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Recombinant HIV-1 Glycoprotein 120 Induces Distinct Types of Delayed Hypersensitivity in Persons With or Without Pre-Existing Immunologic Memory

Florian Hladik,*† Sean Bender,‡ Robert E. Akridge,‡ YuXiang Hu,‡ Christine Galloway,‡ Don Francis,§ and M. Juliana McElrath2*†‡

Induction of T cell help is critical in HIV-1 control and potentially in prevention by immunization. A practical approach is needed to identify HIV-1-specific helper activities in vivo. We explored the feasibility of measuring delayed-type hypersensitivity (DTH) following intradermal injection of recombinant soluble HIV-1<sub>MN</sub> glycoprotein 120 in HIV-1-infected, vaccinated, and exposed individuals. DTH reactions were elicited within 48 h in 16 of 29 untreated, infected patients and in 24 of 30 uninfected vaccinees. Concomitant envelope-specific lymphoproliferation in vitro was undetectable among 9 infected patients tested with positive envelope-specific DTH. By contrast, no 48-h DTH reactions occurred among 25 high risk and 32 low risk, uninfected volunteers. However, 7–12 days after injection, 10 (40%) high risk and 11 (34%) low risk individuals developed induration resembling DTH, and the cellular infiltrates contained monocytes and T cells. Five of 18 examined also developed anti-gp120 Abs. The very delayed time course and lack of correlation with previous Ag exposure clearly distinguish this reaction from DTH. Thus, HIV-1 skin testing can identify persons with HIV-specific recall responses resulting from infection, in the absence of in vitro lymphoproliferation, and from vaccination. In contrast, very late reactivities may signify chemotactic properties of the envelope protein and/or herald the induction of primary HIV-specific Th1-type immunity. The Journal of Immunology, 2001, 166: 3580–3588.

One of the clinical hallmarks of pre-existing Ag-specific T cell immunity is a delayed-type hypersensitivity (DTH) response following intradermal injection with the test Ag. The DTH response manifests clinically as an area of induration with erythema between 24 and 72 h after Ag injection and appears microscopically as a local cellular infiltrate dominated by macrophages and T lymphocytes. The reaction is commonly associated with a Th1 cytokine profile. Identification of DTH responses is used commonly to predict immunocompetence and immunity to infectious agents following previous infections or immunization (1–3).

To date, skin testing for detection of HIV-1-specific DTH responses has not been developed, but has potential utility for several reasons. Measurements of DTH responses to HIV-1 proteins or peptides may correlate with successful induction of an HIV-1-specific T cell memory response in vaccine recipients (4). Likewise, an HIV-1 DTH skin test administered in HIV-infected individuals may predict restoration of HIV-specific CD4<sup>+</sup> Th cell responses when testing antiretroviral or immune-based therapeutic regimens. Furthermore, it is conceivable that a skin test can facilitate identification of persons previously exposed to HIV-1 who fail to seroconvert or manifest overt infection.

In this study we explored the utility of recombinant soluble gp120 based upon the HIV-1<sub>MN</sub> strain (rsgp120/MN) as a skin test reagent. We first established its feasibility in stimulating a classical DTH reaction in HIV-1-infected patients and in recipients of a recombinant HIV-1 gp120 immunogen. To estimate the relative sensitivity of skin testing as a measurement of HIV-1-specific cellular immunity, we compared DTH responses with in vitro proliferative responses of PBLs to HIV-1 envelope proteins. To determine whether the skin test can identify seronegative individuals who have previously been exposed to HIV-1, we administered the Ag to two additional study groups, those at either low or high risk for acquiring HIV-1 infection. Our findings indicate that soluble rsgp120 can elicit a well-defined DTH response in persons with previous HIV-1 antigenic exposure through either infection or vaccination. However, significantly delayed reactions, ≥1 wk after injection, may occur independently of pre-existing immunologic memory. Hence, such atypical reactions represent either unique chemotactic properties of the envelope protein in vivo or, more likely, a strategy to elicit primary Th1-type immunity.

Materials and Methods

Study subjects

The University of Washington human subjects review board approved all aspects of the study, and participants provided informed consent before injection of the test Ag. An initial pilot study was conducted to establish dosage in 18 HIV-1-uninfected persons (12 reporting low risk and 6 reporting high risk HIV-1 activities) and 14 HIV-1-infected persons with CD4 counts ≥500 cells/ml. Assessment of HIV-1 risk was based upon previously reported criteria (5). Subsequently, 29 HIV-1-infected untreated volunteers (19 with CD4<sup>+</sup> T cell counts ≥500 cells/ml, 5 with CD4<sup>+</sup> T cell counts of 200–500 cells/ml, and 5 with CD4<sup>+</sup> T cell counts ≤200 cells/ml)
and 39 HIV-1-uninfected healthy individuals (20 reporting low risk and 19 reporting high risk HIV-1 activities) were tested for DTH responses. All HIV-1-seropositive study participants acquired HIV-1 infection in the U.S., where transmission of clade B subtypes predominates.

