are unavailable; it could provide rapid results in large cohorts, since results are available in 48 h instead of a week (PBMC proliferation) to months (CD4 T lymphocyte line development) of laboratory effort; and it should be relatively inexpensive once it is commercially available.

Figure 2. Delayed-type hypersensitivity reactions to V3 loop peptides and controls 48 h following intradermal injection. A, Measured induration of tetanus toxoid (Tet), LAI-B (B), and LAIA (A) on right forearm of volunteer 1. B, Measured induration of saline control (S), LAI-P18 (P18), LAI-D (D), and candida (Cand) on left forearm of volunteer 1. Ink delineates approximate circumference of induration. C, Punch biopsy of LAI-B site in volunteer 1 demonstrated superficial and deep peri-vascular and periadnexal inflammation (arrows) 48 h following injection (hematoxylin-eosin, original magnification \( \times 50 \)). D, Immunostain of LAI-B punch biopsy site in volunteer 1 demonstrated CD4 T lymphocytes (arrows) as predominant component of inflammatory infiltrate (OPD4, original magnification \( \times 25 \)). Volunteer 2 had similar, albeit less pronounced, infiltrates at LAI-A biopsy site (not shown). Biopsies also taken of 0.9% saline site as negative control and routine recall antigen sites as positive control (not shown).
Figure 3. Average diameter of induration for both volunteers demonstrated concordance between in vitro epitope mapping and this in vivo test.

This technique has the potential to define expanded immunoreactive epitopes of all HIV-1 proteins, allowing rapid correlation of specific cellular immune responses to clinical outcomes, thereby leading to improved vaccine strategies. Furthermore, this technique could provide rapid in vivo data on epitope cross-reactivity and permissivity among persons infected with different subtypes of HIV-1. Other biologic data, such as correlations between CD4 helper activity, CD4 and CD8 cytotoxicity, and cytokine production profiles, may be evaluated ex vivo, at the epitope level, by examining cellular infiltrates of the skin test site. Peptide-induced DTH epitope mapping has potential usefulness in a broad range of clinical research specialties, including infectious diseases, rheumatology, allergy and immunology, oncology, and transplantation immunology, as a tool for diagnostic and therapeutic decision-making.

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