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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 MN 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 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.

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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 Group 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-1MN 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 MDP; Chiron, Emeryville, CA) at 0, 2, 6, and 18 mo (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 gp120 (300 μg) formulated with 50 or 100 μg of QS21 with or without alum (VaxGen, South Francisco, CA) at 0, 1, and 2 mo, and 6 and 12 mo and were skin tested 6–12 mo after the last immunization.

Seroserology for HIV-1 infection was performed by HIV-1 ELISA and Western blot. Serum Abs recognizing recombinant HIV-1MN gp120, the V3 region, and inhibition of gp120 binding to CD4 were measured by enzyme immunoassay 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 contains a fusion protein consisting of the first 27 N-terminal amino acids from HIV type 1 gp120 and 63–483 from the mature native gp120 of the HIV-1gag 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 100-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 upon 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 onto 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-CD4 (OKT4A; Zymed, San Francisco, CA), anti-CD8, C8/144B (Dako, Glostrup, Denmark) at 1/25 dilution; anti-myeloid/histiocyte Ag, MAC 387 (Dako) at 1/100 dilution; anti-GL-3 (Dako) at 1/100 dilution; and anti-Th cell, OPD4 (Zymed) and a coverslip. Slides were viewed on an Olympus BH-2 light microscope (Tokyo, Japan) with a grid to facilitate counting. Photomicrographs were taken with an attached Olympus camera.

**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 influx. 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 with 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 density gradient centrifugation, washed twice with PBS, and resuspended in culture medium. Cells were resuspended in culture medium and distributed at 105 cells/well in 96-well round-bottom microtiter plates. Irradiated 5 × 104 allogeneic PBMC were resuspended in culture medium containing either 2 μg/ml PHA (Sigma) or 10 μg/ml recombinant HIV-1gag 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 HIV-1 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 rIL-2 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-CD44 FITC, and anti-CD8 PE (Becton Dickinson, San Jose, CA).

In brief, 104 cells were incubated with the mAb for 30 min at 4°C, washed twice with PBS, and centrifuged, fixed in 1% paraformaldehyde (Baker, Waltham, MA), and analyzed with a FACScan flow cytometer (Becton Dickinson). Samples were gated using Consort-30 software (Becton Dickinson), and the appropriate isotype IgG controls (Becton Dickinson) were used to define background-staining limits.

**Lymphoproliferative assays**

Mononuclear cells isolated from blood or expanded from skin biopsies were resuspended in culture medium and distributed at 103 cells/well in 96-well round-bottom microtiter plates from quadruplicate biopsies. Cells were incubated under light microscopy. Biopsies yielded 0.5–3 × 106 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 × 104 allogeneic PBMC were resuspended in culture medium containing either 2 μg/ml PHA (Sigma) or 10 μg/ml recombinant HIV-1gag 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 HIV-1 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 rIL-2 and fresh irradiated allogeneic feeder cells and expanded into 24-well tissue culture plates (Costar, Cambridge, MA).
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 immune gp120 and infected with recombinant vaccinia virus encoding HIV-1_MN Env (vP1174; provided by the National Institutes of Health AIDS Reagent Program) were used as HIV-specific stimulator cells. Skin mononuclear cells (5 × 10^4) plated in triplicate into 96-well round-bottom plates, were incubated with 5 × 10^5 fresh irradiated autologous stimulator cells and 100 U/ml RTL-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 ^51Cr (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-1_MN 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-1_MN 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.

**HIV-1-infected individuals mount in vivo DTH, but not in vitro lymphoproliferative, responses to gp120**

Th cell dysfunction occurs early after HIV-1 infection. HIV-1 Env-specific lymphoproliferative responses are uncommonly detected among untreated patients and, to a lesser extent than Gag-specific responses, among patients treated with potent combination antiretrovirals (10–16). Thus, induction of HIV-1 Env-specific DTH responses was unexpected among the HIV-1-infected untreated volunteers in this study (15, 16). To determine whether HIV-1-specific cellular immunity is more readily detected by in vivo skin testing than in vitro, we compared gp120-specific DTH with peripheral blood lymphoproliferative responses in 14 HIV-1-infected patients with CD4+ T cell counts ≥500 cells/ml. Following in vitro stimulation with HIV-1_MN rsgp120, lymphocyte proliferation was not detected above levels observed following stimulation with the control Ag in any of the 14 patients. The SIs averaged 0.97 (range, 0.2–2.7), and no differences in Env-specific lymphoproliferation were noted among those with or without a gp120-specific DTH response (Fig. 1). Thus, patients who mount HIV-1 Env-specific DTH responses failed to respond by lymphoproliferation to the same Ag in vitro.