In addition, 30 healthy HIV-1-uninfected volunteers were skin tested who were participants of AIDS Vaccine Evaluation Unit Protocols 015, 016, and 16A, vaccine studies using recombinant envelope immunogens. These individuals were recruited, enrolled, and followed at the University of Washington AIDS Vaccine Evaluation Unit. Of note, none of these volunteers acquired HIV-1 infection during the course of the study. Three volunteers in protocol 015 received four doses of HIV-1 clade B recombinant gp120 (50 µg) with one of six adjuvants (MF59, MTP-PE with MF59, liposome-adsorbed monophosphoryl lipid A, SAF/2, and SAF/2 plus MPL; Chiron, Emeryville, CA) at 0, 2, 6, and 18 µg (6) and were skin tested a median of 10 mo after the last immunization. Seven volunteers in protocols 016 and 016A received three doses of HIV-1MN, recombinant gp120 (300 µg) formulated with 50 or 100 µg of Q212 with or without alum (VaxGen, South Francisco, CA) at 0, 1, and 2 or 0, 1, and 6 mo and were skin tested 6–12 mo after the last immunization.

SEROLOGIC TESTING FOR HIV-1 INFECTION WAS PERFORMED BY HIV-1 ELISA and Western blot. Serum Abs recognizing recombinant HIV-1 clade B gp120, the V3 region, and inhibition of gp120 binding to CD4 were measured by enzyme immunoassays as previously described (7). All HIV-1–infected and pilot study participants were also evaluated for HSV-type-specific serology by Western blot assay (8). Anticoagulated blood was obtained from the volunteers just before and 1 wk (excluding vaccine study participants) and 1 mo following skin testing.

Study treatment and measurement of DTH responses

The recombinant soluble HIV-1MN gp120, provided by VaxGen, was derived from a genetically modified Chinese hamster ovary (CHO) cell line. The polypeptide is a fusion protein consisting of the first 27 N-terminal amino acids from HSV type 1 glycoprotein D (93% homology to HSV-2 glycoprotein D) fused by a synthetic amino acid linker to amino acid residues 12–483 from the mature native gp120 of the HIV-1MN isolate. The rsgp120 was formulated without adjuvant or preservatives in a buffered sodium succinate vehicle at a concentration of 1500 µg/mL. The stock solution was diluted with 0.9% NaCl to deliver a specified amount of Ag in a 10-µL volume. In pilot studies, DTH responses were induced with 10, 20, 40, and 80 µg of rsgp120. In the subsequent main study, we administered 10 µg of rsgp120 in the vaccine group, 40 and/or 80 µg of rsgp120 in the HIV-1–positive group, and both 10 and 40 µg of rsgp120 in the low and high risk groups. The negative control was 0.9% NaCl, and the positive controls were intermediate strength Candida Ag (Candid; Allermed Laboratories, San Diego, CA) and 0.08 limit of flocculation units of tetanus Ag (Tetanus Toxoid USP; Connaught Laboratories, Toronto, Canada), each administered in 100 µL.

Ags were injected intradermally on the posterior thorax. Responses were evaluated on days 2, 5, 7, and 10 after injection. The mean diameter of induration was calculated as (greatest diameter + perpendicular diameter)/2. Based on established criteria for skin test reactivity to recall Ags in HIV-1–infected and uninfected persons, a positive response was defined as a mean diameter of induration of ≥5 mm in HIV-1–infected patients and ≥10 mm in HIV-1–uninfected persons. Four-millimeter punch biopsies of positive reactions were performed on selected consenting subjects. Biopsies were either placed in transport medium (RPMI containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B Bio-Whittaker, Walkersville, MD) and used for isolation of live cells or placed in fixative (3% paraformaldehyde), paraffin-embedded, sectioned on glass slides, and used for immunohistochemistry and hematoxylin/eosin staining.

Immunohistochemistry

Tissue sections were deparaffinized with xylene, quenched of endogenous peroxidase with methanol containing 3% H2O2 for 10 min, rehydrated in a graded series of ethanol, and washed in distilled water. To detect the expression of cell surface markers, sections were reacted with the following mAbs: anti-CD8 mAb: anti-Th cell, OPD4 (Zymed, San Francisco, CA) applied undiluted; and anti-Th cell, TAL.1B5 (Dako) at 1/100 dilution; and biotin-F(ab')2 goat anti-mouse IgG myeloid/histiocyte Ag, MAC 387 (Dako) at 1/100 dilution; anti-HLA-DR, anti-CD4 FITC, and anti-CD8 PE (Becton Dickinson, San Jose, CA). Populations of skin lymphocytes: anti-CD3 FITC, anti-CD16/anti-CD56 (Becton Dickinson, San Jose, CA). The positive controls were intermediate strength Candida Ag (Candid; Allermed Laboratories, San Diego, CA) and 0.08 limit of flocculation units of tetanus Ag (Tetanus Toxoid USP; Connaught Laboratories, Toronto, Canada), each administered in 100 µL.

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Enumeration of infiltrating leukocyte subpopulations

Three infiltrates in the papillary dermis close to the dermal/epidermal junction were chosen to assess T cell and monocyte infiltrus. Among 200 total cells/infiltrate examined, the number of cells staining with a particular phenotypic marker was recorded, and the mean per 100 total cells was calculated. To enumerate epidermal Langerhans cells, HLA-DR+ cells with the typical morphology were counted within three 100-µm epidermal fields, and the mean number of Langerhans cells per field was calculated.

Mononuclear cell isolation and expansion from blood and skin biopsies

PBMC were isolated from anticoagulated blood by Ficoll-Hypaque gradient centrifugation and washed three times by centrifugation. Skin biopsies were transported in HEPES-buffered RPMI on ice to the laboratory and processed within 3 h of collection. The skin biopsies were gently washed to remove any contaminating blood, pushed with a pestle through a 140-µm pore size screen to obtain a single-cell suspension, and then washed by centrifugation. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation or filtered through a 70-µm cell strainer. The cells were washed twice, and counted by trypan blue (Sigma, St. Louis, MO) exclusion under light microscopy. Biopsies yielded 0.5–3 × 104 viable mononuclear cells. Skin cells were plated in 100 µL of culture medium (RPMI with 10% human AB serum (Biocell, Rancho Dominguez, CA), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL 2-ME (Eastman Kodak, Rochester, NY)) into one well of a 96-well round-bottom microtiter plate. Irradiated 5 × 105 allogeneic PBMC were resuspended in culture medium containing either 2 µg/mL PHA (Sigma) or 10 µg/mL recombinant HIV-1MN gp120 (CHO-derived; VaxGen) and added in a volume of 100 µL to the microwell. After 4 days the medium was exchanged, and a final concentration of 100 µL of human rIL-2 (Chiron, Emeryville, CA) was added. The cultures were provided with fresh medium and IL-2 twice weekly. Cells demonstrating growth after 2 wk were re-stimulated with PHA or rgp120 and fresh irradiated allogeneic feeder cells and expanded into 24-well tissue culture plates (Costar, Cambridge, MA).

Flow cytometric analysis

The following mouse anti-human mAbs were used to characterize subpopulations of skin lymphocytes: anti-CD3 FITC, anti-CD16/anti-CD56 PE, anti-CD4 FITC, and anti-CD8 PE (Becton Dickinson, San Jose, CA). In brief, 105 cells were incubated with the mAb for 30 min at 4°C, washed by centrifugation, and resuspended at a concentration of 1 × 106 cells/mL in culture medium containing either 2 µg/mL PHA (Sigma) or 10 µg/mL recombinant HIV-1MN gp120 (CHO-derived; VaxGen) and added in a volume of 100 µL to the microwell. After 4 days the medium was exchanged, and a final concentration of 100 µL of human rIL-2 (Chiron, Emeryville, CA) was added. The cultures were provided with fresh medium and IL-2 twice weekly. Cells demonstrating growth after 2 wk were re-stimulated with PHA or rgp120 and fresh irradiated allogeneic feeder cells and expanded into 24-well tissue culture plates (Costar, Cambridge, MA).

Lymphoproliferative assays

Mononuclear cells isolated from blood or expanded from skin biopsies were resuspended in culture medium and distributed to 107 cells/well in 96-well round-bottom plates. PBMC were stimulated with 5′-paranucleoside of dC (BACTH) in medium alone). On day 6, wells were pulsed with 2.5 µCi of [3H]thymidine (NEN Products, Boston, MA). After 18 h, the cell suspensions were harvested, and radioactive thymidine uptake was measured with a microplate scintillation counter (Topcount; Packard, Meriden, CT). Lymphoproliferation was expressed as the stimulation index (SI; mean [3H]thymidine incorporation with PBS vs cells stimulated with fixed in 1% paraformaldehyde cells with medium alone). A SI >3 was considered positive, based on our previous findings among vaccine trial participants. In the HIV-1–infected volunteers, SIs from two separate venipunctures were averaged.
Assays of CTL

Cell lines from skin biopsies were tested for cytotoxic activity as previously described (9). In brief, gamma-irradiated (3000 rad) autologous PBMC pulsed with 10 μg/ml recombinant HIV-1 gp120 and infected with recombinant vaccinia virus encoding HIV-1 Env (vP1174; provided by the National Institutes of Health AIDS Reagent Program) were used as HIV-specific stimulator cells. Skin mononuclear cells (5 × 10^5) plated in triplicate into 96-well round-bottom plates, were incubated with 5 × 10^5 fresh irradiated autologous stimulator cells and 100 U/ml rIL-2 for 7 days. On day 6, EBV-transformed B lymphoblastoid cell lines were infected with recombinant vaccinia virus, either vP1174 or vSC-8 containing the control lacZ gene. Targets were labeled with 100 μCi of [3H]Tdr (NEN Products) for 16 h. On day 7, chromium release assays were performed, and the percent specific lysis was calculated as previously described (9).