By contrast, recall Ag-specific in vitro lymphoproliferation was commonly observed in the infected patients, and the level of SIs correlated with DTH responses to the corresponding skin test Ags.

**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)a</th>
<th>Average Induration in mm (range)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 infected CD4+ T cells ≥500</td>
<td>rsgp120 40 μg</td>
<td>8/14 (57)</td>
<td>2</td>
<td>8.5 (5–15.5)</td>
</tr>
<tr>
<td></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></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rgp120SF-2 vaccine recipients</td>
<td>rsgp120 10 μg</td>
<td>18/23 (78)</td>
<td>2</td>
<td>17 (5–34)</td>
</tr>
<tr>
<td></td>
<td>Candida</td>
<td>22/23 (96)</td>
<td>2</td>
<td>14 (7–35)</td>
</tr>
<tr>
<td>rgp120/MN vaccine recipients</td>
<td>rsgp120 10 μg</td>
<td>6/7 (86)</td>
<td>2</td>
<td>26.5 (16–40)</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 Low HIV-1 risk</td>
<td>rsgp120 10 μg</td>
<td>9/20 (45)</td>
<td>12 (7–12)</td>
<td>18 (11–27)</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>

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

b Average induration of subjects with positive responses was defined in Materials and Methods.
were more frequent than CD3\(^+\) T cell anergy. Individuals received rsgp120, Candida and tetanus Ags intradermally, and reactions were assessed after 48 h. PBMC were assayed for lymphoproliferation following stimulation with HIV-1 Env, Candida, and tetanus Ags. Plots show medians (heavy horizontal bar) and 95th percentiles (outer thin bars) of stimulation indices. Responses to Candida and tetanus Ag were combined for statistical analysis.

The mean SI for Candida and/or tetanus Ags was 81 (range, 3.7–237) in patients with DTH responses to these Ags and 17 (range, 1.2–70) in those who failed to mount a DTH response to recall Ags (\(p = 0.02\)). These results confirm our earlier findings (15) that lymphoproliferative responses to recall, but not to HIV-1, Ags are often restored following acute HIV-1 infection. This immunologic recovery is reflected at the level of both in vivo 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\(^-\) NK 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- 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 reactions within 48 h following injection of 10 \(\mu\)g 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 following 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-1 Env rsgp120 vaccine mounted DTH responses to 10 \(\mu\)g 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 \(\mu\)g, 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 \(\mu\)g 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 \(\mu\)g) 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 \(\mu\)g rsgp120 injection sites in 12 individuals and at the 40 \(\mu\)g 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 subjects. Of those responding to the 10-\(\mu\)g 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-\(\mu\)g 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 \(\mu\)g 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 immunity.
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 volunteers with CD4+ T cell counts ≥500 (five from the pilot study and 14 from the main study), 12 uninfected 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 glycoprotein D, the mean SIs 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 II. Ab responses induced by rsgp120/MN intradermal injection**