Statistical analysis

Association between rsgp120-specific and recall Ag-specific DTH responses was tested using the χ² statistic. For lymphoproliferative assays, SIs between groups were compared using the Mann-Whitney U test.

Results

Recombinant gp120 induces a classic memory DTH response in HIV-1-infected individuals

An initial pilot study was performed to establish the optimal dose of HIV-1MN rsgp120 for eliciting a DTH response in HIV-1-infected patients. Seven doses (1, 5, 10, 20, 40, 80, and 120 μg) were tested in groups of two volunteers per dose. Among 14 patients with CD4⁺ T cell counts ≥500 cells/μl, four exhibited ≥5-mm induration, one per group of the 20-, 40-, 80-, and 120-μg dose groups. Thus, the 40 and 80 μg doses were selected for further testing in the HIV-1-infected patients.

The majority of HIV-1-infected, untreated individuals with CD4⁺ T cell counts ≥500 cells/ml demonstrated positive (≥5-mm induration) skin reactions within 48 h of intradermal injection of 40–80 μg of HIV-1MN rsgp120 (Table I). Reactions included both erythema and induration at the injection site and were consistent clinically with a typical DTH response. The average induration was 8.5 (40 μg/site) and 12 mm (80 μg/site) among the responders. These patients mounted HIV-1 envelope DTH responses as commonly as responses to other recall Ags. As shown in Table I, 14 of the 19 (73%) HIV-1-infected individuals had positive DTH reactions to tetanus Ag, and 9 of 19 (47%) responded to the Candida Ag. In addition, there was a trend for persons with envelope-specific DTH to also have a DTH response to both recall Ags (p = 0.06). Thus, the majority of infected, untreated patients with normal CD4⁺ T cell counts were not anergic and demonstrated T cell recognition of HIV-1 envelope in addition to recall Ags.

Skin reactions were also noted within 48 h of intradermal injection of 80 μg of rsgp120/MN in two of five HIV-1-infected individuals with CD4⁺ T cell counts of 200–500 cells/μl (average induration, 8.5 mm) and two of five HIV-1-infected individuals with CD4⁺ T cell counts <200 cells/μl (average induration, 10.5 mm; Table I). Thus, some HIV-1-infected individuals still recognize HIV-1 envelope and mount a visible DTH response despite a decline in circulating CD4⁺ T cells.

Table I. Skin test reactions following intradermal injection of HIV-1 rsgp120/MN or recall Ags

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Test Ag</th>
<th>Responders of Total Tested (%)</th>
<th>Day of Maximum Induration (range)</th>
<th>Average Induration in mm (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 infected</td>
<td>rsgp120 40 μg</td>
<td>8/14 (57)</td>
<td>2</td>
<td>8.5 (5–15.5)</td>
</tr>
<tr>
<td>CD4⁺ T cells ≥500</td>
<td>rsgp120 80 μg</td>
<td>12/19 (63)</td>
<td>2</td>
<td>12 (5–16.5)</td>
</tr>
<tr>
<td></td>
<td>Tetanus</td>
<td>14/19 (73)</td>
<td>2</td>
<td>20 (10–39)</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>9/19 (47)</td>
<td>2</td>
<td>7 (10–39)</td>
</tr>
<tr>
<td>CD4⁺ T cells 200–500</td>
<td>rsgp120 80 μg</td>
<td>2/5 (40)</td>
<td>2</td>
<td>8.5 (7–8)</td>
</tr>
<tr>
<td>CD4⁺ T cells ≤200</td>
<td>rsgp120 80 μg</td>
<td>2/5 (40)</td>
<td>2</td>
<td>10.5 (10–11)</td>
</tr>
<tr>
<td>HIV-1 uninfected</td>
<td>rsgp120SF-2 vaccine recipients</td>
<td>rsgp120 10 μg</td>
<td>18/23 (78)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>22/23 (96)</td>
<td>2</td>
<td>14 (7–35)</td>
</tr>
<tr>
<td></td>
<td>rsgp120MN vaccine recipients</td>
<td>rsgp120 10 μg</td>
<td>6/7 (86)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>7/7 (100)</td>
<td>2</td>
<td>16.5 (10–22)</td>
</tr>
<tr>
<td>HIV-1 uninfected</td>
<td>Low HIV-1 risk</td>
<td>rsgp120 10 μg</td>
<td>9/20 (45)</td>
<td>12 (7–12)</td>
</tr>
<tr>
<td></td>
<td>rsgp120 40 μg</td>
<td>10/20 (50)</td>
<td>12 (7–12)</td>
<td>21 (10–35)</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>18/20 (90)</td>
<td>2</td>
<td>16 (9–24)</td>
</tr>
<tr>
<td>HIV-1 high risk</td>
<td>rsgp120 10 μg</td>
<td>3/19 (16)</td>
<td>11 (9–12)</td>
<td>13 (9–16)</td>
</tr>
<tr>
<td></td>
<td>rsgp120 40 μg</td>
<td>5/19 (26)</td>
<td>9 (7–12)</td>
<td>17 (11–25)</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>18/19 (95)</td>
<td>2</td>
<td>16 (5–30)</td>
</tr>
</tbody>
</table>

* Responses in vaccine recipients and responses to recall Ags were measured at day 2 only.

* Average induration of subjects with positive responses was defined in Materials and Methods.
were more frequent than CD3

Candida

T cell anergy. Individuals received rsgp120, for lymphoproliferation following stimulation with HIV-1MN rgp120, (Table I), and 22 (96%) responded to Candida to the recall Ags (17 (range, 1.2–70) in those who failed to mount a DTH response (Fig. 1). The mean SI for

dida

reactive cells to HIV-1 Env, within 48 h of the intradermal injection, we failed to recover Ag-

insert (data not shown). Thus, although DTH reactions occurred expanded by stimulation for 14 days with irradiated PBMC in-
detected in T cell lines or clones derived from the biopsies and

Candida

to HIV-1 envelope when tested in a thymidine incorporation assay the skin mononuclear cell lines or clones proliferated in response

DTH responses and in vitro immune reactivity to recall Ags.

In an attempt to isolate envelope-specific T cells from the DTH reaction, lymphocytes from biopsies of reactive rsgp120 skin test sites were expanded in vitro by both gp120/MN Ag and mitogen stimulation in seven patients. In six of seven biopsy cultures, CD3

T cells predominated (mean, 76%; range, 62–90%), as determined by flow cytometry, and the CD4/CD8 ratios ranged from 0.01–25 (median, 1.8). In one volunteer, CD16

/CD56

1

T cells were more frequent than CD3

T cells (data not shown). None of the skin mononuclear cell lines or clones proliferated in response to HIV-1 envelope when tested in a thymidine incorporation assay (mean SI, 0.8; range, 0.04–1.9). Similarly, we were unable to detect Candida-specific and tetanus-specific lymphoproliferation from mononuclear cells isolated from skin biopsies of Candida- and tetanus-positive DTH reaction sites (data not shown). In addition, no HIV-specific CTL activity recognizing HIV-1 Env was detected in T cell lines or clones derived from the biopsies and expanded by stimulation for 14 days with irradiated PBMC infected with recombinant vaccinia containing the HIV-1 Env gene insert (data not shown). Thus, although DTH reactions occurred within 48 h of the intradermal injection, we failed to recover Ag-reactive cells to HIV-1 Env, Candida, or tetanus from the sites following in vitro amplification.

Induction of DTH responses following HIV-1 envelope subunit immunization

Among 23 low risk, HIV-1-uninfected subjects who received four immunizations with rgp120/SF-2, 18 (78%) developed DTH re-
tions within 48 h following injection of 10 µg of rsgp120/MN (Table I), and 22 (96%) responded to Candida Ag (Table I). The gp120-specific responses were detected a median of 10 mo follow-
ing the last immunization, and no responses were detected among three placebo recipients in the clinical trial. The average diameter of induration was 17 mm (range, 5–34 mm). In a second vaccination protocol (AVEG 016, 016A), six of seven volunteers receiving three doses of HIV-1MN rgp120 vaccine mounted DTH responses to 10 µg of rsgp120/MN, with an average induration of 26.5 mm (range, 16.5–40.5 mm) at 48 h (Table I). Similarly, the two vaccine placebo recipients failed to develop induration at the site of the skin test injection. Responses to the Candida skin test reagent were also noted in both vaccine (Table I) and placebo recipients. The vaccinated uninfected subjects exhibited a greater frequency of positive responses and a larger diameter of induration than the HIV-1-infected patients with normal CD4

T cell counts despite receiving a lower dose of test Ag (10 vs 40–80 µg, respectively; Table I). Thus, intradermal application of rsgp120 induces a memory DTH response in persons with previous exposure to HIV-1 or its gene products through either infection or vaccination.

Rsgp120 induces a very late DTH-like reaction without pre-existing immunity

To determine whether HIV-1-seronegative persons who engage in high HIV-1 risk sexual activities develop envelope-specific DTH responses, we initiated a pilot study contrasting responses among 18 volunteers with either HIV-1 low (n = 12) or high (n = 6) risk activities. None of the volunteers demonstrated a DTH reaction at 48–72 h to either 40 or 80 µg of rsgp120 test Ag. Surprisingly, however, one individual in the lower risk group (8.3%) and five individuals in the higher risk group (83%) developed a large area of induration (average, 20 mm; range, 11–28 mm) at the test site after a median of 8.5 days (range, 5–9; Table II). Of note, these very delayed responses were not observed in the HIV-1-infected or HIV-1-uninfected immunized individuals.