<table>
<thead>
<tr>
<th>HIV-1 Risk (Volunteer No.)</th>
<th>rsgp120 Dose (μg)</th>
<th>Induration (day of response)</th>
<th>Anti-gp120 Abb</th>
<th>Anti-V3 Abb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (30)</td>
<td>40</td>
<td>28 mm (Day 5)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (31)</td>
<td>40</td>
<td></td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (32)</td>
<td>40</td>
<td></td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (33)</td>
<td>40</td>
<td></td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (34)</td>
<td>40</td>
<td></td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (35)</td>
<td>80</td>
<td>11 mm (day 7)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (36)</td>
<td>80</td>
<td>21 mm (day 9)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (40)</td>
<td>80</td>
<td>25 mm (day 9)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (41)</td>
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<td>20 mm (day 8)</td>
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<td>&lt;1.7</td>
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<tr>
<td>Low (42)</td>
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<td>16 mm (day 9)</td>
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<td>&lt;1.7</td>
</tr>
<tr>
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<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (47)</td>
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<td></td>
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<td>&lt;1.7</td>
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<tr>
<td>High (37)</td>
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<td>&lt;1.7</td>
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<tr>
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<td>&lt;1.7</td>
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<tr>
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<td>25 mm (day 9)</td>
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<td>&lt;1.7</td>
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<tr>
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<td>&lt;1.7</td>
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<tr>
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</tr>
<tr>
<td>High (47)</td>
<td>80</td>
<td></td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Low (43)</td>
<td>80</td>
<td></td>
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*Induration was not detected at 48 h. Late responses shown represent mean diameters.

**Table III. Correlation of DTH responses to HIV-1 MN rsgp120 and herpes simplex virus serostatus**

<table>
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<tr>
<th>HIV-1/HSV-2 Serology</th>
<th>DTH Response</th>
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<th>+/−</th>
<th>−/+</th>
<th>−/−</th>
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<td>5</td>
<td>1</td>
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<td></td>
<td>Negative</td>
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<td>0</td>
<td>6</td>
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<tr>
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<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

* 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).

**FIGURE 2.** Very late DTH-like skin reaction on day 10 after intradermal injection of rsgp120 (10 and 40 μg) in an HIV-1-seronegative individual at low risk for HIV infection.
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<sup>+</sup> T cells (Fig. 4B), CD8<sup>+</sup> 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<sup>+</sup> Langerhans cells was similar in Candida and very late rsgp120 lesions (Fig. 4E). CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> T cell counts were 59/100 cells (range, 56–60), mean CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells were more frequent than CD8<sup>+</sup> 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-1<sub>MN</sub> gp120 by day 28 following injection of the rsgp120/MN skin test. Moreover, HIV-1<sub>MN</sub> 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<sup>+</sup> 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. 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.](http://www.jimmunol.org/)
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. We did not observe envelope-specific DTH 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. We did not observe envelope-specific DTH 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
gp120$_{MN}$ or the reduced and carboxymethylated gp120$_{MN}$ (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 it appeared to be the case as well in the HIV-1 Gag-specific CD4$^+$ T cells are commonly detected in persons whose HIV disease fails to progress in the absence of treatment (long term nonprogressors) (12, 25, 26) and in those who receive combination antiretroviral therapy particularly early in their disease course (12, 16). Finally, rgp120-specific DTH responses may in part be mediated by CD8$^+$ T cells (27–29), which are less likely to proliferate in response to gp120 stimulation in vitro. Thus, the inability to detect HIV-1 envelope-specific CD4$^+$ T cells in vivo may be circumvented by the in vivo DTH skin testing, which reflects memory-driven recruitment of T cells and macrophages over the 48-h period (3, 30, 31).