We considered the possibility that the very late responses to rsgp120 injection may reflect previous exposure to HIV-1 despite its unusual time course. To explore this further, we initiated a larger trial among 39 HIV-1-uninfected healthy adults, 20 low HIV-1 risk, and 19 high HIV-1 risk persons. Each subject received two doses (10 and 40 µg) of rsgp120 intradermally. Candida and mumps Ag were also injected at separate sites as positive controls and the test Ag diluent (buffered saline) as a negative control. All volunteers mounted responses to either Candida (Table I) or mumps Ags (data not shown), but no response to rsgp120/MN, after 48 h. However, after 7–12 days, erythema and palpable induration developed at the 10 and 40 µg rsgp120 injection sites in 12 individuals and at the 40 µg site only in three others (Table I and Fig. 2). Unlike findings in the pilot study, responses were more often observed in the lower rather than the higher HIV-1 risk sub-
jects. Of those responding to the 10-µg dose, 9 were low risk and 3 were high risk volunteers; similarly, 10 low risk and 5 high risk subjects were positive responders to the 40-µg rsgp120 dose (Table I).

The very late skin reactions resembled DTH responses clinically and were not subtle, as depicted in Fig. 2 in a low risk individual whose clinical reaction was typical. Erythema was common, and the average inductions of the very late responses were 17 and 20 mm at the 10 and 40 µg rsgp120 injection sites, respectively (Table I details responses by risk group). Repeated questioning of risk behavior among study participants with very late responses failed to elucidate information altering their original risk behavior classification. These observations suggest that rsgp120 can induce a local, very late inflammatory response independent of pre-existing
immunologic memory, and we sought an explanation for why these reactions occur.

**DTH responses to injection of rsgp120/MN are not caused by recognition of the short HSV leader sequence within the recombinant gp120 molecule**

The rsgp120 skin test reagent contains a leader sequence of 27 N-terminal amino acids from glycoprotein D of herpes simplex virus. To exclude the possibility that the very delayed DTH responses to the skin test were caused by HSV-specific reactivity, we compared rsgp120 DTH responses with HSV serostatus in 19 HIV-1-infected healthy individuals at lower risk for HIV-1 infection, and six uninfected healthy individuals at higher risk for HIV-1 infection. A correlation between DTH responses to rsgp120 and the HSV serostatus was not apparent within those groups. For example, eight HIV-1+ individuals did not respond to the rsgp120 skin test despite being HSV positive (Table III). Six of these individuals responded to recall Ag, however, indicating the general ability to mount a cellular immune response (data not shown). In addition, one HIV-1+ individual lacked both HSV-1 and HSV-2 Abs, but had a positive DTH response to rsgp120 (Table III). Moreover, none of the nine healthy HIV-uninfected and HSV-positive individuals demonstrated a classical DTH reaction to rsgp120 within 5 days after injection (Table III). A lack of correlation with HSV serology was also observed for the very late rsgp120 reactions between days 7 and 12 (Table III). For example, five HIV-uninfected and HSV-positive individuals did not develop a very late rsgp120 skin reaction, while two HSV-1- and HSV-2-negative individuals did respond to the rsgp120 skin test.

When 14 HIV-1-infected individuals with CD4+ T cell counts ≥500 were evaluated for in vitro proliferation of PBLs to HSV-2 glycoprotein D, the mean SI s were comparable in individuals with (mean SI, 30; range, 1.2–133) and without (mean SI, 21; range, 2.8–65) skin reaction to rsgp120 (p = 0.8; data not shown). These results indicate that DTH responses to injection of rsgp120/MN do not correlate with HSV immunity and do not result from recognition of the short HSV leader sequence within the recombinant gp120 molecule.

| Table III. Correlation of DTH responses to HIV-1 MN rsgp120 and herpes simplex virus serostatusa |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | HIV-1/HSV-2 Serology |
|                  | +/+  | +/− | −/+  | −/− |
| HIV-1 infected  |       |     |      |     |
| Positive        | 3    | 2   | 5    | 1   |
| Negative        | 2    | 0   | 6    | 0   |
| HIV-1 uninfected|       |     |      |     |
| Positive        | 0    | 2   | 2    | 2   |
| Negative        | 0    | 5   | 0    | 7   |

* Responses were measured 48 h after intradermal injection of rsgp120 in HIV-infected subjects and a median of 8 days after injection of rsgp120 in HIV-1-uninfected subjects (no reactions were observed within 5 days after injection in the HIV-1-uninfected subjects).
Inflammatory infiltrates in Candida-induced memory DTH responses and in rsgp120-induced very late DTH-like responses are similar

To determine whether mononuclear inflammatory cells typical of a DTH response migrated into the sites of the very late rsgp120 reactions, we compared the cellular infiltrates of biopsies taken from day 7–12 rsgp120-induced skin reactions with those of 48-h Candida Ag reactions in seven individuals (six low risk and one high risk). Tissue sections stained with hematoxylin/eosin revealed mononuclear infiltrates surrounding small vessels in the papillary dermis and occasional infiltration of lymphocytes through the basal membrane into the epidermis. Neutrophils and eosinophils were not present. This morphologic pattern was commonly observed in both Candida DTH and day 7–12 rsgp120 very late responses (Fig. 3). In addition, the cellular phenotypes identified by immunocytochemistry in rsgp120 reaction sites were similar to those in the Candida reaction sites and consisted primarily of T cells and macrophages (Figs. 4 and 5). In the dermis, both lesions exhibited comparable distribution of CD4+ T cells (Fig. 4B), CD8+ T cells (Fig. 4C), and macrophages (Fig. 4D). The majority of dermal mononuclear cells were activated, as demonstrated by strong and abundant staining of both smaller (presumably lymphocytes) and larger (presumably macrophages and dermal dendritic cells) cells with anti-HLA-DR Abs (Fig. 4E). In the epidermis, the distribution of HLA-DR+ Langerhans cells was similar in Candida and very late rsgp120 lesions (Fig. 4E). CD4+ and CD8+ T cells were only occasionally observed within the basal and squamous cell layers of both reactions (Fig. 4, B and C).

To determine whether the types and relative frequencies of cells migrating into the skin test site were similar, T cells and macrophages, defined by mAb staining patterns, were enumerated within three representative dermal infiltrates and compared between the two biopsies for a given donor. Cell counts for CD4+ and CD8+ T cells and macrophages in the day 2 Candida lesions were similar to the cell counts in day 7–12 rsgp120 lesions in all six of the low risk volunteers (Fig. 5A, volunteers 1–6). For these volunteers, mean CD4+ T cell counts were 59/100 cells (range, 56–60), mean CD8+ T cell counts were 21 (range, 15–24), and mean macrophage counts were 24 (range, 13–45) in the Candida lesions. In the rsgp120 very late lesions, the counts were 62 (range, 53–70), 25 (range, 18–36), and 22 (range, 13–45), respectively. The one high risk volunteer biopsied (Fig. 5A, volunteer 7) demonstrated higher macrophage numbers in the Candida response (37 vs 10 cells/100 cells counted) and higher CD8+ T cell counts in the rsgp120 response (28 vs 16 cells/100 cells counted). However, the individual cell counts were well within the range of counts observed for the other six volunteers.

In the epidermis, the numbers of HLA-DR+ Langerhans cells were compared in biopsies of Candida and very late rsgp120 reaction sites of six volunteers (Fig. 5B). Mean Langerhans cell counts per 100 μm of epidermis were 36 (range, 25–45) in Candida lesions and 30 (range, 22–37) in rsgp120 very late lesions. Although differences in frequency of Langerhans cells were noted between Candida and rsgp120 very late lesions in individual volunteers, no significant trend in either direction was apparent. Thus, the cellular infiltrate was similar in both types of skin reactions, the classic DTH response and the very late DTH-like response. The infiltrates were dominated by macrophages and T lymphocytes, and CD4+ T cells were more frequent than CD8+ T cells. The immunohistochemical analysis therefore confirms that rsgp120 injected intradermally in HIV-1-negative, unvaccinated individuals induces a very late DTH-like skin reaction that is not driven by pre-existent immunologic memory.

Induction of serum Abs to HIV-1 gp120 by intradermal rsgp120 injection

These results suggest that intradermal injection of rsgp120 in uninfected nonvaccinated persons may elicit a primary immune response, manifested by a predominant Th1-type delayed DTH response. To determine whether envelope-specific Abs were also elicited, sera stored before and 28 days following skin testing were evaluated for binding to gp120/MN in the first 18 volunteers enrolled (12 low risk and 6 high risk uninfected). As shown in Table II, none of the 18 volunteers had anti-gp120 Abs before skin testing. However, five of the 18 (27.8%) developed serum Abs that recognized HIV-1MN gp120 by day 28 following injection of the rsgp120/MN skin test. Moreover, HIV-1MN anti-V3 Abs were also detected in two of the five with anti-gp120 Abs, but none of the sera from the five responders was capable of blocking CD4 binding to gp120/MN (data not shown). Of note, induction of anti-gp120 Abs followed either the 40- or 80-μg injection and in the small sample size was not associated with HIV-1 high risk activities. Only one of the five Ab responders simultaneously exhibited the very late DTH-like response. Thus, these results suggest that one intradermal rsgp120 injection in HIV-1-negative, unvaccinated individuals may lead either to a local cellular response characterized by a very late DTH-like reaction and/or to a low level Ab response.