Identification of Th activities induced by immunization is a key step in determining correlates of protection in vaccine efficacy trials. One of the simplest approaches, particularly in field testing, is measurement of DTH responses following skin testing. A reagent such as rsgp120 or a recombinant soluble Gag Ag spanning known Th epitopes may be useful in this application. In support of the rsgp120 test Ag, we demonstrated here that DTH responses can be detected in the majority of volunteers who received a recombinant envelope vaccine based upon either the homologous (HIV-1$_{MN}$) or heterologous (HIV-1$_{3p2}$) strain. DTH cross-reactivity between HIV-1$_{MN}$ skin test reagent and HIV-1$_{3p2}$ vaccine suggests the feasibility of administering this test successfully in a wider range of HIV-1 envelope-based vaccine trials. However, a number of issues must be considered to move this approach forward. Although DTH responses have been commonly considered Th1-type responses mediated by IFN-γ and migration inhibition factor, in IFN-γ knockout mice DTH responses can be mediated by Th2-type cells (32). Moreover, in virus-specific DTH responses, both Tc1 and Tc2 CD8$^+$ T cells can mediate responses (27), and CD8$^+$ T cells may function primarily during the early phase of reaction (28). Thus, measurement of induration alone at the site of injection will not be sufficient in specifically elucidating the phenotypic properties of responding T cells, and their proportions relative to other inflammatory cells will remain obscure. Identification and quantitation of Ag-specific CD4$^+$ T cells by techniques such as intracellular cytokine expression using flow cytometry will be necessary to precisely define the Th responses, and comparative studies of the in vitro and in vivo activities may be useful in subjects who demonstrate DTH responses. There are always practical considerations, including consistent injection intradermally and correct measurement of induration rather than erythema at the appropriate time after application. Nevertheless, when performed and read by trained persons according to established guidelines, in conjunction with comparative in vitro studies in a subset of subjects, the DTH assay may have merit in assessing immunogenicity and immune correlates of protection in large scale HIV vaccine efficacy trials.

In addition to the classic memory-driven DTH response, we discovered that HIV-1 gp120 can trigger pronounced inflammation independently of pre-existing immunity. This local response was observed 7–12 days after intradermal rgp120 injection in 21 of 57 HIV-uninfected healthy individuals, with approximately half of the responders clearly classified at low risk for HIV infection. Although its clinical and immunohistologic features resembled DTH (33), the mechanisms and kinetics of this inflammation were fundamentally different from those of DTH. Distinguishing clinical features were its very delayed time course, the high magnitude of induration, and the lack of correlation with previous Ag exposure.

Presently, we cannot ascertain whether inherent chemotactic properties of the gp120 molecule or de novo priming of naive T cells led to the very late responses. In addition, we cannot formally exclude the possibility that a contaminant induced these responses. If this were the case, one might expect persons who exhibited the classic DTH responses to have also mounted very late responses, and this was not observed. In vitro experiments have demonstrated that HIV-1 gp120 can induce chemotaxis of CD4$^+$ and CD8$^+$ T cells as well as macrophages (34–37), and this may relate to interactions with CXC chemokine receptor 4 expressed on dermal T cells and macrophages (38). However, it is not known whether this occurs in vivo, nor are the kinetics of this reaction known. In contrast, it is conceivable that local persistence of gp120 or its cleavage products not only triggers the afferent (from dermis to draining lymph nodes), but also the efferent, arm of cellular immunity. It is well recognized that in contact allergy, a typical type IV DTH reaction, sensitization and establishment of immunologic memory typically occur within 10–14 days (39–42). The induction of serum anti-gp120 Abs in some volunteers after skin testing suggests that naive T and B cell priming does occur, albeit Ab responses did not correlate with DTH responses. Previous studies demonstrate that proteins injected into the skin can be detected locally for only 1 day (29, 43). Investigations addressing the fate of intradermally injected rsgp120 as well as detection of gp120-specific cells within the infiltrate by newer methodologies such as peptide-MHC dim-Ing or class II MHC-peptide tetramers will help elucidate these important issues. Furthermore, repeated skin testing of HIV-1-seronegative very late responders will clarify whether intradermal gp120 injection indeed triggers a memory response in HIV-1-naive individuals. If so, kinetics of the reaction should significantly accelerate during sequential intradermal gp120 exposures.

In conclusion, our studies provide evidence that both classic DTH and very late inflammatory reactions are triggered by intradermal injection of HIV-1 rgp120, and the two responses are clearly distinguishable. The interesting property of rsgp120 to trigger very late inflammatory responses independently of pre-existing immunologic memory therefore does not preclude its use as a skin-testing agent for detection of HIV-specific immunity in clinical and vaccine settings. The value of skin testing for assessment of disease progression and response to treatment as well as of vaccine-induced immunity will require further validation and should therefore be investigated in larger study populations. Finally, these findings may also have uncovered an approach to examine either unique chemotactic properties of HIV-1 envelope or in vivo induction of a primary HIV envelope-specific immune response.

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References