Discussion

The skin test for DTH is the only in vivo assay available for measurements of cellular immunity in man, is dependent upon the presence of Ag-specific T cells, and typically is associated with Th1-type CD4+ T cell responses. Although a few studies have reported the use of an HIV-1 skin test (4, 17–19), this is the first to provide an in-depth investigation to establish potential broad utility in defining the induction and persistence of T cell responses associated with HIV-1 exposure, infection, and immunization. Our

![FIGURE 3.](http://www.jimmunol.org/) Candida Ag-induced DTH and HIV-1 rsgp120-induced very late DTH-like reactions similarly consist of perivascular mononuclear infiltrates in the papillary dermis. Sections are stained with hematoxylin-eosin and are representative of all seven HIV-negative individuals studied. Biopsies were taken from an HIV-1 low risk seronegative volunteer on day 2 at the Candida Ag-reactive site, on day 10 at the 40 μg HIV-1 rsgp120 injection site, and at the nonreactive normal saline injection site.
studies demonstrate that rsgp120/MN administered intradermally elicits DTH responses in patients with HIV-1 infection and in persons immunized with an HIV-1 envelope vaccine. Within 48 h of rsgp120 injection, a classical DTH reaction occurs in both HIV-1-infected and rgp120-vaccinated individuals, but not in HIV-1-seronegative individuals who have not been vaccinated or have received the placebo control. We did not observe envelope-specific DTH in HIV-1-seronegative individuals who have not been vaccinated or have received the placebo control. Our results suggest that persons with HIV-1 infection can mount HIV-1 envelope-specific DTH reactions despite the inability to detect lymphoproliferative responses to the same Ag in vitro. Several factors may contribute to this discrepancy. We acknowledge that a relatively high Ag dose was used in the HIV-1-infected cohort (40–80 μg), compared with the vaccine (10 μg) and the uninfected low and high risk groups (10–40 μg). However, we have been unable to detect in vitro proliferative responses with higher Ag doses (10–15 μg/ml) of either the recombinant

![Figure 4](image.png)

**FIGURE 4.** Influx and distribution of mononuclear cells are similar in the *Candida* DTH response (left panel) and the HIV-1 rgp120-induced very late DTH-like reaction (right panel). Staining patterns are representative of all six low HIV-1 risk individuals tested. Biopsies were obtained on day 2 (*Candida* Ag injection site) or day 10 (40 μg HIV-1 rgp120 injection site), fixed, and paraffin-embedded, and sections were stained by standard immunohistochemistry (see Materials and Methods). A, Isotype controls. B, Th cell staining. C, CTL staining. D, Macrophage staining. E, HLA-DR-positive Langerhans cells, dendritic cells, and activated mononuclear cell staining.

![Figure 5](image.png)

**FIGURE 5.** A, Mononuclear infiltrates in the papillary dermis of *Candida* Ag-induced DTH and HIV-1 rgp120-induced very late DTH-like skin lesions contain comparable fractions of CD4+ T cells, CD8+ T cells, and M387+ macrophages. Skin sections shown were obtained from six low HIV-1 risk individuals (volunteers 1–6) and one high HIV-1 risk individual (volunteer 7) on day 2 (*Candida* Ag injection site) or day 10 (40 μg HIV-1 rgp120 injection site). Bars show fractions of CD4+ T cells, CD8+ T cells, and macrophages of three different infiltrates and 600 total cells counted (see text). B, Epidermal Langerhans cell counts in skin sections obtained from five low HIV-1 risk individuals (volunteers 1–5) and one high HIV-1 risk individual (volunteer 6) on day 2 (*Candida* Ag injection site) or day 10 (40 μg HIV-1 rgp120 injection site). Langerhans cells were detected by HLA-DR expression and counted over three spans of 100 μm of epidermis. Bars show mean cell numbers per 100 μm of epidermis.
gp120MN or the reduced and carboxymethylated gp120MN (unpublished data). These in vitro findings are consistent with our previous investigations and reports by others (15, 20, 21). It is well recognized that induction of apoptosis of T cells may occur in vitro in the presence of HIV-1 envelope (20, 22). This may explain our failure to detect peripheral blood envelope-specific Th cells in vitro in contrast to Th cells recognizing other HIV-1 and recall Ags. Likewise, this may account for the inability to identify envelope-specific T cells from the DTH skin test sites following in vitro Ag stimulation, although by the time induration is apparent, bystander cells recruited into the lesions may predominate (which appeared to be the case as well in the Candida- and tetanus-reactive sites). Moreover, even with suppression of plasma viremia in Ags. Likewise, this may account for the inability to identify envelope-specific T cells from the DTH skin test sites following in vitro Ag stimulation, although by the time induration is apparent, bystander cells recruited into the lesions may predominate (which appeared to be the case as well in the Candida- and tetanus-reactive sites). Moreover, even with suppression of plasma viremia in Ags.

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References


